Already published in this series:

OLFACTION AND TASTE *Edited by* Y. Zotterman, 1963.

LIGHTING PROBLEMS IN HIGHWAY TRAFFIC *Edited by* E. Ingelstam, 1963.

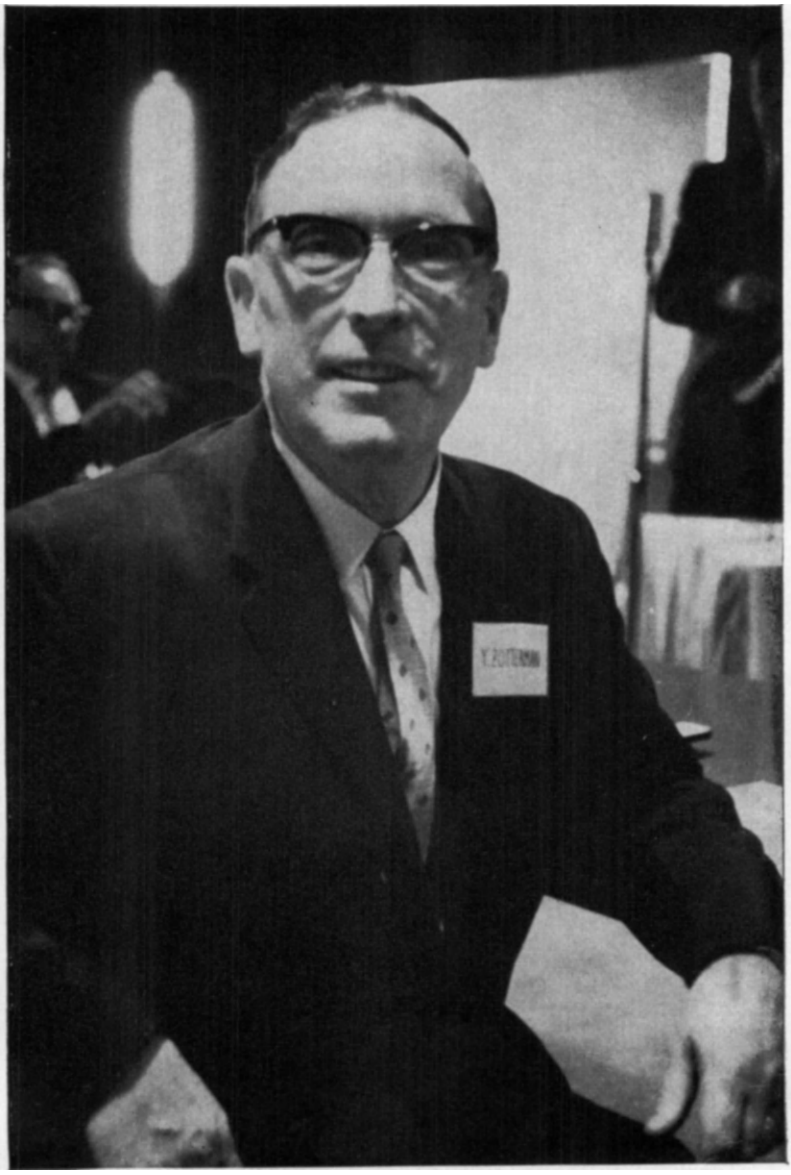
THE STRUCTURE AND METABOLISM OF THE PANCREATIC ISLETS *Edited by* S. E. Brolin, B. Hellman and H. Knutson, 1964.

TOBACCO ALKALOIDS AND RELATED COMPOUNDS *Edited by* U. S. von Euler, 1965.

MECHANISMS OF RELEASE OF BIOGENIC AMINES *Edited by* U. S. von Euler S. Rosell and B. Uvnäs, 1966.

COMPARATIVE LEUKAEMIA RESEARCH *Edited by* G. Winqvist, 1966.

THE FUNCTIONAL ORGANIZATION OF THE COMPOUND EYE *Edited by* C. G. Bernhard, 1966.



YNGVE ZOTTERMAN

OLFACTION AND TASTE II

Proceedings of the Second International Symposium held in Tokyo September 1965

EDITED BY

T. HAYASHI

SYMPOSIUM PUBLICATIONS DIVISION

PERGAMON PRESS

OXFORD · LONDON · EDINBURGH · NEW YORK
TORONTO · SYDNEY · PARIS · BRAUNSCHWEIG

Pergamon Press Ltd., Headington Hill Hall, Oxford
4 & 5 Fitzroy Square, London W.1.
Pergamon Press (Scotland) Ltd., 2 & 3 Teviot Place, Edinburgh 1
Pergamon Press Inc., 44–01 21st Street, Long Island City, New York 11101
Pergamon of Canada, Ltd., 6 Adelaide Street East, Toronto, Ontario
Pergamon Press S.A.R.L., 24 rue des Écoles, Paris 5e
Vieweg & Sohn GmbH, Burgplatz 1, Braunschweig,
Pergamon Press (Aust.) Pty. Ltd, 20–22 Margaret Street, Sydney, New South Wales

First Edition 1967

Library of Congress Catalog Card No. 63-1222

(2681/67)

OPENING ADDRESS

YNGVE ZOTTERMAN

DURING a meeting on Sensory Coding at Eddicot House outside Boston, 1959, Dr. Paffmann, Dr. Beidler and I met and they agreed with me that we should arrange a symposium on Olfaction and Taste in Stockholm in 1962 in conjunction with the International Physiological Congress at Leyden that summer. This meeting was highly successful and resulted in a book *Olfaction and Taste* Vol. 1 in the Wenner-Gren Center International Symposium Series. At the symposium, it was also decided that we should meet again this time in Japan in conjunction with the 23rd Physiological Congress.

We are greatly indebted to Professor Takashi Hayashi that he kindly offered his services to act as organizer of this Second International Symposium. It is also a great pleasure to find that so many of those who took an active part in the Stockholm Symposium are attending this meeting in addition to quite a few new members who are heartily welcome. Judging from the program we will have a very busy time listening to all the communications but I do hope we will have time for discussions. For that reason I have to appeal to the speakers not to exceed their time allotment.

At our last meeting Lord Adrian ventured the opinion that olfaction and taste have been rather neglected by physiologists compared to other senses like vision and hearing but now it is evident judging from the very rich program of this meeting that this is no longer the case. Each of us here who has devoted some time to studying any of the chemoceptive organs will I am sure feel that his endeavours have been rewarded by interesting new data concerning the chemoceptors or the chemoceptive parts of the nervous system.

It is also becoming evident that the study of chemoception in different animals will give new insights on animal behaviour making us understand many hitherto obscure relations. I am also confident that the study of the chemoceptive organs and their related parts of the nervous system will be helpful in the analysis of the function of other sensory organs and their neural connections just as we profit of the results of other sensory physiologists.

Our task is certainly not the easiest. In spite of all our endeavours it is easier to tell what we do not know than what we know. Let us start at the

uttermost periphery of the chemoceptors, the olfactory surface or the microvilli of the gustatory cells of the taste buds. We know that the stimuli set up an electrical potential in the receptor cell which leads to a discharge of signals in the nerve fibre but we know as yet very little of the real nature of the processes taking place on these surfaces. Our talented electron-microscopist friends have shown us the intimate and wonderfully complicated structure of these reactive surfaces and given us hopes that one day we will find the relation between structure and function.

There are many intricate problems like that of the specificity of the receptors. The fact that the receptor cells have a rather short span of life while the peripheral neurons are there all the life time of the individual, raised at our previous meeting a very interesting discussion about the influence of the neuron on the undifferentiated epithelial cells growing in from the periphery of the taste bud replacing the ageing gustatory cells. Now at this conference new data will be presented showing that the epithelium of the front of the tongue has other properties than that of the back of the tongue as far as the conversion of its cells into gustatory cells is concerned under the influence of the outgrowing gustatory nerve fibres.

Next comes the problem which we share with all other sensory physiologists, i.e. how the coded information from the receptors is transmitted and decoded in the central nervous system. Even there we expect quite a few new elucidating data as we are collecting more and more facts but it is rather intriguing that we still are in the dark as concerns the projection of the gustatory system to the cerebral cortex although we know quite exactly the localization of the gustatory relays in the thalamus. Until we have been able to find this out we lack objective data as concerns the central interaction and analysis of the gustatory information and it is only of a very minor consolation that we now know more about interaction of gustatory stimuli on the receptor level.

Our study comprises the chemoception in insects, other invertebrates and vertebrates from fishes to the monkey. In all of these, recordings have been made of the electrical response to chemoceptive stimuli as well as of the behavioural response of the animal. To this we have recently been able to add a few data concerning the correspondence between the electrical response from human taste nerves and the psychophysical estimate of the subject.

We often differ in our approach to our subject which only adds more variety and colour to our discussions. Also we may come to different opinions. But it is not the possession of truth but the seeking of truth which characterizes the cultured scientist.

CORRELATIONS BETWEEN STEREOCHEMICAL ASSESSMENTS AND ORGANOLEPTIC ANALYSIS OF ODOROUS COMPOUNDS

JOHN E. AMOORE and DELPHA VENSTROM

Western Regional Research Laboratory, Western Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture, Albany, California

INTRODUCTION

A CENTRAL problem in olfaction research is the relationship between the chemical constitution of odorous molecules and the qualitative character of the sensation produced. It has long been the contention of the senior author (Amoore, 1952) that the overriding determinant of odor type is the stereochemical configuration of the molecule concerned. In recent years this "Stereochemical Theory of Odor" has been thoroughly investigated. The results are published in a series of five detailed papers (Amoore, 1962*a*, *b*, 1964; Amoore and Venstrom, 1966; Amoore, 1965*a*).

The most recent of these articles (Amoore, 1965*a*) describes a combined stereochemical and organoleptic survey of 40 odorous compounds. Five chemicals were chosen as representing five distinctive classes of odor: ethereal, camphoraceous, musky, floral and minty. The odorous qualities and the molecular sizes and shapes of these 40 compounds were measured quantitatively in terms of their degrees of similarity to the 5 chosen standard compounds. It was concluded that for each odor class there is a highly significant correlation ($P < 10^{-3}$) between molecular shape and odor quality.

The present paper describes the extension of this study to a further 67 odorants, bringing the grand total of compounds surveyed to 107. The quantitative data on all 107 compounds are recorded in tabular and graphical form. This paper is primarily a record of detailed experimental results. Amplification of the methods and rationale can be found in the preceding paper (Amoore, 1965*a*), and further discussion of the results and conclusions is being presented elsewhere (Amoore, 1965*b*).

MATERIALS

The 107 chemicals employed as test compounds were generally reagent grade. They were used as received from the suppliers without attempting further purification. The sources of the odorants are indicated in the second column and footnotes of Table 3. The 4 compounds listed by their trade names have the following structures: Lilial[R] is *p-tert.* butyl-α-methyl-hydrocinnamaldehyde, Phantolid[R] is 6-acetyl-1,1,2,3,3,5-hexamethylindane, Tonalid[R] is 7-acetyl-1,1,3,4,4,6-hexamethyltetralin and Versalide[R] is 1,1,4,4-tetramethyl-6-ethyl-7-acetyl-1,2,3,4-tetrahydronaphthalene. The eight pentyl acetate isomers, cyclopentyl acetate and *n*-butyl propionate were gas chromatographically fractionated samples furnished by Dr. Roy Teranishi of this laboratory.

METHODS

Standard Odorants

The 5 compounds used as the principal standards in this work are listed in Table 1. (Also included are the pungent and putrid standards, but these

TABLE 1. ODOR STANDARD SOLUTIONS
The compounds were dissolved in triple-distilled water

Standard odor	Representative compound	Concentration (ppm)
Ethereal	1,2-Dichloroethane	800
Camphoraceous	1,8-Cineole	10
Musky	15-Hydroxypentadecanoic acid lactone	1
Floral	*d, l*-β-Phenylethylmethylethyl carbinol	300
Minty	*d, l*-Menthone	6
Pungent	Formic acid	50,000
Putrid	Dimethyl disulfide	0·1

two classes of odor do not depend on molecular shape, and are not further considered in this paper.) The 5 standard odorants were included among the 107 test compounds presented as "unknowns".

Organoleptic Analyses

All assessments of odor were made by the same panel of 29 judges (13 ♂ and 16 ♀) as employed previously. Before the unknown compounds were offered to the panel for judgment of odor similarity, the concentrations were adjusted to equalize the intensity of odor, using a 1 ppm solution of 15-hydroxypentadecanoic acid lactone as the reference standard (Amoore and Venstrom, 1966). The resulting "matched concentrations" of the odorants are shown in the third column of Table 3.

Each unknown odorant was then presented to the panel for assessment of odor quality. The method is basically the "matching standards method" of Schutz (1964) with modifications by Amoore and Venstrom (1966). The unknown odor was compared by the judge with each of the standard odorants in turn (Table 1). The judge expressed the degree of similarity between the unknown and the standard on a scale from 0 to 8 (Table 2).

TABLE 2. SCALE OF SIMILARITY FOR ODOR JUDGMENTS

Similarity	Rating
Extremely similar	8
	7
Very similar	6
	5
Moderately similar	4
	3
Slightly similar	2
	1
Not similar	0

Each compound was presented twice to the panel, about 25 judges attending a given session. Thus the average degree of similarity of odor represents the mean of about 50 judgments on each compound. The "similarities of odor to standards" are entered in columns 4 through 10 of Table 3.

Stereochemical Analyses

The measurements of molecular size and shape were based on scale models built from atomic units. The models were put into their most probable configuration, placed in a standard orientation, and photographed in silhouette (Amoore, 1964). Three photographs were taken from direc-

tions mutually at right angles to produce the "top", "front" and "right" silhouettes of the molecule.

Each unknown compound was then assessed as to molecular shape by the "shadow-matching" method described in the preceding paper

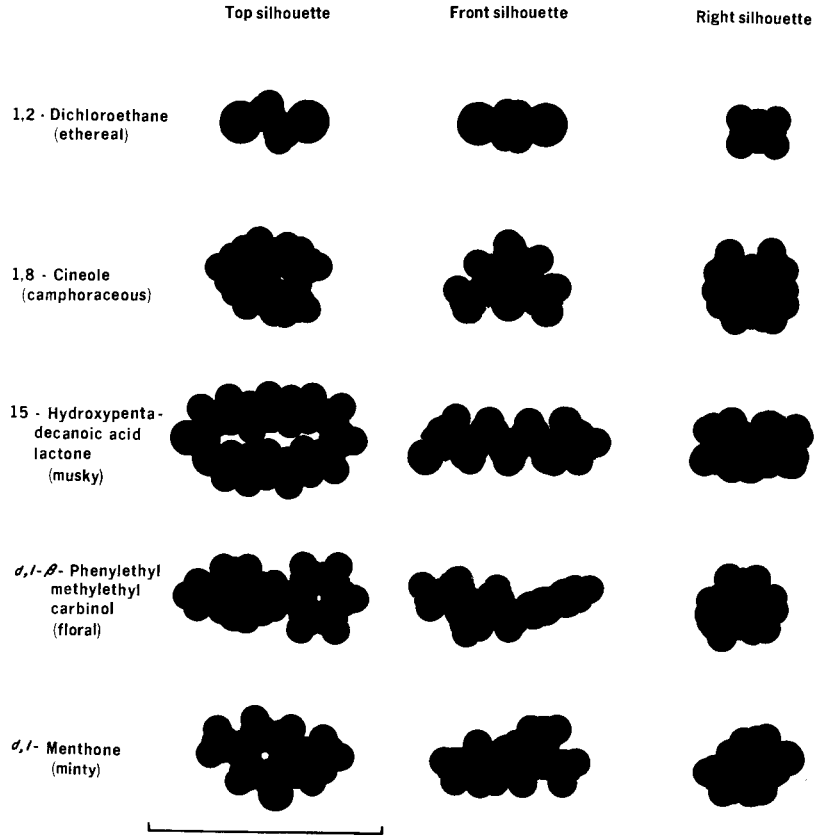


FIG. 1. Molecular model silhouettes of the five standard odorants. The scale marker shows 15 Å.

(Amoore, 1965*a*). The molecular silhouettes of each unknown were compared with the corresponding silhouettes of each of the standard odorants (Fig. 1). This was done by comparing the corresponding radii drawn from the center of gravity of the silhouettes to the periphery. The difference in length between corresponding radii of the two compounds (ignoring sign) is a measure of the difference in molecular size and shape (Fig. 2). The measurements were made every 10° of arc around all three silhouettes, and

an average value was found for this pseudo-vectorial difference in molecular radius ($\bar{\Delta}$). The reciprocal of this difference is a measure of the similarity of molecular shape. However, arithmetically it was more convenient to use the expression: Similarity of shape $= 1/(\bar{\Delta}+1)$. The average degree of similarity of shape represents the mean of 108 measurements on each compound. These "similarities of shape to standards" are entered in columns 11 through 15 of Table 3.

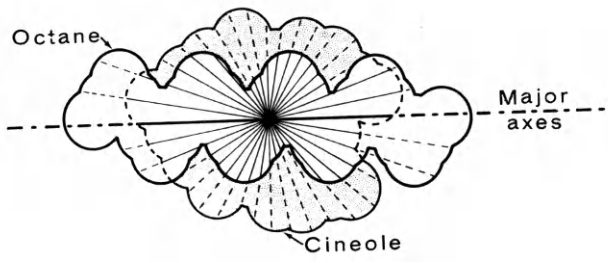


FIG 2. Top silhouette photograph of the elongated molecule of *n*-octane superimposed on that of 1,8-cineole (the globular camphoraceous standard). The centers of gravity and main axes of the silhouettes are co-aligned. The differences in the lengths (i.e. the overlap) of the corresponding radii (broken lines) measure the difference in molecular size and shape between these two compounds.

Statistical Correlations

The handling and interpretation of this mass of data (Table 3) was much facilitated by punched card and electronic computer techniques. For each unknown compound 5 cards were prepared, one for each of the standard odors. Each card was punched with the compound's serial number, the standard odor designation, the average radial difference ($\bar{\Delta}$) between unknown and standard molecular silhouettes, and the degree of similarity between unknown and standard odors. These cards were put through an IBM 1620 computer for printing the conversion of $\bar{\Delta}$ to $1/(\bar{\Delta}+1)$, and punching a new batch of cards with this reciprocal function, but with the other information and data unaltered.

The new batch of cards was separated into 5 sets of 106, each set representing all the comparisons of 106 test compounds with one given standard odorant. (The self-comparison of the standard odorant with itself was omitted from the statistical analysis, which reduces the total, N, from 107 to 106.) Each set of punched cards was put through an IBM 7094 computer programmed to perform a complete straight-line regression analysis by the method of least squares, using the "SNAP" object deck from

TABLE 3. ORGANOLEPTIC ANALYSES AND STEREOCHEMICAL ASSESSMENTS OF 107 ODOROUS COMPOUNDS

The compounds are listed alphabetically, ignoring prefixes. The standard odors are abbreviated thus: ETHereal, CAMphoraceous, MUSky, FLOral, MINty, PUNgent, PUTrid. For further information see text and footnotes

Compound	Source*	Matched concn.[a] ppm	Similarity of odor to standards							Similarity of shape to standards				
			ETH	CAM	MUS	FLO	MIN	PUN	PUT	ETH	CAM	MUS	FLO	MIN
Acetone	3	30,000	4·42	1·04	0·63	0·70	1·83	0·37	0·27	0·687	0·460	0·378	0·429	0·453
Acetophenone	4	25	0·71	2·00	2·08	3·24	1·55	0·13	0·27	0·584	0·567	0·461	0·524	0·554
Acetylene tetrachloride	4	50	4·32	1·05	0·57	1·29	1·27	0·57	0·37	0·647	0·573	0·464	0·469	0·534
Adamantane	16	10,000, w/v[c]	1·41	1·95	0·64	0·39	0·87	0·74	1·59	0·508	0·690	0·449	0·501	0·561
tert. Amyl alcohol	4	300	2·16	1·84	0·67	1·19	1·67	0·84	0·78	0·634	0·597	0·445	0·512	0·571
Δ^{16}-Androsten-3α-ol	13	2, w/v[d]	0·65	0·70	2·71	0·59	0·66	0·66	0·76	0·364	0·523	0·613	0·529	0·558
Anisic aldehyde	4	30	0·77	2·53	1·73	2·81	1·34	0·15	0·31	0·579	0·538	0·506	0·562	0·580
Anisole	4	6	2·65	1·44	0·91	0·75	1·25	0·57	0·92	0·630	0·554	0·468	0·531	0·555
Benzaldehyde	4	20	1·27	1·73	1·14	2·67	2·14	0·11	0·09	0·643	0·513	0·433	0·478	0·504
Benzene	2	200	3·62	1·10	0·70	0·81	0·93	0·29	0·35	0·639	0·493	0·403	0·444	0·477
Benzonitrile	4	8	1·14	2·54	1·32	2·16	1·35	0·09	0·26	0·640	0·506	0·431	0·478	0·509
Benzophenone	4	55,000, w/v[c]	1·18	1·79	0·99	1·93	1·45	0·76	0·48	0·453	0·528	0·460	0·529	0·513
Benzyl acetate	8	100	1·05	1·42	1·18	3·92	1·78	0·22	0·02	0·505	0·539	0·501	0·614	0,557
Benzyl alcohol	4	40,000	1·65	1·69	1·68	2·29	1·78	0·17	0·57	0·623	0·567	0·469	0·437	0·558
l-Borneol	7	15, w/v	1·23	2·06	1·40	1·10	1·74	0·42	0·87	0·455	0·644	0·472	0·479	0·574
Bornyl chloride	9	7000, w/v[c]	1·76	3·39	1·30	1·50	2·02	0·08	0·32	0·453	0·680	0·480	0·496	0·582
tert. Butylcarbinol	1	80, w/v	1·16	1·27	0·89	0·98	0·54	0·65	2·60	0·614	0·592	0·433	0·481	0·551
n-Butyl propionate	17	37	1·32	1·47	1·17	3·53	2·34	0·27	0·11	0·599	0·460	0·478	0·550	0·520
d, l-Camphor	4	40, w/v	1·60	5·42	1·29	0·90	2·27	0·32	0·18	0·469	0·662	0·466	0·488	0·558
Carbon tetrachloride	3	30,000[c]	4·73	0·81	0·48	0·77	1·58	0·69	0·31	0·569	0·522	0·387	0·456	0·486
l-Carvone	10	25	0·82	1·78	0·81	2·10	4·60	0·13	0·17	0·514	0·586	0·524	0·599	0·664
Cedryl acetate	8	20, w/v[b]	1·21	2·39	1·72	1·35	1·74	0·28	0·44	0·360	0·528	0·584	0·511	0·526
Chloretone	4	1500, w/v[b]	1·57	3·10	1·17	1·17	2·01	0·33	0·52	0·576	0·671	0·448	0·503	0·564
Chlorobenzene	4	5	2·16	1·47	1·10	1·22	1·10	0·34	0·39	0·668	0·518	0·430	0·480	0·507
Chloroform	11	1300	6·69	0·60	0·24	0·65	0·93	0·45	0·00	0·645	0·488	0·388	0·450	0·477

1,8-Cineole	4	10	0·82	(5·23)	1·00	1·21	2·25	0·18	0·06	0·484	(1·000)	0·503	0·534	0·628
Coumarin	4	100, w/v	0·89	1·87	1·96	2·36	1·47	0·17	0·17	0·586	0·507	0·483	0·511	0·542
Cyclododecanone	1	25, w/v	1·46	2·21	1·55	1·01	3·06	0·43	0·33	0·453	0·589	0·655	0·574	0·643
Cyclohexane	4	40,000	3·66	1·78	0·62	1·30	1·43	0·57	0·51	0·609	0·593	0·448	0·503	0·558
Cyclohexanol	4	1000	1·51	2·25	0·74	1·03	2·03	0·68	0·89	0·618	0·617	0·469	0·527	0·587
Cyclohexanone	4	150	1·91	2·06	1·17	1·85	1·89	0·28	0·30	0·627	0·614	0·465	0·523	0·565
Cyclooctane	1	10,000[c]	2·57	1·08	0·80	0·78	1·04	1·13	1·62	0·566	0·659	0·519	0·521	0·599
Cyclooctanone	1	450	1·55	3·28	0·29	0·87	2·36	0·34	0·59	0·545	0·658	0·520	0·535	0·617
Cyclopentadecanone	1	2·5, w/v[b]	0·35	0·42	5·46	2·52	0·96	0·12	0·14	0·411	0·527	0·793	0·588	0·579
Cyclopentanone	1	1800	2·33	2·05	0·87	1·77	1·65	0·33	0·67	0·698	0·539	0·427	0·469	0·515
Cyclopentyl acetate	17	52	0·91	1·75	1·68	4·20	1·46	0·04	0·00	0·580	0·599	0·509	0·610	0·624
Dibenzyl	4	80,000, w/v[c]	1·01	2·38	1·75	3·06	2·47	0·32	0·29	0·473	0·477	0·565	0·648	0·498
Di-*n*-butyl ether	4	45	1·29	0·87	1·14	2·83	2·73	0·07	0·32	0·582	0·435	0·471	0·545	0·496
p-Dichlorobenzene	4	3, w/v	1·69	1·92	0·93	1·77	0·97	0·23	0·23	0·613	0·506	0·449	0·495	0·534
1,2-Dichloroethane	4	800	(6·25)	1·09	0·54	0·87	1·09	0·30	0·08	(1·000)	0·484	0·398	0·463	0·484
cis-1,2-Dichloroethylene	9	1000	5·45	0·71	1·67	0·69	0·60	0·34	0·20	0·672	0·455	0·374	0·434	0·448
trans-1,2-Dichloroethylene	9	1000	3·41	1·26	0·74	1·19	0·81	0·43	1·08	0·793	0·450	0·372	0·428	0·444
Diethyl ether	11	200	5·12	1·86	0·69	1·40	1·80	0·26	0·10	0·725	0·490	0·412	0·487	0·502
Diethyl sulfate	4	800	1·86	1·90	1·32	2·45	2·07	0·23	0·20	0·531	0·551	0·517	0·610	0·625
Dimethylbenzylcarbinol	8	500	1·02	1·48	1·25	3·29	1·88	0·29	0·09	0·504	0·633	0·535	0·624	0·618
Dimethylbenzylcarbinyl acetate	8	35	1·07	2·30	1·14	2·44	3·56	0·09	0·05	0·457	0·607	0·565	0·641	0·638
2,2-Dimethylpropyl acetate	17	24	1·34	2·35	1·54	3·51	1·97	0·83	0·22	0·546	0·582	0·478	0·575	0·616
2,6-Dinitro-3,5-dimethyl-4-acetyl-*tert.* butylbenzene	9	6000, w/v[c]	0·43	0·91	3·94	2·76	1·03	0·32	0·38	0·391	0·504	0·632	0·530	0·552
2,4-Dinitro-3-methyl-6-*tert.*butylanisole	9	23,000, w/v[c]	0·30	1·24	3·57	1·49	1·22	0·20	0·31	0·497	0·534	0·570	0·504	0·567
Diphenyl ether	4	1	1·14	1·07	1·04	2·83	2·03	0·49	0·71	0·503	0·566	0·485	0·582	0·581
Ethyl acetate	2	500	3·48	1·23	0·67	1·23	1·17	0·73	0·37	0·698	0·503	0·423	0·490	0·506
Eugenol	8	45	1·28	1·96	1·55	1·76	2·74	0·20	0·05	0·498	0·535	0·557	0·552	0·597
Geraniol	9	40[b]	0·88	1·80	2·18	4·38	2·08	0·28	0·04	0·466	0·529	0·509	0·633	0·558

Table 3. cont.

Compound	Source*	Matched concn.[a] ppm	Similarity of odor to standards							Similarity of shape to standards				
			ETH	CAM	MUS	FLO	MIN	PUN	PUT	ETH	CAM	MUS	FLO	MIN
1,1,1,2,2,3,3-Heptachloropropane	9	2500[c]	1·31	2·16	0·92	2·15	2·06	0·34	0·24	0·497	0·674	0·467	0·533	0·618
γ-*n*-Heptyl-γ-butyrolactone	5	20	0·78	1·26	1·18	1·96	1·12	0·48	0·76	0·468	0·468	0·509	0·590	0·506
Hexachloroethane	4	10, w/v	1·14	2·09	0·63	0·91	0·67	0·51	1·92	0·595	0·638	0·429	0·489	0·550
Δ^9-Hexadecenolactone	8	2[b]	0·45	0·85	3·83	1·65	0·87	0·02	0·11	0·407	0·488	0·748	0·551	0·528
Hexamethylethane	1	20,000, w/v[c]	1·26	2·76	0·95	0·71	0·94	0·82	1·69	0·544	0·686	0·451	0·506	0·572
n-Hexane	9	40,000[c]	3·90	0·92	0·53	1·19	1·22	0·55	0·75	0·729	0·493	0·445	0·524	0·550
15-Hydroxypentadecanoic acid lactone	8	1, w/v	0·52	0·77	(5·66)	2·27	1·11	0·22	0·03	0·397	0·500	(1·000)	0·559	0·546
α-Irone	8	1	0·46	1·21	1·56	3·61	1·81	0·02	0·09	0·394	0·516	0·527	0·501	0·515
Isopropylcarborane	15	20,000[c]	0·83	2·47	2·08	1·30	1·43	0·24	0·25	0·415	0·635	0·547	0·549	0·599
Isopulegol	1	17	0·61	1·20	0·48	4·18	2·11	0·21	0·17	0·499	0·632	0·583	0·603	0·764
Isopulegyl acetate	8	22	0·68	1·60	0·98	3·64	1·96	0·16	0·16	0·418	0·542	0·567	0·508	0·539
Isoquinoline	4	20	0·86	1·89	2·04	2·12	1·85	0·18	0·32	0·588	0·506	0·469	0·489	0·530
Lilial[R]	8	70[b]	0·97	1·22	1·70	2·06	1·57	0·52	0·78	0·413	0·525	0·566	0·606	0·545
d-Limonene	4	5000[c]	0·98	1·34	0·95	3·22	1·92	0·20	0·00	0·495	0·619	0·505	0·581	0·634
Linalyl acetate	8	10	1·13	1·79	1·65	3·09	1·69	0·30	0·06	0·439	0·506	0·532	0·601	0·569
l-Menthol	7	75, w/v	0·63	3·81	0·61	1·67	3·38	0·05	0·00	0·486	0·635	0·567	0·587	0·698
d, *l*-Menthone	9	6	0·61	1·23	0·61	1·82	(6·60)	0·16	0·00	0·484	0·628	0·546	0·593	(1.000)
Menthyl acetate	7	10	0·88	2·50	0·81	2·30	3·41	0·19	0·12	0·406	0·534	0·536	0·501	0·538
Methyl anthranilate	8	500	0·89	2·42	1·89	2·35	2·53	0·12	0·31	0·542	0·517	0·516	0·510	0·568
d, *l*-2-Methyl-1-butyl acetate	17	25	1·60	1·78	2·00	3·62	1·99	0·23	0·03	0·567	0·534	0·493	0·560	0·599
2-Methyl-2-butyl acetate	17	100	1·98	2·80	1.10	1·90	2·84	0·52	0·27	0·556	0·587	0·495	0·592	0·630
3-Methyl-1-butyl acetate	17	22	1·40	1·63	1·58	3·43	1·94	0·35	0·31	0·569	0·525	0·510	0·589	0·634
d, *l*-3-Methyl-2-butyl acetate	17	48	1·71	1·85	1·52	3·19	1·70	0·34	0·18	0·539	0·612	0·537	0·570	0·656
Methyl cyclopropyl ketone	1	500	3·77	1·42	0·91	1·26	1·53	0·33	0·32	0·654	0·553	0·430	0·499	0·531
Methylene chloride	4	4000	6·27	0·33	0·42	0·22	0·72	0·45	0·23	0·736	0·448	0·368	0·426	0·440
Methylphenylcarbinyl acetate	8	35	1·45	1·62	1·04	1·86	1·70	0·77	0·77	0·475	0·561	0·564	0·618	0·617

Naphthalene	6	1, w/v	1·77	2·77	0·74	0·88	1·20	0·59	0·62	0·587	0·588	0·474	0·492	0·532
Nitrobenzene	4	5	1·26	1·87	1·59	1·53	1·93	0·25	0·32	0·636	0·521	0·440	0·483	0·528
n-Octane	9	140,000[c]	1·16	1·60	0·88	2·71	1·89	0·38	0·54	0·619	0·451	0·476	0·549	0·512
1-Pentyl acetate	17	40	1·43	1·72	1·75	3·49	2·38	0·33	0·12	0·595	0·469	0·489	0·577	0·547
2-Pentyl acetate	17	60	3·02	2·09	1·08	1·73	2·25	1·16	0·36	0·566	0·554	0·507	0·589	0·628
3-Pentyl acetate	17	80	2·40	1·89	1·12	1·97	2·50	0·87	0·40	0·519	0·596	0·520	0·486	0·583
Phantolid[R]	14	5, w/v[b]	0·52	0·66	5·12	1·70	1·02	0·06	0·09	0·372	0·497	0·577	0·518	0·523
Phenetole	4	3	2·39	1·14	1·04	0·68	1·12	0·38	0·70	0·590	0·526	0·475	0·535	0·548
Phenylacetic acid	4	150, w/v	0·75	0·96	1·98	1·75	1·05	1·08	0·98	0·565	0·575	0·488	0·575	0·587
β-Phenylethyl alcohol	4	400	0·49	0·85	2·00	3·38	1·88	0·55	0·35	0·573	0·559	0·488	0·565	0·561
2-Phenylethyl benzoate	4	30,000[c]	0·68	1·02	1·56	2·34	1·02	0·94	0·74	0·418	0·434	0·480	0·528	0·483
d, *l*-β-Phenylethyl-methylethyl carbinol	8	300	0·43	1·23	1·63	(6·32)	1·53	0·09	0·04	0·463	0·531	0·553	(1·000)	0·593
Pinacol	4	(1000 mg)[e]	0·65	2·27	1·30	0·93	1·32	0·96	0·80	0·573	0·683	0·457	0·523	0·598
d, *l*-Piperitone	10	80	1·09	2·12	0·79	1·21	3·41	0·15	0·06	0·493	0·648	0·577	0·609	0·662
Piperonal	8	800, w/v	0·90	1·34	1·74	2·60	1·62	0·07	0·19	0·573	0·506	0·491	0·534	0·577
Propanol-(1)	4	8000	2·28	1·31	1·04	2·03	1·46	0·39	0·18	0·840	0·478	0·391	0·451	0·473
Propanol(-2)	3	35,000	5·11	1·02	0·74	1·13	1·29	0·14	0·39	0·672	0·497	0·387	0·458	0·489
Pulegone	7	4	0·78	1·79	0·64	1·15	5·43	0·04	0·12	0·497	0·653	0·564	0·594	0·632
Salicylaldehyde	4	10	1·82	2·07	0·97	0·85	1·55	0·53	0·83	0·618	0·517	0·453	0·487	0·520
Santalol	8	35[b]	0·84	1·96	1·98	1·36	1·17	0·53	0·54	0·392	0·491	0·532	0·539	0·519
1,1,2,2-Tetrabromoethane	6	25	3·47	1·27	0·59	1·31	1·93	0·50	0·02	0·584	0·559	0·477	0·467	0·545
Thymol	3	100, w/v	1·93	2·34	0·91	1·30	1·79	0·75	0·87	0·500	0·559	0·527	0·568	0·590
Tonalid[R]	14	4, w/v[b]	0·47	0·69	4·17	1·68	0·72	0·17	0·31	0·360	0·486	0·574	0·508	0·521
Trichloroethylene	4	200	4·58	0·93	0·76	1·01	1·09	0·18	0·10	0·681	0·491	0·401	0·448	0·483
2,2,4-Trimethylpentane	4	200,000[c]	1·84	3·05	0·65	1·17	2·60	0·28	0·38	0·549	0·687	0·513	0·571	0·612
2,4,6-Trinitro-1,3-dimethyl-5-*tert*.butylbenzene	9	14,000, w/v[c]	0·37	0·72	5·06	2·77	0·96	0·06	0·21	0·402	0·537	0·626	0·519	0·566
Vanillin	12	200, w/v	0·67	1·06	1·47	2·89	1·25	0·00	0·13	0·562	0·541	0·509	0·563	0·591
Versalide[R]	8	2, w/v	0·48	0·71	5·30	2·30	0·78	0·07	0·03	0·353	0·489	0·558	0·461	0·504

* The source of each compound is indicated by a number, as below:

1. Aldrich Chemical Co.
2. Allied Chemical.
3. J. T. Baker Chemical Co.
4. Eastman Organic Chemicals.
5. Firmenich, Inc.
6. Fisher Scientific Co.
7. Fluka A.G.
8. Givaudan-Delawanna, Inc.
9. K. and K. Laboratories
10. L. Light & Co. Ltd.
11. Mallinckrodt Chemical Works.
12. Matheson, Coleman and Bell.
13. Medical Research Council (Prof. W. Klyne).
14. Soflor, Ltd.
15. Thiokol Chemical Corporation.
16. University of California (Prof. W. G. Dauben).
17. Western Regional Research Laboratory (Dr. R. Teranishi).

[a] The concentration of each compound is in parts per million (v/v) in water, except where indicated otherwise.
[b] Aqueous colloidal suspension.
[c] Solution in mineral oil.
[d] 120 μg. androstenol dissolved in 10 μl. isopropanol and dispersed in 60 ml. water.
[e] 1000 mg. powdered pinacol, without solvent.
[R] See Materials section for chemical name.

Mr. Edison Lowe of this Laboratory. The mean parameter values and axial intercepts obtained were used to plot the regression lines on each graph (Figs. 3–7). Each analysis was performed twice, first assuming that the molecular shape was the independent variable (shallower line), and secondly taking odor similarity as the independent variable (steeper line). The computer also yielded the correlation coefficient r and various other statistics.

RESULTS

The data on qualitative odor similarity and molecular size and shape similarity on all 107 compounds are consolidated in Table 3. Although only two significant figures would be realistic for an individual compound, the values are recorded to three significant figures to retain precision in subsequent computations.

The experimental results are also displayed in graphical form (Figs. 3–7). Each graph shows the relationship between molecular shape and odor for a given standard odor. The same set of 107 compounds was assessed for similarity to each standard odorant: ethereal, camphoraceous, musky,

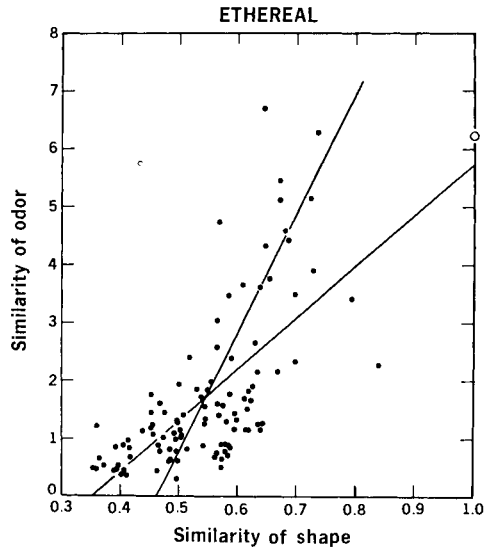


FIG. 3. Graph of the "ethereal" data from columns 4 and 11 of Table 3. The open circle represents the standard odorant itself, 1,2-dichloroethane. The other points show for each of the 106 test odorants the correlation between the similarity of odor quality to the standard and the similarity of molecular shape to the standard. The calculated regression lines are shown.

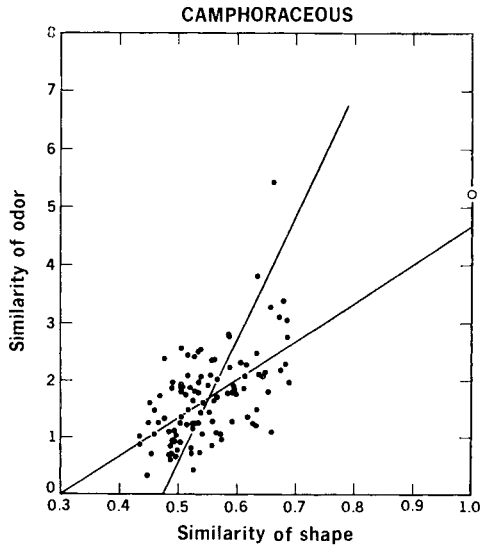


FIG. 4. Graph of the "camphoraceous" data from columns 5 and 12 of Table 3. The standard odorant is 1,8-cineole (open circle). The test odorants are the same in all five graphs.

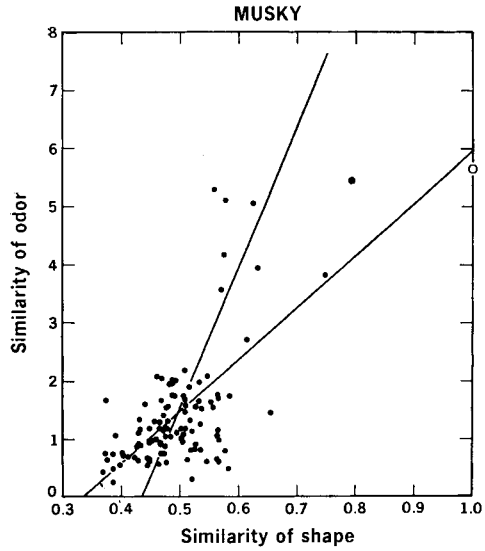


FIG. 5. Graph of the "musky" data from columns 6 and 13 of Table 3. The standard odorant is 15-hydroxypentadecanoic acid lactone. The points appear to have a somewhat sigmoid distribution.

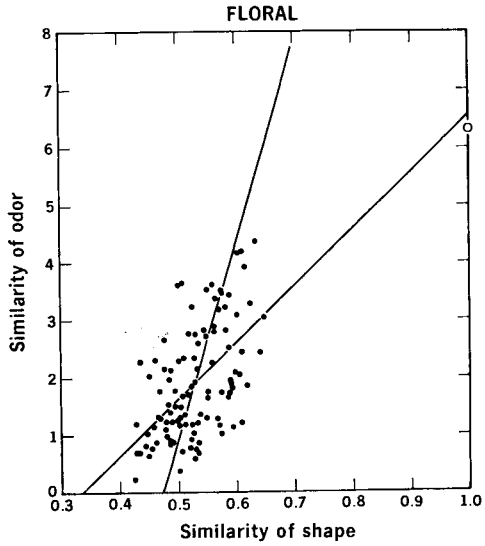


FIG. 6. Graph of the "floral" data from columns 7 and 14 of Table 3. The standard is *d*, *l*-β-phenylethylmethylethyl carbinol.

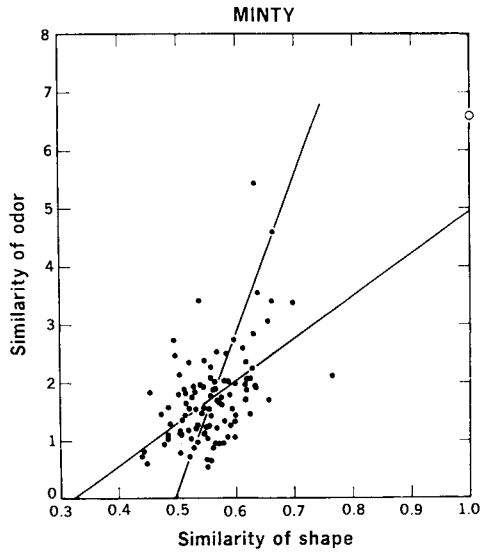


FIG. 7. Graph of the "minty" data from columns 8 and 15 of Table 3. The standard is *d*, *l*-menthone.

floral and minty. Hence the same unknown compound appears on all five graphs. (It proved impracticable to label the graphs with the serial number of each compound, but any desired point may be found by selecting its coordinates from Table 3.) It may be mentioned that each point represents the mean of about 160 measurements; the five graphs together summarize the results of about 85,000 measurements of odor quality and molecular shape.

DISCUSSION

The original choice of compounds for the survey represents a compromise among odorous variety, chemical diversity and commercial availability. Every compound admitted to the investigation has been completely surveyed and the results presented (Table 3, Figs. 3–7).

The statistical survey showed that for all five classes of odor there is a highly significant correlation between molecular size and shape and odor quality. The correlation coefficients (r) are shown in Table 4. For comparison the correlation coefficients obtained for the first 39 compounds surveyed (Amoore, 1965*a*) are included. The correlation coefficient should be independent of the number of observations. In accordance with this expectation, the bringing of 67 more compounds into the survey caused

TABLE 4. STATISTICAL SIGNIFICANCE OF THE RESULTS

Odor class	Correlation coefficient (r)	
	First 39 compounds	All 106 compounds
Ethereal	0·576	0·660
Camphoraceous	0·626	0·559
Musky	0·716	0·621
Floral	0·696	0·543
Minty	0·590	0·516

little change in the correlation coefficients. The mean r value for all five odor classes merely decreased slightly from 0·641 to 0·580.

For a given value of the correlation coefficient (r), when the number of observations (N) is increased, the probability (P) decreases markedly, that the observed distribution of points is a sample from a population of compounds in which molecular size and shape are independent of odor quality. To estimate the probabilities (for the least favorable of the results) we made use of the lowest correlation coefficients observed in the 39 compound and the 106 compound surveys (0·576 and 0·516 respectively). Both correspond with P values well below the lowest level (0·001) found in common statistical tables. However, Mr. Harold Cogswell in this Laboratory has evaluated upper limits for P in the two special cases of $N = 39$ and $r = 0{\cdot}57$, and of $N = 106$ and $r = 0{\cdot}51$. The results were respectively $P < 10^{-5}$ and $P < 10^{-9}$.

We may conclude that expanding the survey from 39 to 106 compounds has decreased the probability of randomness about ten thousand-fold. Furthermore the overall odds that the correlation is due to sheer chance are infinitesimally small, less than one in a billion. Finally, the quantitative results demonstrate indisputably that a strong positive correlation exists between molecular size and shape and odor quality.

It may be noted that this very encouraging degree of correlation was obtained in spite of a number of unfavorable factors which would tend to obscure the conclusion. The presence of contaminant by-notes in some of the chemicals, the inclusion of certain judges with an indiscriminating sense of smell, and the noncomprehensive measurements of molecular shape, would all detract from the height of the final correlation. It is anticipated that the value of the correlation coefficient could be substantially raised by attending to detailed improvements in methodology (Amoore, 1965*a*, *b*).

SUMMARY

The stereochemical theory of olfaction was systematically tested by means of detailed experimental measurements on 107 compounds. Each compound was assessed by a panel of judges to determine its odor quality, and by measurements on silhouettes of its molecular model to determine its molecular size and shape. The experimental data, presented in full in tabular and graphical form, were processed by computer. For each of the five odor classes examined (ethereal, camphoraceous, musky, floral and minty), a very highly significant correlation was observed between odor quality and molecular shape ($P < 10^{-9}$).

ACKNOWLEDGMENTS

We are very grateful to the members of the odor panel for their services, to Mr. E. Lowe for guidance with computer programming, to Mrs. Jean Harris and Mr. Richard Larock for assistance, and to Dr. D. Althausen and Mr. N. Cogswell for advice.

Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

REFERENCES

AMOORE, J. E. 1952. The stereochemical specificities of human olfactory receptors. *Perfumery Essent. Oil. Record* **43**, 321–323, 330.

AMOORE, J. E. 1962*a*. The stereochemical theory of olfaction. 1. Identification of the seven primary odours. *Proc. Sci. Sect. Toilet Goods Assoc.* Supplement to No. 37, 1–12.

AMOORE, J. E. 1962*b*. The stereochemical theory of olfaction. 2. Elucidation of the stereochemical properties of the olfactory receptor sites. *Proc. Sci. Sect. Toilet Goods Assoc.* Supplement to No. 37, 13–23.

AMOORE, J. E. 1964. Current status of the steric theory of odor. *Ann. N.Y. Acad. Sci.* **116**, 457–476.

AMOORE, J. E. 1965*a*. Psychophysics of odor. *Cold Spring Harbor Symposia Quant. Biol.* **30**, 623–637.

AMOORE, J. E. 1965*b*. Stereochemical theory of olfaction. *In* Symposium on Foods: The Chemistry and Physiology of Flavors, H. W. Schultz and E. A. Day, editors. Avi Publishing Co., Westport, Conn., in press.

AMOORE, J. E. and VENSTROM, D. 1966. Sensory analysis of odor qualities in terms of the stereochemical theory. *J. Food Sci.*, **31**, 118–128.

SCHUTZ, H. G. 1964. A matching-standards method for characterizing odor qualities. *Ann. N.Y. Acad. Sci.* **116**, 517–526

EXPERIMENTS ON THE SPECIFICITIES OF HUMAN OLFACTION. PART 2*

JAMES W. JOHNSTON, JR.

Department of Physiology and Biophysics
Georgetown University
Schools of Medicine and Dentistry
Washington, D.C.

THE critical, olfactometric experimental tests of the Stereochemical Theory of Olfaction, described herein, had their inception in 1961 at a time when recent evidence had not been produced.[9] A great deal of exploratory blending in liquid phase had to be completed in order to find rational practical formulations for the original blending in vapor phase because this work was carried out with a pair of 6-channel olfactometers that were designed for this investigation.[3,10] A progress report was read to the Conference on Surface Effects in Detection over a year ago, in which the unfinished testing of one experimental formulation indicated a possibility of producing a blend, in strict conformance with the theoretical limitations, that would match reasonably well the odor of the natural isolate, sandalwood oil. After testing two more formulations in vapor phase that were developed after additional exploratory blending in liquid phase, it was decided that a reasonable amount of testing of the prediction by Amoore[1] had been completed. This article is an advanced progress report of the most critical method of testing a postulated mechanism of 7 primary odors on human subjects.

Amoore had predicted that the organoleptic quality of the odor of cedarwood is a subtle natural blending of 4 of his 7 alleged primaries: camphoraceous, floral, musky and pepperminty. Inasmuch as sandalwood oil exemplifies the quality that perfumers call "woodiness" equally as well as cedarwood oil, both of these natural isolates were imitated by blending various pure odorant chemicals until it was discovered that the less oily odor of sandalwood was the better practical model. The more oily odor of cedarwood seemed to be difficult to match because of this component.

* This work was supported in part by a grant from the Givaudan Corporation, Delawanna, N.J., and in part from the Warner–Lambert Pharmaceutical Company, Morris Plains, N.J.

However, it is important to note that the prediction was deduced from a study of the size and shape of a few species of molecules that were reported to smell like the odor of cedarwood; e.g. cyclotridecanone.[1] In short, the deduction had to be based on steric considerations of single chemical compounds in 1950–1951 because the two isolated natural oils have complex chemical compositions. Inasmuch as the overall perceptions of sandalwood and cedarwood odors are similar, it was decided to attempt to match sandalwood by blending in vapor phase not more than 4 compounds drawn from each of the relevant "primary" classes.

This essential condition of the experimental design was dictated by the need to start from theoretical first principles and attempt to produce an odor similar in organoleptic quality to an existing odorant product even though the 4 components did not exhibit singly a trace of the odorant model. The resultant complex neural signals received by the brain from a blend of 4 components would be similar, according to the steric concept of a "complex" odor, to those signals elicited by a pure compound that smells "cedar" or "sandalwood" because such odorivectors were thought to possess 4 different qualities.

During the 17 months that elapsed from the late autumn of 1963 until May 1965, while the following subexperiments were performed, other investigators have produced experimental evidence that the concept of 7 primary odors is inadequate to explain the perception of odor quality. However, the blends in vapor phase had indicated that something was missing from the first principles by September 1964. It remained to complete the several parts of the experimental design in order to have a cohesive argument.

Amoore[2] stated that tests of his primaries that correlated measurements of subjective judgments with site fitting as measured by means of the Archimedes principle showed that the rigid receptor sites for ethereal and camphoraceous were untenable. A new method has led him to state the principle that the odorant molecular shape and size are correlated with the odor similarity scores of a panel of human subjects. At approximately the same time, Lettvin and Gesteland[12] stated that electrophysiological investigation of the frog demonstrated that one smell may excite several olfactory receptor cells and inhibit others. These effects were graded to impassivity and made it clear that, for the array of odors employed, specific primary receptors did not exist. Dravnieks[6] held that different current theories of olfaction seem to conflict, but in the final analysis they reduce to energetics at the molecular level and actually may supplement each other. A considerable amount of critical experimentation is needed

to supply the evidence that might unify the better theories. In the meantime, it is necessary to postpone serious discussion of a finite number of primary odors. As the number 7 is not an integral part of the stereochemical theory, the relation of molecular size and shape to organoleptic quality is very likely to be an essential part of the ultimate general theory. A concept of poorly differentiated receptor units is upheld by electrophysiological experiments on small mammals.[14] This result suggests that a precise determination of specific sensor units or sites may remain forever indeterminate. And, it has been speculated[5] that the olfactory sensor units form a continuum over the olfactory organ. Nevertheless, there are recognizable subjective odor classes which are related to the cross-sectional area of the odorivectors and the energy of desorption from the lipid–water interface to water.

The work reported herein brings to the next to last phase an exhaustive investigation into the possibility of making a woody odor like sandalwood from only 4 pure chemicals. The characterization of the odor quality of the final blend in conjunction with a G–L chromatographic quantification of the component stimuli in molecules of odorivector per cubic millimeter of air provides information about intensity of pure odorants in mixtures. Such information cannot be obtained from the proportions of these odorants in liquid phase. This is because the latter quantities are not directly related to the actual intensities of the stimuli. The appraisal of the prediction based upon rigid receptor or sensor sites indicates the direction that odor analysis should take by means of rating scales.

EXPERIMENTAL DESIGN

In order to prove a successful match of experimental blend and natural model, it will be necessary to compare the pure compounds that are used in the blends as well as the latter with the model. This idea was taken a step farther by comparing the sandalwood oil against itself and the floral Linalool against itself. As a pair of olfactometers were available for controlled appraisal of the olfactory stimuli, a given test compared the qualities within a pair of stimuli. The subjects' judgment was based upon perceptions of 5 pairs of reference standards that had been evaluated in large sample experiments with naive subjects. The reference standards "anchored" alternate points on an original modification[10] of Schutz's rating scale for characterizing odor qualities.[16] The modification was required by the judgment within pairs technique, as the working standards contained in stoppered Erlenmeyer flasks could not be opened in the odor-neutral test

room containing the olfactometers. This laboratory is constantly flushed by clean air and extraneous odors are barred from it. Therefore, the subjects smelled the reference standards prior to each session from flasks immediately outside of the olfactometry laboratory.

The modified rating scale had 9 fixed points ranging from "extremely similar" to "extremely dissimilar" with the "8, 6, 4, 2 and 0" values each exemplified by a paired reference standard. This technique reinforced the naive judges' memory of the odor distances at the alternate points. The distance was nil at point "8" where the odors were essentially identical. They were perceived as "8 and 7" for most judgments. The distance was relatively very great at point "0" which represented the antithesis of identity.

This method of expressing the organoleptic qualities did not produce a characterization or "signature" of the unknowns, one at a time. The method gave precise comparisons in terms of quantitative judgment values against a definite background of 9 odor distances. When group mean scale ratings were drawn from the samples, it was possible to interpret them in respect to agreement or non-agreement with the theoretical expectations of similarity and dissimilarity.

Inasmuch as the sessions at the paired olfactometers permitted only one subject at a time, a complementary and parallel experiment was performed on 8 subjects who had experienced the longest service in the olfactometric investigation (see Materials and Methods). In order to test these people as a group, the paired unknown odorants were placed in coded and stoppered Erlenmeyer flasks like the reference standards. A sufficient duplication of the unknowns was done so that each subject could work separately. In this experiment, the subjects were permitted to make a direct comparison with the appropriate reference standard during the judging of a given pair. This change of protocol from the olfactometry work eliminated the reliance upon memory of the odor distances. The sessions of this experiment were held in a large, well-ventilated room where the people sat at large tables. The principal advantage was the testing of 8 pairs of unknowns per session, so that additional information about odorants related to the investigation could be obtained before the completion of the olfactometry.

Each pair of unknowns belongs in one of 5 groups according to its chemical composition and odor quality. There were identical and non-identical pairs of pure compounds, identical and non-identical pairs of natural isolates, and non-identical pairs comprised of a blend and the model (sandalwood oil). All of these pairings are stipulated below (see Experimental Reagents).

The method of characterizing odor quality produced two sets of the group's judgments that were analyzed by the Index of Similarity, D,[15] as it was possible to use many vials instead of two olfactometers, and satisfy the requirement of two isolated sets of large samples of judgments of a given unknown. This treatment of the result satisfied the requirement of a non-parametric or "distribution free" method for data of an unknown frequency distribution. As each olfactometric test was given 8 times and each flask test was given 10 times to all of the subjects, different statistical treatments had to be selected for the present study.

The first step in this procedure was to determine the frequency distributions of the pooled judgments for each unknown pair of odorants in the flask experiment. If the Gaussian distribution were shown, the Analysis of Variance would be indicated. The pooled judgments were transformed to their cumulative percent frequencies and plotted on normal (probability) graph paper.[18] The results clearly indicated that the experimental data was an unknown, non-Gaussian distribution. Inasmuch as the protocols of the flask and olfactometric experiments were very similar, it was concluded that the latter result was also distributed in an unknown way.

Since both experiments produced data of the two-sample form, it was possible to select two-sample rank tests[17] and a test of homogeneity when the expected values are small.[8] The decision to rank the mean judgments was based upon the assumption that the validity of the actual differences in the rating scale units is not known for the relationships and processes that were assessed. This current status enables one to translate the individual mean judgments into ranks with few qualms in the testing of differences.[13] Therefore, the two-sample tests explained below obviated the risk of making the assumptions that would have been made had the Analysis of Variance been applied to the data.

The test of homogeneity of the judgments answers the question: "Which subjects are more consistent in using a small range of judgments rather than a wide one?" The test analyzes the individual subjects. For example, if the value of Chi Square is small, it can be concluded that the frequency distribution of each individual is similar to the others. Since the Chi Square is evaluated by means of a *t*-test, a significant value of *t* indicates that the individual frequency distributions are likely to be dissimilar. (A computer program was devised by the Georgetown University Computer Center and is available through the 1620 Users Group, Library Program No. 6.0.182, under the title: exact mean and variance of Chi Square when expectations are small.)

The Friedman test is a two-way Analysis of Variance by ranks[17] which answers the question: "Are there different responses by the judges to the several pairs of unknown odorants?" This treatment of the results would determine whether all of the subjects responded to the paired reagents in the same way. When the result statistic shows that the group is not responding in the same way, the data can be scrutinized in order to identify those subjects who differ from the group's performance. The Friedman test is applicable to the mean judgments of the group and the individual judgments of each subject.

The Kendall Coefficient of Concordance[17] answers a question similar to Friedman's test, but the former applies either when the subjects judged the unknown odorant pairs to be different or not different. The question answered by this test is: "Do the subjects agree independently that the pairs of unknowns are different in their effects?" Hence this test was applied only to the group's mean judgments.

MATERIALS AND METHODS

The subjects were volunteers of both sexes who were known to have a fair to good perception of odor qualities, but who were naive in respect to the new rating scale and the reference standards that "anchored" 5 alternate points of that scale. In fact, as the investigation progressed from early subexperiments with pairs of pure species of chemicals to the blends and sandalwood oil, replacements were added. These new people were virtually untried in psychophysical experiments. All were accepted on the assumption that they were representative of the North American subpopulation in the region of Washington, D.C.

During the 17 months when the experiment was in progress, a total of 23 men and women were tested. They ranged in age from 18 to 28 years. There were two hiatuses during that interval while complementary, shorter experiments on human subjects were completed. Subject withdrawals occurred because of graduation and illness, thus causing a turnover. However, no person withdrew before giving the required judgments during the last subexperiment in which he worked. Of the 23 people, 5 of them were studied in all subexperiments. Seven men and 10 women were available in all but the last two subexperiments. Three men and 9 women worked in the special tests with three pairs of pure chemicals, while 4 men and 6 women worked in the experiment on the final blend and sandalwood oil. In short, these were good sized samples for olfactometric experiments when compared to the samples of other investigators.

This method of recruitment made it possible that defective persons could be accepted at the start. As all subjects were investigated during the last three subexperiments without selection on the basis of performance, it is not known definitely that there were not any cases of limited partial anosmia. It seems most probable that several physiologically representative strains were obtained which possessed significant variations of sensitivity to some odorants. This recruitment without prior screening makes it possible for independent workers to recapitulate the experiment.[2]

In addition to determining mean judgments for all subjects, a novel method was devised for separating the judges into categories of performance before their means were analyzed. Consequently, these subgroups were not selected according to a theory of olfaction like the entire group. No one was culled from the original volunteers, but a rational classification was produced on the basis of three criteria. The mean judgments of the two subgroups were calculated.

The first criterion was suggested by the finding that 39·1 per cent of the subjects could not perceive odorant identity among the paired unknowns. To an apparently lesser extent, Amoore found this defect using the odor characterization technique.[2] A strict requirement had to be satisfied for tentative classification in the subgroup (*A*) of people who could perceive that both compounds of 4 paired unknowns were the same material. These subjects had mean judgments of "very similar" (7·0) to "extremely similar" (8·0). The subjects who had one or more means less than 7·0 were assigned to a different performance subgroup or category (*A'*).

Then tentative Subgroup *A* was examined by the individual scores against a background of the tests of homogeneity and treatment effects; e.g. Haldane's and Friedman's methods of analysis. For each subexperiment it was known whether the subjects were judging a given odorant pair about the same way or not, and whether the group revealed significantly different responses to the treatments or not. When the Friedman's test of individual judgments showed lack of conformity to the group response, such people were classified as *A'*. For example, if the group did not show a treatment effect, but a certain individual did so, he was removed from tentative Subgroup *A*.

For the individuals whose Friedman scores conformed to the group, a scrutiny was made of the standard deviation of all judgments. These values were studied for all of the subexperiments to determine their frequency of occurrence and upper and lower limits. Then an arbitrary cut-off of 1·0 was selected and subjects whose standard deviations exceeded 1·0 were assigned to Subgroup *A'*.

The resultant Subgroup *A* was comprised of subjects whose judging of all the paired unknowns gave presumptive evidence that they were not partially anosmic to certain odorants. Two other possible factors that may have contributed to excessive variation were guessing and ambiguous perception of certain odor qualities. The first factor was infrequently suggested by a subject giving rating scale values within a pair at different sessions that ranged from a "6" for moderately similar to a "2" for moderately dissimilar. The latter factor can only be surmised, as clear evidence of its occurrence was not forthcoming.

The selection of the odoriferous compounds (reagents), solvent and defoaming agent is the result of a number of years of reviewing the ideas of perfumers and odor chemists. The final decisions about the most suitable chemicals to subserve the two principal goals were reached after scores of determinations of odor quality by the investigator. On the one hand, several pure odorants from 4 organoleptic categories (camphoraceous, floral, musky and pepperminty) were tried in exploratory blending work with Erlenmeyer flasks. On the other hand, odorants of varied qualities without regard for "primaries" were tested during the quest for pairs of valid reference standards of similarity, neutrality and dissimilarity. Inasmuch as the concentrations of certain of these reagents differed between the olfactometry and flask experiments, two statements are given below. While the concentrations of the reagents in the olfactometric experiments are given under "Experimental Reagents", the liquid phase concentrations of the flask experiment are in the footnote to Table 5.

Each compound is represented by a symbol in the statement of the composition of blends *A*2, *A*3, and *A*4 (symbols of constituent compounds are stated in the order: camphoraceous, floral, musky and minty), and the tabulated results. For instance, "ca" symbolizes synthetic camphor, "ad" adamantan, "m" menthone, and"pi" Piperitone. It will be noted that the dilutions are stated where the concentrations had to be reduced in the sparging vessels ("saturators") of the olfactometers. The flask experiment contrasted with the other as the liquid phases were on odor test strips (chemically pure blotters) to a controlled distance of one inch from an end.

An impression of the amount and complexity of the exploratory blending to find promising formulations before placing reagents in an olfactometer may be gained from the following facts. Approximately 100 trials with the liquid phase mixtures had been made when blend *A*2 was tried in vapor phase. Then 84 additional trials of liquid phases were performed after July 1, 1964, in order to select the constituents of blends *A*3 and *A*4. This final

EXPERIMENTAL REAGENTS

Symbol	Odorant chemicals and combinations	Source	Liquid phases
s:s	sandalwood oil vs. same	Givaudan Corp.	undiluted
1:1	δ-3,7-dimethyl-1,6-octadien-3-ol (syn. Linalool) vs. same	Givaudan Corp.	pure
d:1	dimethylbenzylcarbinyl acetate vs. synthetic Linalool	Givaudan Corp.	reagent 40%, Dow Corning 200 fluid[1] (50 viscosity), 10% in mineral oil[2]; same as in 1:1
d:m	dimethylbenzylcarbinyl acetate vs. *d, l*-menthone	Givaudan Corp.	same as in d:l; reagent 4% conc. in mineral oil
ca:t	synthetic camphor vs. 1,1,3,4,4,6-hexamethyl-7-acetyl-tetralin	Givaudan Corp.	saturated solution[3] in mineral oil;
ph:e	phenylethylmethylethylcarbinol vs. 1,1,2,2,-tetrabromoethane	Givaudan Corp. Eastman Organic Chemicals	pure; reagent 98%, Dow Corning 200 fluid (50 viscosity) 2%.
ca:ci	synthetic camphor vs. 1,8-cineole	Givaudan Corp. Givaudan Corp.	saturated solution in mineral oil; reagent 10%, Dow Corning 200 fluid (50 viscosity), 2% in mineral oil
ce:s	cedarwood oil vs. sandalwood oil	Fisher Sci. Co. Givaudan Corp.	undiluted undiluted
ad	adamantan	Columbia Organic Chem.	25% of sat. sol.[3] in mineral oil
pi	*p*-menth-1-en-3-one (Piperitone)	Givaudan Corp.	reagent 4%, Dow Corning 200 fluid (50 viscosity) 14% in mineral oil

[1] A defoaming agent that is essentially odorless.
[2] Humble Oil and Refining Company's Primal No. 325-heavy (U.S.P.)
[3] ca: 0·0740 g/ml; t: 0·1621 g/ml; ad: 2·1690 g/ml.

Blend	Composition of blends
*A*2:	ca + ph + t + e
*A*3:	ad + l + t + m
*A*4:	ad + d + t + pi

exploratory phase was aided by the acquisition of several new floral and pepperminty odorants that were selected and donated by Dr. Julian Dorsky, the Givaudan Corporation.

Olfactometry

The pair of 6-channel olfactometers were designed expressly for the fundamental investigation of the "woody" blends reported herein. As these instruments have been described in detail[10] only the salient facts need be reiterated. The instruments are of all glass construction except for the differential pressure reducers, upstream of the manifold, and the three-way solenoid valves, downstream of the mixing vessels. The liquid phases of the experimental odorants are contained in sparging vessels (or "saturators"). The working quantities are 25 ml of reagent, and each liquid is isolated from the others. A continuous air-flow system conducts the purified air at 25°C through spargers where it separates into small bubbles that pick up the odoriferous vapors. These pass through sensitive flowmeters and thence into the mixing vessels, where the vapors are diluted and homogenized by a stream of pure air. The resultant mixture is transported, by the continuous flow principle, to the three-way solenoid valves. Here the air stream is either diverted through total flowmeters to the ceiling exhaust unit, or it is directed straight forward to glass nose cones.[11] These fit snugly against the face of a subject, who smells the effluents one at a time. It is necessary for the operator to press an instantaneous push button when the subject is ready to inhale from a given olfactometer. The odoriferous air is directed into a nose cone only long enough for 3 to 4 gentle inhalations. The exhalations are vented into a tightly fitted mouth piece and thence through a tube into the ceiling exhaust unit. There is a sixth channel, the bypass line, that is sometimes used to add pure air to the odoriferous stream just before it flows into the nose cones.

The olfactometers are capable of operating with only one sparging vessel in the line, or with 2, 3, or 4 in position. It is possible to change the experimental vapors within a 30 min session by flushing the mixers between tests with pure air. For the precise work with pure odorants only one vessel was installed per olfactometer. All glass parts were muffled at 390°C whenever a change of odorants was desired. Total flow at the effluent end never exceeded 1·98 l./min. With one vessel in the line, certain odorants were sparged at approximately 0·5 l./min. When 4 of them were in the line, the flowmeters inside the airbath registered the flowrate of odoriferous vapors going to the mixing vessel. This was the means of reproducing the

proportions of the constituents. They had to be varied in order to ascertain a precise formulation. The mixture was sought that would elicit the scale rating closest to "6" or better. Like the exploratory blending, this final stage with the vapors required many minute changes of the proportion of each constituent. An average of 20 variations of flow were tried for each blend in order to develop an official protocol. The protocols were then randomized for the 8 presentations to each subject.

The odor-neutral laboratory was maintained at an ambient temperature of 23–24°C, one subject was investigated during a given session of 30 min, and 4 discrete formulations were presented for judgment of similarity and dissimilarity of quality within pairs. The vapor concentrations of the unknowns were adjusted to approximately equal subjective strength in order to eliminate negative errors.[10] Their individual differences of sensitivity, especially to synthetic camphor, might have affected this essential step. The formulations were generalized to some extent for replication in respect to sensitivities because the sample mean was needed to test the prediction of woodiness.

As stated above, when a subject entered the laboratory, he had already reinforced his memory for the "odor distances". When he sat before the opaque screen, he saw on it a copy of the definitions of the points on the rating scale. He could not see the operator, but the latter viewed the judge in a "rear-view" mirror. Neither person knew the chemical identity of the liquids in the sparging vessels, and only the operator knew the vapor concentrations being presented to the subject. After each test, the subject rested for 5–8 min depending upon the required mechanical adjustments. This interval permitted the flow systems to re-equilibrate and the subject to recover from any tendency to nasal fatigue (if he developed it). The judgment of scale value was entered in the record after smelling alternately from each nose cone two or three times.

A brief comment about the possible effect of the mineral oil and defoaming agent upon organoleptic quality will suffice because these occasional additions to the experimental reagents will be discussed below under G–L chromatography. Not one of the odors was even slightly changed by these additions to the pure chemicals. The solutions and pure liquids were replaced by fresh materials before chemical changes could affect their odorant qualities.

Flask Method

In respect to the shorter, complementary experiment, 125 ml Erlenmeyer flasks were deodorized by muffling at 250°C and stoppered with aluminum foil-wrapped corks. The 5 pairs which contained the reference standards were identified by the scale values they epitomized in black numbers. The 8 pairs of unknown odorants were identified by alphabet code in red letters. The code was changed between each session. The subjective strengths of all reagents were made approximately equal within paired unknowns as for the olfactometric experiment. The liquid phases were freshly prepared as often as was needed to insure the characteristic organoleptic qualities.[4] The reagents were stored at ambient temperature. Table 5 gives the concentrations, solvent or diluent.

The 8 subjects who participated had been investigated in the olfactometric subexperiments that were completed prior to the last two. They were available for the second experiment. As the same rating scale was employed in both situations, and the flask experiment was modelled after the other one for presenting the unknown stimuli, the principal difference was the judging of 8 pairs of unknowns per session. These odorants contrasted in quality more often than in the case of the olfactometers. The subjects were instructed to judge each pair as an independent entity without comparing it to the others. The similarity or dissimilarity was judged solely in respect to the gamut from "extremely similar" to "extremely dissimilar". Subjects entered the independent judgments on the record form and rested 1 min between pairs. All unknowns were presented in 10 consecutive 30 min sessions twice weekly.

Quantification of Stimuli

The quantification of the odorants presented by the olfactometers was designed to sample the vapors in essentially the same state as they were inhaled by the subjects. The measurements of stimulus-intensity will be expressed as the number of molecules of odorant per cubic millimeter of pure air. An original sample collector was used to conduct a given pure vapor from the effluent end of a total flowmeter to the injection block of a Jarrell-Ash G-L chromatograph.[10]

The sample collector was tested prior to its use for measurement of intensity in respect to the method of injecting the standards. Four and 6-hundredths μg were injected with a Hamilton microsyringe into the injection block and for comparison, into the copper tube of the sampler near its upstream (proximal) end. The liquid volatilized in the 75°C tube and

was carried by Argon to the injection block. Here, the vapor was collected by condensation in the cold block which was maintained at dry-ice temperature. The procedure is summarized below.

A test of the efficiency of the vapor sampler was performed with *d, l*-menthone. The injection volumes were 0·2 μl of this compound in

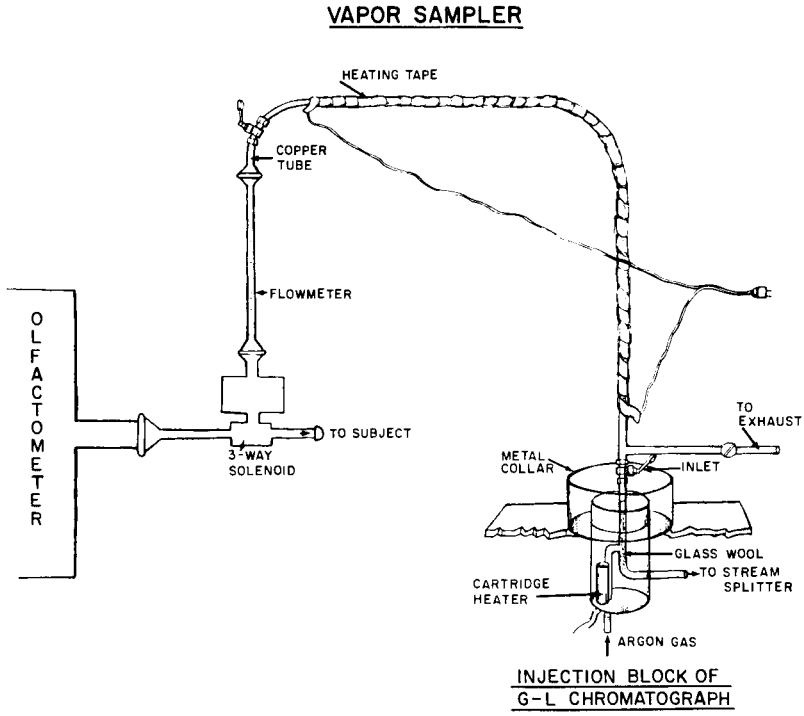


FIG. 1

which 0·1015 g were diluted to 5 ml in n-hexane. The expected chromatographic load was 4·06 μg as a result of injection into the block, in the usual way. In separate trials, the 0·2 μl were injected into the upstream end of the heated tube. The detected loads were 3·76 μg, 3·83 μg and 4 μg.

The special tests proved that the several odorant vapors could be collected when equilibrated because losses due to possible minute leaks or adsorption on the inner walls were either trivial or not present. The detector loads showed that tube collection differed from liquid injection without condensation by 1·48–7·59 per cent. As it is impossible to simulate the constant flow system from the sparging vessels into the condensation section, appraisal of the method is limited by the assumption that injection

of liquid standards is representative of collection by the condensation of vapor.

The chromatograph was prepared for sampling by bringing the packed column and detector ovens to the requisite temperatures (Table 8). Then the injection block was cooled by packing dry ice into a metal ring which surrounded the condensation section. The sampling tube was connected with the olfactometer 15 min prior to collection. The bone dry air carrying the vapor was thus brought to equilibrium inside the tube while being vented to the ceiling exhaust unit. When the temperature of the injection block was about −20°C, the stream of odoriferous air was directed into the condensation unit for a time interval (2–25 min). When enough condensate had formed so as to insure a detectable load for the chromatograph, the proximal Swagelok valve was closed. Then the Argon was directed into the injection block for 10 sec to flush away the remaining air. Now flow was stopped briefly to remove the dry ice and heat the injection block to that of the column. As soon as this operating condition was satisfied, the Argon was turned on again and the "slug" of vaporized condensate was carried into the column. Timing of the chromatographic analysis was started at this point.

Chromatograph

Experimental and standard operations of the G–L chromatograph were the same. The conditions were:

Coiled glass column packed with diethylene glycol adipate, 5% by wt., on 80/100 mesh "Gaschrom P" (Applied Science Laboratories)	209 cm × 4·10 mm dia.
Column Argon pressure	25 psi
Purge Argon pressure	15 psi
Column temperatures	Table 8
Detector temperatures (Argon diode ionization)	25° + column *T*.
Detector voltage	1200 V
Sensitivity	10^{-7}

In all tests, the several reagents were homogeneous as evidenced by one characteristic peak in the chromatograms. The quantities were determined by peak-area evaluations using a Librascope continuous integrator. These quantities were converted into the intensities of the stimuli (vapor concentrations). The chromatograms of the standards were the units of calibration.

In order to round out the quantification of the stimuli, the percentage of total flowrates at which each component was sparged for the three blends have been calculated. These values will approximate the true proportions of the components that are being determined. The adamantan presented a special case as the adipate is not an effective stationary phase. The little known adamantan will require research on other stationary phases before it can be quantified.

RESULTS

The results of the test of homogeneity afford an opportunity to evaluate the individual subjects similar to a comparison of standard deviations. A significant value of t would be evidence that the s's would exhibit noticeable variation between subjects in respect to all subexperiments. This possibility was found in 7 subexperiments with the olfactometers and not found in 3 of them. For instance, ce : s (cedarwood vs. sandalwood) showed little variation between subjects. Possibly the refined natural blending of the odorants in these oils accounts for this small degree of variation. On the other hand, ph : e (phenylethylmethylethylcarbinol vs. 1,1,2,2-tetrabromoethane) had a very significant t. The variation between subjects seems to be due, in part, to the complex quality of the ethane compound. The other subexperiment (ca : ci) that contained one odorant with a complex odor, 1,8-cineole, had a similar very significant t. It appears that the presence of a complex quality within nonidentical pairs caused the greatest observed variation. In general, the other nonidentical pairs of pure compounds exhibited significant variation to a lesser degree. Blend $A4$: s belongs in this group, too, as the only pair with mixed chemicals.

The other blends vs. sandalwood odor, $A2$: s and $A3$: s, showed little variation between subjects like the nonidentical natural isolates. This is a particularly interesting result as it proved that the simpler, synthetic blends were not complicating the judging as much as the pure compounds with complex qualities! It has been observed that the hedonic effect is an integral part of the response to organoleptic quality[7] suggesting that the e and ci may have affected the subjects differently than the blends and oils.

The tests of homogeneity for the flask experiment showed variation between subjects for all subexperiments. Even subexperiments s : s and 1 : 1 had very significant t's with this technique and the smaller sample of subjects. The smaller t's with $0{\cdot}05 > P > 0{\cdot}01$ were derived for ph : e and pa : ca. The former combination has the complex ethane odor and the latter has the natural isolate, patchouly oil. Therefore, there is a marked

contrast with the olfactometry which is probably due to a less efficient control of stimulus-intensity in the flasks.

In respect to Friedman's test of the group's responses to the olfactometric experiment, it should be noted in Table 1 that the minor variations

TABLE 1. FRIEDMAN'S TEST OF RANKS (X_R^2)

All subjects

	X_R^2	P	N
s : s	1·4	$0{\cdot}62 > P > 0{\cdot}49$	21
1 : 1 d : 1 d : m	24·9	$P < 0{\cdot}001$	48
ca : t	2·9	$0{\cdot}50 > P > 0{\cdot}30$	40
ph : e	6·2	$0{\cdot}70 > P > 0{\cdot}50$	80
ca : ci	2·5	$0{\cdot}80 > P > 0{\cdot}70$	60
ce : s	3·8	$0{\cdot}24 > P > 0{\cdot}15$	24
*A*2 : s	0·14	$1{\cdot}00 > P > 0{\cdot}96$	14
*A*3 : s	0·6	$P \cong 0{\cdot}67$	24
*A*4 : s	1·0	$P \cong 0{\cdot}90$	50

of 8 subexperiments did not produce significant X_R^2's. The subexperiment with the two floral and one minty compounds, as was anticipated, yielded a very significant result statistic. This is proof that the minor changes in the intensity of the stimuli lacked a treatment effect, while the contrasting qualities of the special case elicited different judgments from all judges. Nevertheless, as shown in Table 4, the largest group mean is presented for each subexperiment.

Even though most subexperiments fail to show a treatment effect, when the Friedman tests of individual judgments are examined, there is a considerable variation in subexperiments 1 : 1 and ca : t. Table 2 gives a very significant X_R^2 for Subject No. 94 and a borderline statistic for Subject No. 9 in the case of the identical floral pair. A similar, though more extreme, difference is to be noted for two other subjects in the case of the nonidentical pair with camphoraceous and musky odors. The blend *A*3 : s, on the other hand, exhibits a range of variation in the X_R^2 that is well within the "not significant" portions. The surprising result is in the 1 : 1 case where No. 94 was quite variable in respect to the identical, while No. 9 was not variable. It should be borne in mind that the compound was taken from

one stock bottle and placed in each olfactometer, and that the subjective strengths had been equalized for the group. This identical pair seems to have caused more difficulty for the subjects than *A*3 : s. Once more, the blend and natural isolate appear to have caused a dependable result.

TABLE 2. FRIEDMAN'S TEST OF RANKS (X_R^2)
Selected subjects and subexperiments

	X_R^2	P	N
ca : t			
86♂	6·8	$0{\cdot}04 > P > 0{\cdot}005$	12
61♂	0·6	$P = 0{\cdot}93$	12
1 : 1			
94♂	60·9	$P < 0{\cdot}001$	28
9♀	7·7	$0{\cdot}10 > P > 0{\cdot}05$	32
*A*3 : s			
88♂	2·2	$0{\cdot}53 > P > 0{\cdot}36$	9
91♀	0·15	$1{\cdot}0 > P > 0{\cdot}94$	12

As was anticipated, the Friedman test of the group in the flask experiment showed a very significant treatment effect. The $X_R^2 = 42{\cdot}96$, $P \ll 0{\cdot}001$. All tests of the individual's judgments likewise proved that the treatments caused significantly different judgments.

Against this background, the *W*'s for the olfactometric data showed outstanding to fair concordance of judgments in 7 subexperiments. There was significant discordance in the special case with the two florals and the minty compound. In this subexperiment, $W = 0{\cdot}738$, which shows that the qualities between pairs were different. While 1 : 1 is an identical pair, there are a nonidentical floral pair and a floral-minty pair to cause differences in the judging. Table 3 should be consulted for these values as well as for comparisons of the entire group, *G*, with Subgroups *A* and *A'*.

These performance categories reveal the effect of smaller samples, *N*, upon the value of *W*. The most frequent effect is for *W* to increase as *N* decreases, but the differences in respect to the group's *W*'s do not contradict the latter. An exception is subexperiment ca : t where Subgroup *A'* shows concordance like the group, while Subgroup *A* shows less of it. In the 7 subexperiments with small to medium *W*'s, all of the probabilities are greater than 0·05 because the *N*'s are not quite large enough to detect the concordance. Nevertheless, it is evident that the subjects judged these pairs in approximately the same way.

TABLE 3. KENDALL'S TEST OF CONCORDANCE (W)

Subexp.	*Group*			*Subg. A*			*Subg. A'*		
	W	P	N	W	P	N	W	P	N
s : s	0·118	> 0·05	7			too small			too small
1 : 1 } d : 1 } d : m	0·738	< 0·01	12	0·720	< 0·01	6	0·813	< 0·01	6
ca : t	0·165	> 0·05	8	0·509	> 0·05	4	0·096	> 0·05	4
ph : e	0·100	> 0·05	10	0·186	> 0·05	6	0·263	> 0·05	4
ca : ci	0·075	> 0·05	10	0·144	> 0·05	6	0·059	> 0·05	4
ce : s	0·293	> 0·05	8			too small			too small
*A*3 : s	0·041	> 0·05	8	0·111	> 0·05	3	0·133	> 0·05	5
*A*4 : s	0·188	> 0·05	10	0·369	> 0·05	6	0·539	> 0·05	4

W was calculated for all subexperiments of the flask experiment with Subgroup A only. $W = 0{\cdot}829$ and $P < 0{\cdot}01$ when $k = 5$. This result proved that the subjects judged the similarity and dissimilarity between pairs in the same way and that there was significant contrast of degree of similarity between certain pairs.

Greatest confidence is to be placed in the group means of the identical pairs, s : s and 1 : 1, and the nonidentical ones ca : ex and pa : ca. The first two subexperiments had ranges of 4 scale units from "5" to "8" with clearly defined peaks at "8" in both cases. The second two had ranges of 5 scale units from "2" to "6" with peaks at "4" (ca : ex) and "3" (pa : ca). The subjects appear to have had their greatest difficulty with subexperiment ce : v. There was a range of 7 scale units from "2" to "8" with peaks at "3" and "5". The remaining three subexperiments show less than outstanding consistency and some doubt must be attached to the dependability of their means.

Mean Judgments

The official or mean judgments of the degree of similarity and dissimilarity within pairs of unknowns for the olfactometric experiment are stated in Table 4. The group judged the identical pairs differently, with the s : s between extremely and very similar and the 1 : 1 between very and moderately similar. In both cases, Subgroup A was greater than the group and Subgroup A' was less than the group. The opposite result obtains for the three blends ($A2$, $A3$, $A4$) vs. sandalwood, where the mean of Subgroup A was less than the group and Subgroup A' was greater than the group. While this evidence is too limited to support a generalization, it is

TABLE 4. MEANS OF JUDGMENTS

Rating scale	Combinations of unknowns										
	s : s	ca : t	ph : e	ca : ci	s : *A*2	ce : s	s : *A*3	s : *A*4	l : l	l : d	d : m
	*G A A′	G A A′	G A A′	G A A′	G A A′	G A A′	G A A′	G A A′	G A A′	G A A′	G A A′
8											
	7·3								7·3		
7	7·1 7·0										
						6·5			6·6		
6						6·2 6·2			6·2		
5		5·1		4·9 5·1			4·8				
		4·3	4·6	4·6	4·3		4·5	4·3		4·5 4·6 4·4	
4					3·9		3·9				3 9 4·0 3·8
		3·5	3·7					3·6			
3			3·1					3·2			
					2·7						
2											
1											
0											

* *G* = all subjects.
A = subjects who perceived identical qualities and judged all combinations with small variations.
A′ = other subjects (see text for explanation).

of interest to note that the subjects who perceived the identities judged less similarity between the synthetic blends and the natural model. The closest match was achieved by *A*3 : s, where the group indicated the degree of very slight similarity. The other two matches were rated slightly less than the point of psychological neutrality, "4".

Of over-riding interest is the judgment of better than moderately similar for ce : s. During the exploratory phase of blending, it was decided that sandalwood odor was a more practical choice as a model than cedarwood[9] because the former was considerably less resinous. The present measurement proved that the two odors were close enough to permit the substitution as a test of the prediction of the composition of the cedarwood odor.

Two Sample Ranks Tests

Another requirement was the determination of the dissimilarity of the constituent odorants. All of them were judged slightly dissimilar, while the two camphoraceous compounds, ca : ci, were found to be slightly similar in odor. In order to ascertain whether the individual odorants were significantly unlike the sandalwood model, tests of two-samples by ranks[13] were made of the differences between the individual subject's means. This treatment was developed by Wilcoxon and White and is almost as sensitive as a precise test of measurement data. The range of variation in the judgments of both samples, as well as their relative values on the rating scale, affect the test of the difference.

The following comparisons of subexperiments show that the group did not differ significantly in the ratings: s : s vs. ce : s, s : s vs. 1 : 1, s : s vs. *A*4 : s, ca : t vs. ph : e, ca : t vs. ca : ci, and ph : e vs. d : m. The $P > 0{\cdot}10$ for these two-sample cases. The expectancy that no factor would cause a difference was upheld for the identical pairs whose means are 7·1 and 6·6, respectively. The nonidentical pairs of pure compounds are very close because they are either very slightly dissimilar or virtually at the neutral point of 4·0. The ca : ci is in the circumneutral zone and hence is not significantly different from the others. The surprise result is the comparison of the identical sandalwood sample and blend versus the model. The expectancy was that the difference of 3·5 scale units would reflect a real difference. This result may be due to a sampling effect caused by small, unequal *N*'s of 7 and 10.

Several other comparisons proved to be significantly different with $0{\cdot}05 > P > 0{\cdot}01$. They are s : s vs. *A*3 : s and ce : s vs. ca : t. One comparison,

s : s vs. ca : t, was borderline with $0{\cdot}10 > P > 0{\cdot}05$. In these cases, the expectancy of a real difference was upheld, and is of great interest in respect to the *A*3 : s subexperiment. The result shows that the largest mean, 4·5, of the three blends was only very slightly similar to the naturally occurring model (sandalwood oil). The difference between ce : s and ca : t shows that the oils are significantly different from means in the circumneutral zone. Oddly enough the s : s vs. ca : t difference cannot be accepted as a good risk.

Three comparisons proved to be very significantly different with $P < 0{\cdot}01$. They are s : s vs. *A*2 : s, ce : s vs. *A*3 : s and 1 : 1 vs. 1 : d. The results prove that blend *A*2 is not like the model and that blend *A*3 probably does not match cedarwood any better than sandalwood. The nonidentical florals, 1 : d, are rather different when compared with the identical florals even though the two odorants are very slightly similar.

Inasmuch as it was reported that 30 subjects often gave the gamut of judgments at a given session from "0" to "8", [2] it is worthwhile contrasting those results using 125 ml Erlenmeyer flasks and the present results with two olfactometers. Judgments in subexperiments s : s and ce : s had a range of 3 scale units, the 1 : 1, *A*2 : s, and *A*3 : s increased to 5 units, the ca : ci and 1 : d varied between 6 units, the ph : e, ca : t and d : m varied within 7 units, while the judgments of blend *A*4 : s embraced 8 units. Not in any subexperiment did the subjects require the full 9 units of the rating scale, and in the case of s : s and ce : s the small ranges did not cross the neutral point. Therefore, it is held that the continuous flow technique utilized by the olfactometers gives a better control over variation than the most carefully handled flask technique.

The olfactometric blends produced odors that were "woody", but lacked the more or less oily and resinous components of the natural oils. In an effort to characterize blend *A*4, the last to be studied, the author was tested in a literally blindfold experiment with 10 standards of non-resinous, native woods. The specimens were collected in February 1965 at an ambient temperature of $-3°$ to $-4°$C from a large sawmill at Hughesville, Maryland (U.S.A.). Clean chunks were cut from either the heartwood or both heartwood and sapwood, of logs of various species that were from 1 to 5 months old. The specimens were placed in deodorized jars and stored in the dark at $-2°$C. Two species, white oak, *Quercus alba*, and black locust, *Robinia Pseudo-Acacia*, were judged to have very slightly similar odors to *A*4. The blend was sweeter smelling than both woods, and they had a pungent nuance that was lacking in blend *A*4. The judging was done on 6 different sessions with rests of 5–7 days after each one. These rests eliminated memory of the previous ratings of the 10 standards. The 9-point rating

scale was used in conjunction with the 5 pairs of reference standards. As the blend may better match a dry lacquer synthesized with natural substances, an effort will be made to find a more helpful characterization. The restorer of finished wooden articles at the Smithsonian Institution could help to obtain various lacquers.

TABLE 5. MEANS OF JUDGMENTS
Complementary experiment with flasks

Combinations	Group	Subg. *A*	Subg. *A'*
1 : 1*	7·54	7·8	7·1
s : s*	7·46	7·9	6·7
ce : v	4·56	4·5	4·7
m : h	4·34	3·9	5·1
ca : ex	3·98	4·1	3·7
ph : e	3·70	3·7	3·7
pa : ca	3·62	3·6	3·7
f : ph	1·55	1·6	1·5

Additional symbols

ce : v cedarwood oil, 5% conc. in mineral oil, vs. vetivert, 50% conc. in diethyl phthalate.

m : h *d*, *l*-menthone, 0·009% conc. in mineral oil, vs. 4-*t*-butyl cyclohexanone, 0·0715 g/1·92 ml of mineral oil.

ca : ex syn. camphor, 3% of saturation in diethyl phthalate, vs. Exaltone, sat. sol. (13·8033 g/18 ml. mineral oil).

ph : e phenylethylmethylethyl carbinol, 30% conc. in mineral oil, vs. 1,1,2,2-tetrabromoethane, 5% conc. in mineral oil.

pa : ca patchouly oil, 8% conc. in mineral oil, vs. syn. camphor, 75% of saturation in mineral oil.

f : ph formic acid, 30% aqueous, vs. phenylethylmethylethyl carbinol, 38% conc. in mineral oil.

The Exaltone was obtained from Firmenich & Co., the formic acid from Fisher Sci. Co., and all the other reagents from the Givaudan Corporation.

* Same concentrations as in olfactometric experiment.

The mean judgments obtained by the complementary flask experiment are presented in Table 5 where a brief format replaces the rating scale arrangement of Table 4. It should be noted that 1 : 1 is much closer to expectancy (7·5) than it was in Table 4, and that in all subexperiments the difference between means for the entire group, Subgroup *A*, and Subgroup *A'* are small in comparison with the values of the major experiment. Among those paired odorants for which there was an expectancy based upon previous experimental results, all were upheld by the results. The identical

pairs were close to "nearly extremely similar", ca : ex (the reference standard of "4") was neither similar nor dissimilar, and f : ph was less than moderately dissimilar (1·6). Among the unknowns for which there could not be an expectancy, because they were being judged for the first time, were ce : v (two natural isolates), pa : ca (the first is a natural isolate and the second a pure compound), and three non-identical pairs of pure compounds.

The individual means of selected subexperiments or combinations were analyzed by the two-sample ranks tests.[13] Two comparisons, s : s vs. 1 : 1 and ca : ex vs. ph : e, were not significantly different as $P > 0{\cdot}10$. Thus, the two identical unknowns were extremely similar, while the floral : minty pair were essentially neutral like the reference standard. (This unknown was present, also, among the 5 pairs of reference standards to which the subjects referred at each test.) Two other comparisons, ph : e vs. f : ph and ce : v vs. pa : ca, were significantly different with $0{\cdot}05 > P > 0{\cdot}01$. This result is rational for ph : e vs. f : ph on intuitive grounds, but it is especially interesting to note that the other two samples have a mean difference of 0·9 scale unit in Table 5. Therefore, the vetivert has a woody quality that is very slightly similar to cedarwood, and the patchouly oil (another borderline woody quality) is dissimilar to one of its constituents, camphor gum. Finally, two comparisons are very significantly different with $P < 0{\cdot}01$. They are s : s vs. ca : ex and s : s vs. ce : v. The first comparison upheld the expectancy while the second one was a new finding. The very slight similarity of cedarwood and vetivert is unimpressive when tested against the identical unknown. One may doubt that the latter smells woody.

Although the range of variation of the individual judges for the 8 subexperiments that comprised the flask experiment were discussed under the test of concordance, it is necessary to refer to them again. The subjects did not use 9 units of the scale even with Erlenmeyer flasks. This experiment had 8 subjects with 8 unknowns per session, and 10 judgments per unknown. This uniform design made the analysis of variation simpler and is based upon slightly larger samples than the olfactometry. Combinations s : s and 1 : 1 varied but one scale unit, but the other combinations exceeded 3 units. Furthermore, the judgments were above and below the neutral point (4·0) in all cases. Combination pa : ca embraced 5 units, ca : ex and ph : e 6 units, ce : v and f : ph 7 units, and only m : h embraced 8 units. In general, it may be said that the flask technique controlled variation less well than the olfactometry, but that under no circumstance was there an excessive degree.

Quantification of Stimuli

The choice of flowrates of purified air through the sparging vessels was dictated by different requirements according to whether a pure odorant or a blend of 4 odorants were desired. Table 6 presents the total effluent flow for each olfactometer and the percentages of it through the upstream parts when pairs of pure chemicals were tested. Table 7 presents the total effluent flow and percentage of it when one olfactometer was used to blend the theoretically selected odorants. Notice that sometimes dilution of the odoriferous air was made either with the dilution line to a mixing vessel or the bypass line, but that both lines were required for blend *A*2.

TABLE 6. PERCENTAGE AIR FLOWS

Combinations	Total flow (ml/min)	Percent			Total flow (ml/min)	Percent		
		By-pass line	Dilution line	Saturator		By-pass line	Dilution line	Saturator
s : s	970	65·0	0·0	35·0	970	65·0	0·0	35·0
1 : 1	1820	0·0	72·0	28·0	1820	0·0	80·8	19·2
*d : 1	1480	0·0	88·5	11·5	1480	0·0	88·5	11·5
d : m	1480	0·0	46·7	43·3	1480	0·0	77·7	22·3
ca : t	640	0·0	0·0	100·0	640	0·0	0·0	100·0
ph : e	640	0·0	0·0	100·0	640	46·8	0·0	53·2
ca : ci	750	0·0	0·0	100·0	970	97·4	0·0	2·6
ce : s	1480	0·0	80·4	19·6	1320	50·0	0·0	50·0

* The nonidentical combinations arer reported in direct juxtaposition to the flow values for the two olfactometers. For instance, combination d : 1 has the data for dimethyl benzyl carbinyl acetate in columns 2 to 5 and Linalool in colums 6 to 9.

TABLE 7. PERCENTAGE AIR FLOWS

Multiple odorant channels

Combinations	Total flow (ml/min)	Percent						Total flow (ml/min)	Percent	
		By-pass line	Dilution line	Camphor	Floral	Musk	Mint		By-pass line	Sandalwood
*A*2 : s	1980	8·1	62·4	4	4	18·7	2·8	1480	65	35
*A*3 : s	1085	0·0	56·2	18·9	2·3	21·7	0·9	1480	44·6	55·4
*A*4 : s	1660	0·0	59·6	15·6	0·9	22·4	1·5	1480	39·1	60·9

The paired odorants were required to be of a subjective strength that was somewhat above the recognition threshold of all subjects. This procedure allowed latitude for each member of a pair, so additional adjustments were made to equalize the strengths for the group. The subexperiments on blending had more complex requirements. The overall strength was determined by the blend as it was weaker than the sandalwood oil. This was diluted to equality with the weaker unknown. The flows through the sparging vessels to the mixing vessel were dictated by those rates that were found by trial to produce a balanced, overall quality.

It developed that all of the flowrates through the sparging vessels were within a range that yielded the same unit of vapor concentration for any given chemical. Thus, the concentration was stable for the chemicals, but the actual quantities delivered to the mixing vessels were unequal. This was due to the different rates of flow during a given mixing operation.

The vapor sampler and G–L chromatograph measured the homogeneity of reagents and intensity of the stimuli. In respect to homogeneity, the chromatograms had only one spike for each reagent. This indicated that the mineral oil diluent and defoaming agent (200 Fluid) did not adulterate certain of the experimental vapors. In respect to the intensity, Table 8

TABLE 8. STIMULUS INTENSITY

Reagent	Column temp. °C	Lead (μg)	Retention time/min (t_r)	10^8 molecules per mm^3
ca	125	3·2	4·50	121·00*
ph	150	2·3	3·30	1·92*
l	125	1·9	2·80	27·80*
d	135	2·5	3·65	24·80†
t	185	3·7	8·20	1·72*
e	150	4·5	2·45	3·31*
m	100	5·2	3·15	18·80*
pi	100	4·8	4·40	16·50‡

* Analysis completed.
† Analysis incomplete; estimate based upon 2 to 4 chromatograms.
‡ Analysis incomplete; estimate based upon interpolation from completed work on similar odorants.

presents the chromatographic operating conditions and vapor concentrations in molecules of odorant per cubic millimeter of air. These values apply to the subexperiments with pure compounds as well as the blends.

The second, third and fourth columns state the operating conditions of the G–L chromatograph. The fifth column states the intensities. These apply to the various flowrates used for any given compound as they did not alter the concentrations.

The real differences between the reagents are shown by the intensities because they were obtained after the air passed through the liquid phases. Nevertheless, the synthetic camphor was in a saturated solution while the *d*, *l*-menthone was a 4 per cent solution. This comparison shows to what extent the strong odors had to be diluted prior to sparging in order to permit a gentle total flowrate. Therefore, synthetic camphor had to be the most concentrated in the combinations where it was used, while the three peppermints had to be rather diluted. They were of somewhat different intensities in the blends. Two of the 3 floral odorants were intermediate in intensity, but ph was low and the musk was least concentrated.

The adamantan is not reported because an effective stationary phase for the packed column is being sought. This quantification will be published as soon as possible. The floral smelling reagents will soon be completed, while the work on Piperitone is getting started. Breakdowns in the chromatograph's detector due to a short circuit in the heater at the higher temperature required for the musk (*t*) have delayed this work. In order to round out the report, estimates of the incompletely analyzed reagents are shown.

In Table 9 the method of relating stimulus intensities to flowrate of air is shown. Since the work thus far permitted an estimation of the propor-

TABLE 9. PROPORTIONS OF CONSTITUENTS IN VAPOR PHASE

Reagent	Sparging* flowrate	Conc. to† mixer (w/w)	Total† flowrate	Blended† conc.
ca	80	26·8	1980	1·08
t	370	0·625	1980	0·117
e	55	1·62	1980	0·045
ph	80	0·297	1980	0·012
m	10	4·06	1085	0·365

* ml/min.
† ppm.

tions of the constituents of blend *A*2 only, the second column states the rates through sparging vessels for that subexperiment. Included, also, is the completed analysis of *d*, *l*-menthone even though it was a constituent of blend *A*3. Each vessel was placed in 2 to 3 channels in order to ascertain if they affected the flowrate. No difference between channels was found. The third column reflects the intensities of Table 8, column 5. The fifth column states the original values of the amounts of odorants that were presented to the subjects' external nares after they were homogenized in the mixing vessel. The values for Linalool and dimethylbenzylcarbinyl acetate will be obtained when the last three peak-areas have been measured on the chromatograms.

On the basis of the concentrations in column 5, it can be calculated that 1·254 ppm in air is the total mixture. Inspection of the constituent values shows that synthetic camphor was the largest fraction, tetralin musk next in quantity, 1,1,2,2,-tetrabromoethane third, and phenylethylmethylethyl carbinol the smallest fraction. Reference to Table 7 shows a contrast to these measured proportions. Note that the camphor and carbinol compound have equal percentage flowrates in blend *A*2 (left-hand part of *A*2 : s) while the ethane compound has the least flow and the musk has the largest one. These percentages do not hold true for blends *A*3 and *A*4. Consequently, it will be interesting to determine their relationship with the measured concentrations. The dilution with air is about the same percentage in all cases. This result shows the large quantity of air introduced into the olfactometers in order to achieve an overall odor that was not dominated by any of the constituents.

DISCUSSION

The results of blending odorants drawn from certain subjective classes of organoleptic quality prove that one formulation was similar to the sandalwood odor. It was shown, also, that the constituent reagents were somewhat dissimilar to each other. It follows that not all of them could be similar to the sandalwood in quality. It seems likely that when the last phase of the project is completed, both theoretical requirements will have been satisfied so that the prediction will be upheld. That one may regard a match of *very slightly similar* as a positive result is best understood from the following considerations. The scores of people with varied sensitivities to the reagents tended to cancel each other algebraically thereby bringing the group's mean judgments closer to the neutral point and so "blunt" the composite perception of similarity. It was shown that when the subjects

were divided into two performance categories, prior to the analysis of their means, the less heterogeneous Subgroup *A* gave sharper discrimination of similarities and dissimilarities within pairs. Another consideration is that the prediction of the composition of woodiness depended upon old qualitative descriptions of allegedly pure compounds that smelled like cedarwood. Inasmuch as it was proved that the sandalwood and cedarwood qualities approximated *very similar*, in the olfactometry experiment, the pragmatic substitution of the former as the experimental model does not detract from the test. Had the blend been a close match with sandalwood a strong case would have resulted for the prediction!

When we consider that the odors of both natural isolates are probably caused by an interaction of at least 15 chemicals, the pure compounds that smelled like cedarwood were an approximation of the finely blended natural odors. This approximation made it probable that the blend would be a simplified copy of the complex model. And a final consideration is related to the camphoraceous and floral categories. Even though the rigid receptor site for camphoraceous molecules is untenable, the original statistical survey of odorant chemicals provided a useful prediction from the first principles. In fact, it is the subjective floral class that seems to have been the weakest factor for the deduction of woodiness. Although this problem needs to be investigated by means of the matching-standards method of odor-characterization, our qualitative knowledge of the rather different floral odorants indicates the existence of three or more important subjective categories.

The experiment shows that 4 "primary" odors do not interact to yield a close match with sandalwood (e.g. $\geqq 6{\cdot}3$). More than 4 contrasting odorants would be needed, perhaps many more than 4, in order to achieve a rational satisfactory degree of similarity. Therefore, retraction of the concept of 7 primary odors is substantiated.

It was shown that the deduction failed to account for the organoleptic qualities of "oiliness" and "resinous" that are weak characteristics of sandalwood odor. (These qualities are stronger in the cedarwood.) On the basis of the present result, it is predicted that blending pure odorants to match a resinous woody quality requires consideration of this component. Otherwise, a good match is unlikely.

Evidence from electro-olfactograms of small vertebrates suggests that every receptor neuron has many sensor units or ultramicroscopic sites. The neurons differ in respect to the character of the sites based on maximum sensitivities to specific odorants. Consequently, it is problematical whether there are pure odorivectors that stimulate one type of sensor unit. If this

is so, and human judgments of quality are the interaction of four or more dimensions, new experimental techniques are needed to resolve the nature of the resinous stimulus. Neurophysiologists have yet to produce this kind of odor analysis at the primary level of the neural pathways. Therefore, odor characterization by measurement of human responses to a large array of standards (20–50) offers promise of indicating a resolution of the problem concerning the allegedly "pure" odorivectors.

A second major result of this experimentation was the success of reference standards to define 5 of the odor distances in the rating scale. The representative sample of humans was reasonably consistent in respect to ranges of judgments. This conclusion is supported by the degree of concordance shown for all cases where an expectancy was determined by previous studies. The tendency of the entire group to distribute its judgments on both sides of the neutral point for nonidentical pairs accounts for some of the means in the circumneutral zone of the rating scale ($4{\cdot}0 \pm 1$). Were it possible for humans to make finer distinctions, there would be less overlapping. Another cause was the selection of compounds for the study of woodiness. Woody compounds, natural isolates, and certain of the former "primary odor standards" happened to include more *slightly dissimilar* pairs than *very* and *extremely dissimilar* ones. As to the relative scarcity of *very* and *extremely similar* nonidenticals, the result upholds what seems to have been the general opinion of odor specialists. They indicated that nonidentical pairs of greater than *moderate similarity* are scarce!

These considerations of measurable odor distance should have a bearing upon the concept of *odor space*. Whether psychophysical space conforms to the three-attribute impression at the primary level[12] or needs more than three attributes will become interesting as subjective measurements are produced. The problem would be aided by determination of how the rating scale is used. Since all subjects appear to have their greatest difficulty judging odorants which are *slightly similar* and *dissimilar*, a test of linearity of a large sample of judgments is needed. Certain authors have stated that humans judge odor quality in essentially the same way, but the subjects in Subgroup A' could not perceive the identities of some of the pairs reported above. This finding raises the question of what is a representative sample?

The goals of experiments on olfactory cross adaptation and partial anosmia can be subserved by comprehensive odor characterization. The last two subjects are allied because a major investigation of partial anosmia could utilize matching with many slightly different smells in order to decide whether very discrete partial anosmia exists. Presumably, such a defect

would leave the rest of the olfactory organ receptive to other odorant qualities. Should it develop that partial anosmia always involves more than one discrete sensor unit, an indication of the number of primaries would be produced. However, measurement of odor distances by means of the olfactometric method could establish the number of functional subjective categories within each of Amoore's general odor classes. Although this technique will not prove the existence of a given number of primaries, it will improve the usefulness of the general classes. The putrid and floral classes may need major reclassification due to several important groupings. The synthetic musks may go into two or three discrete categories that are very close together in the odor space. On the other hand, if there is a continuum of specificities, the gradual change of qualities would be clearly demonstrated.

It is clear that something caused the significant, smaller differences of certain paired unknowns. By screening naive subjects for a minimum sensitivity to a gamut of odors, a less heterogeneous and more representative sample could be studied. If it were possible to achieve agreement among investigators on the minimum sensitivity and the selection of test odorants, human samples representative of physiologically adequate types could be recruited at independent laboratories. By this technique, defective volunteers would be eliminated. Olfactory defectives are analogous to the partially color blind and their presence would reduce the precision by making the sample heterogeneous.

The characterization of a simple blend as a nonresinous woody odor by means of olfactometry and G–L chromatography has shown the way to the elusive correlation of the physical parameter, odorant intensity, and the psychological dimension, subjective (odorant) strength. When the blend of adamantan, Linalool$^{(R)}$, tetralin musk, and *d*, *l*-menthone has been so quantified, an approximation of sandalwood odor will be added to this neglected aspect of olfaction. It may be said that the overall effect of the blends reduced the inherently strong odorants to the mildness of the camphor or musk. Therefore, the values in parts per million of the constituents are measurements of the actual strengths of these odors.

Evidence is accruing that the prediction of the resinous, woody quality from the first principles of the Stereochemical Theory of Olfaction is not without cogency. By learning how to apply the knowledge of odor intensity to the new shadow-matching technique of comparing the size and shape of odorant molecules, it should become possible to formulate complex quality with much greater precision. Then this information could be utilized to investigate the relationship between the multidimensionality of odors

and the hypothetical sensor units or sites on the receptor membrane. Such an advance could lead to an insight into the nature of the stimulus after physical adsorption to the receptor membrane.

SUMMARY

1. The concept of 7 rigid, primary olfactory receptor sites was tested by means of extensive blending experiments. A prediction that the woody organoleptic quality, exemplified by the odors of cedarwood and sandalwood oils, is a mixture of certain camphoraceous, floral, musky and peppermint compounds was found to be only partially true.

2. A rating scale was used to measure the human judgments of similarity and dissimilarity which had 5 alternate points of the 9 "anchored" by means of experimentally determined reference standards. The scale measured the "distance" between odor qualities from extremely similar to extremely dissimilar. Analyses of the performances of 23 subjects showed this method to minimize variation of judgments within pairs of odorants. The double unknown method was used with coded odors.

3. Two 6-channel olfactometers, a new method of sampling the olfactometric vapors, and a G–L chromatograph were used to quantify the stimuli in terms of the number of molecules per cubic millimeter of air. By applying these measurements to the differential flows through the liquid phases of the constituent odorants, a true determination of the proportions in the blends was achieved. This is a direct method of studying the relationships between stimulus-intensity and subjective odorant strength.

4. The degree of similarity achieved between an experimental blend and the natural model indicates that the deduction from the first principles has cogency. The blend possessed a nonresinous, nonoily woodiness. As the constituent odorants had different qualities, it is already clear that most of them, if not all, will be proved dissimilar to sandalwood odor.

5. It should be possible to improve the prediction of complex odor quality by applying the results of quantified constituent strengths to Amoore's new "shadow-matching" method of relating the size and shape of molecules to the characterization of their odors.

ACKNOWLEDGMENTS

It is a pleasure to acknowledge the technical assistance of Armando Sandoval in the design of the vapor sampler. He and Marshall Andrews perfected the technique of sampling the olfactometric effluents and Mr. Andrews performed the chromatographic analyses. Albert B. Parks helped to select the non-parametric statistical methods.

REFERENCES

1. AMOORE, J. E., 1952. The stereochemical specificities of human olfactory receptors. *Perf. & Essen. Oil Record*, **43**, 321.
2. AMOORE, J. E. 1965. Psychophysics of odor. *Cold Spring Harbor Symposium* XXX. Sensory Receptors. In press.
3. AMOORE, J. E., JOHNSTON, J. W., JR., and RUBIN, M., 1964. The stereochemical theory of odor. *Sci. Amer.*, **210**, 42–49.
4. AMOORE, J. E. and VENSTROM, D., 1965. Personal communication, Feb. 1965.
5. DAVIES, J. T., 1965. A theory of the quality of odors. *J. Theoret. Biol.*, **8**, 1–7.
6. DRAVNIEKS, A., 1965. Current status of odor theories. Flavor Symposium, Am. Chem. Soc. April. In press.
7. ENGEN, T., 1962. The psychophysical similarity of the odors of aliphatic alcohols, Psychol. Lab., Univ. of Stockholm, Rept. No. 127.
8. HALDANE, J. B. S., 1939. The mean and variance of Chi Square when used as a test of homogeneity when expectations are small. *Biometrika*, **31**, 346–355.
9. JOHNSTON, J. W., JR., 1963. An application of the steric odor theory. *Georgetown Med. Bul.*, **17**, 40–42
10. JOHNSTON, J. W., JR., 1965. Experiments on the specificities of human olfaction. *Surface Effects in Detection:* Ed. by J. I. Bregman & A. Dravnieks: Washington, D. C. Spartan Books, Inc.
11. JOHNSTON, J. W., JR., and SANDOVAL, A., 1962. The stereochemical theory of olfaction. 4. The validity of muskiness as a primary odor. *Proc. Sci. Sec.*, Toilet Goods Assn., No. 37, suppl., 34–45.
12. LETTVIN, J. Y., and GESTELAND, R. C., 1965. A summary of a theory for olfactory receptor action. *Cold Spring Harbor Symposium* XXX. Sensory Receptors. In press.
13. MAINLAND, D., 1963. *Elementary medical statistics.* Philadelphia, W. B. Saunders Co.
14. MOULTON, D. G., and TUCKER, D., 1964. Electrophysiology of the olfactory system. *Ann. N.Y. Acad. Sci.*, **116**, 380–428.
15. OSGOOD, C. E., SUCI, G. J., and TANNENBAUM, P. H., 1957. *The measurement of meaning.* Urbana, Univ. of Ill. Press.
16. SCHUTZ, H. G., 1964. A matching-standards method for characterizing odor qualities. *Ann. N.Y. Acad. Sci.*, **116**, 517–526.
17. SIEGEL, S., 1956. *Non-parametric statistics for the behavioral sciences.* New York, McGraw-Hill Co.
18. WORTHING, A. G., and GEFFNER, J., 1943. *Treatment of experimental data.* New York, John Wiley & Sons, Inc.

THE FREQUENCY COMPONENT HYPOTHESIS IN RELATION TO THE CODING MECHANISM IN THE OLFACTORY BULB*

JOHN R. HUGHES and D. EUGENE HENDRIX

Division of Neurophysiology and EEG
Department of Neurology and Psychiatry
Northwestern University Medical School
Chicago, Illinois

INTRODUCTION

One of the most important questions facing the neurophysiologist is to determine the way in which information is transmitted within the central nervous system. This question of the "coding" used by the CNS is important in the field of olfaction as it is in all other sensory modalities. Our goal is to determine the language used by the olfactory bulb to inform the higher centers of the nature of the olfactory environment. Our hypothesis is that the coding found within the bulb essentially consists of the admixture of various frequency components; furthermore, we are utilizing our hypothesis to investigate neurophysiologically the Amoore Stereochemical Theory of Olfaction, which maintains that the sense of smell is based mainly in the geometry of molecules in the stimulus (Amoore, 1963). The data in this paper supply the evidence for the "Frequency-Component Hypothesis" and also provide the means for a neurophysiological investigation of the Stereochemical Theory.

TECHNIQUE AND APPARATUS

The technique in this study involves the chronic implantation within the olfactory bulbs of stainless-steel macroelectrodes (Type 316), insulated except for an exposed one mm. tip, and the recording of responses to odoriferous stimuli in 5 unanesthetized, conscious white rabbits. At times, the stimuli have been presented by the simple, direct technique of placing a piece of gauze soaked by a given amount of stimulus at a given distance

* This study was supported by grants No. NB-04775 and 05377 from USPHS (NINDB)

from the nose of the animal. Also, the stimuli have been presented via nasal catheters with the use of Heidbrink flowmeters to measure the flow of an odorless gas (O_2 or N_2) as it carried the odoriferous stimulus to the olfactory epithelium. Recently, we have utilized flowmeters to present a flow-rate of O_2 (400 cc/min), through an odorless plastic tube, the end of which was held 4 in. from the nose of the experimental animal. The gas was passed over a piece of gauze soaked with 10 drops of the liquid stimulus held 2 in. from the nose. A second stimulus was not presented until time (up to 1 hour) was taken to clear the experimental room of any existing odor from the previous stimulus. Thus, different stimuli were independently presented under stable physiological conditions with a given flow rate of odorless gas passing over a given amount of odoriferous substance.

Responses from the implanted electrodes, usually 4 in a given animal, have been recorded on the electroencephalographic machine (Model III D, Grass Inst. Co.), but for storage and analysis these responses have also been recorded on a 4-channel FM Mnemotron tape system (Type 204) at the speed of $1\frac{7}{8}$ in. per second.

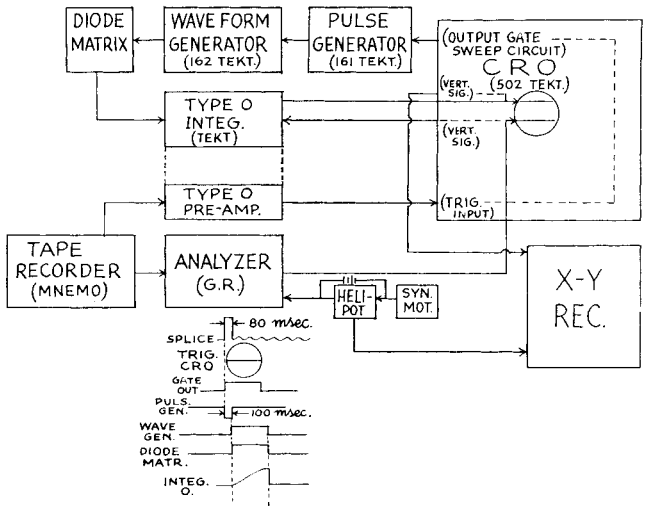


FIG. 1. Apparatus for analysis of olfactory bulb responses recorded on magnetic tape. The responses are analyzed by a frequency analyzer, integrated by operational amplifiers, with an automated write-out by an *X–Y* recorder. See text for further details.

Figure 1 shows the system of analysis of these responses, which tend to be rhythmical in character. Tapes with 30 sec of responses are looped for continuous playback at a speed of 15 in. per second or 8 times the re-

cording speed in order to increase the accuracy of the analysis. The responses are analyzed by a Heterodyne Wave Analyzer (Type 1900 A, General Radio Co.) whose frequency is precisely determined by a Digital Frequency Meter (Type 1150 A, General Radio Co.). For amplitude determinations responses are integrated by Operational Amplifiers (Type 0, Tektronix Co.) whose output is viewed on one channel of a dual beam oscilloscope (Type 502, Tektronix Co.). Some of the data presented in this

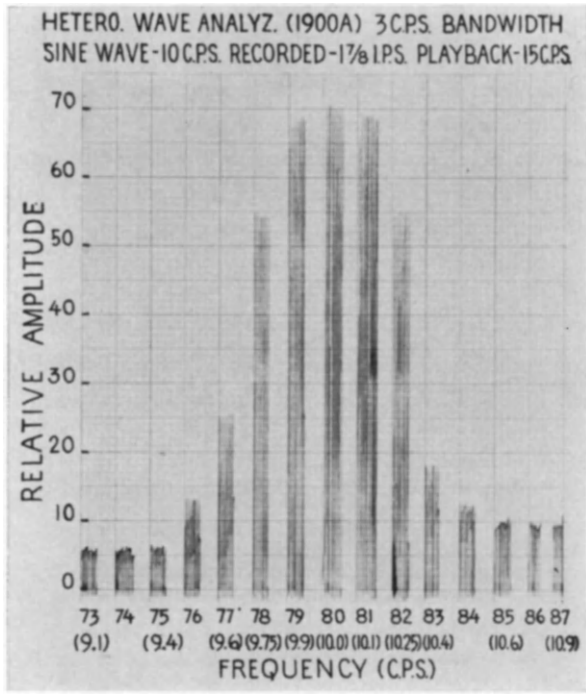


FIG. 2. Example of accuracy of Heterodyne Wave Analyzer. A 10/sec sine wave was recorded at $1\frac{7}{8}$ in. per second and played back at the speed of 15 in. per second, resulting in a 80/sec waveform. The write-out at frequencies between 73–87/sec (9·1–10·9/sec) is shown with conversion to original frequencies appearing in parentheses. See text for further details.

paper have been gathered with the use of readings from the oscilloscope, but within the past few years the technique of analysis has become automated. The output from the Operational Amplifiers is now recorded on the *Y*-axis of an *X*–*Y* recorder (Type 2D–2A, Moseley Co.). The *X*-axis is a measure of frequency and is driven by the output from a Helipot. The center arm of the Helipot drives the frequency dial of the analyzer and is driv-

en by a synchronous motor, which revolves very slowly at 1 revolution per hour. The X–Y recorder therefore writes out an extremely accurate frequency spectrum in which each pen deflection represents a $\triangle$ frequency of approximately 1/10 cycle per second. Since an important factor in the use of operational amplifiers is the time over which the responses are integrated, the technique that has been developed for the precise timing of integration involves the use of pulse generators, waveform generators and diode matrices for the precise initiation and termination of integration (note bottom of Fig. 1).

Figure 2 represents an example of the accuracy with which the Heterodyne Wave Analyzer with a 3-cycle bandwidth analyzes a 10-cycle waveform. Differentiation can be made within 1 cycle of 80/sec or within approximately 0·1 cycle after the conversion back to the "original-frequency" at 10/sec. At 0·25 cycles away from 10/sec the relative amplitude is down to 77 per cent of that seen at the center frequency.

RESULTS AND DISCUSSION

The type of rhythmical responses recorded from the rabbit's olfactory bulb is seen in Fig. 3. Clear differences can be seen between the responses

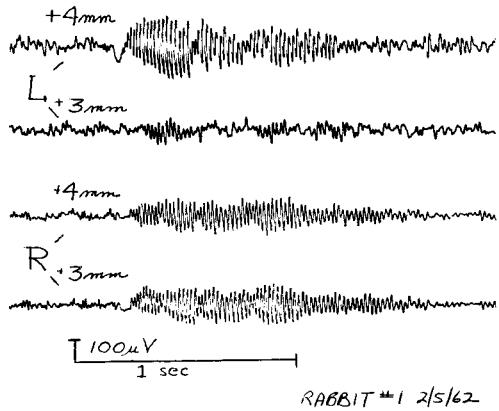


FIG. 3. Unanalyzed responses to cigarette smoke from the olfactory bulb of the rabbit. Channels 1 and 2 are recordings from left bulb at a depth of 4 mm and 3 mm (respectively); channels 3 and 4 are recordings from the right bulb at a depth of 4 mm and 3 mm (respectively).

from electrodes separated by only 1 mm. in distance, as seen in the first 2 channels. Other unanalyzed responses, like those seen in the last 2 channels from the right bulb, may appear similar to each other, but frequency

analysis reveals important differences between them and also between those from the left and from the right bulb.

In the frequency analysis of responses from the olfactory bulb, prominent activity is usually seen at the lower frequencies. Figure 4 shows a typical analysis of the lower frequency components (plotted from readings from the cathode-ray oscilloscope). Usually one large peak of activity is

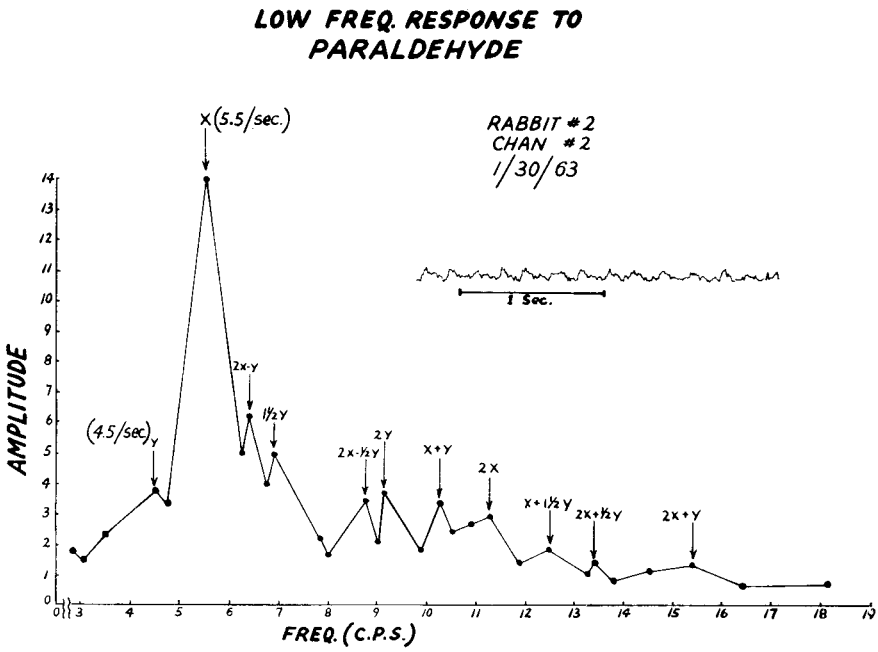


Fig. 4. Low frequency (analyzed) response to paraldehyde. Abscissa: frequency in cycles per second; ordinate: relative amplitude determined by readings from the oscilloscope. Insert is an example of the unanalyzed record showing rhythms at approximately 4·5/sec and also 5·5/sec, as the main sniffing rates, marked by the arrows, *X* and *Y*, in the analysis. The smaller peaks marked by arrows can be explained by assuming that the two main frequency components (*X* and *Y*) combine in various harmonics and difference-frequencies.

seen and frequently a nearby smaller peak is also noted; the large peak likely is related to respiration and represents the main sniffing rate of the animal and the smaller peak seems to represent a secondary sniffing rate (see insert of Fig. 4). This activity at these lower frequencies is similar to the slow potential of Ottoson (1959, 1963) and can be viewed as a modulated DC-type of response, which seems to follow the respiration rate. Figure 4 shows other smaller peaks between 6–16/sec. These peaks can

usually be explained by the admixture of various amounts of the 2 frequency components representing the 2 main sniffing rates. These smaller peaks are equivalent to the harmonics, difference and summation-tones that exist when 2 different pure tones are presented to the ear; in particular, this portion of the analyzed response from the olfactory bulb can be considered the olfactory analog of the known auditory phenomenon in which many different combination tones are found to be represented in the cochlear response of an animal presented with 2 different fundamental tones (Stevens and Davis, 1938). Since the peaks under approximately 20/sec in the analyzed response appear to be related to respiratory rates, these frequency components will now be disregarded in the further discussion, which will deal with the rhythmical activity of higher frequencies as the response of the bulb to a given odor.

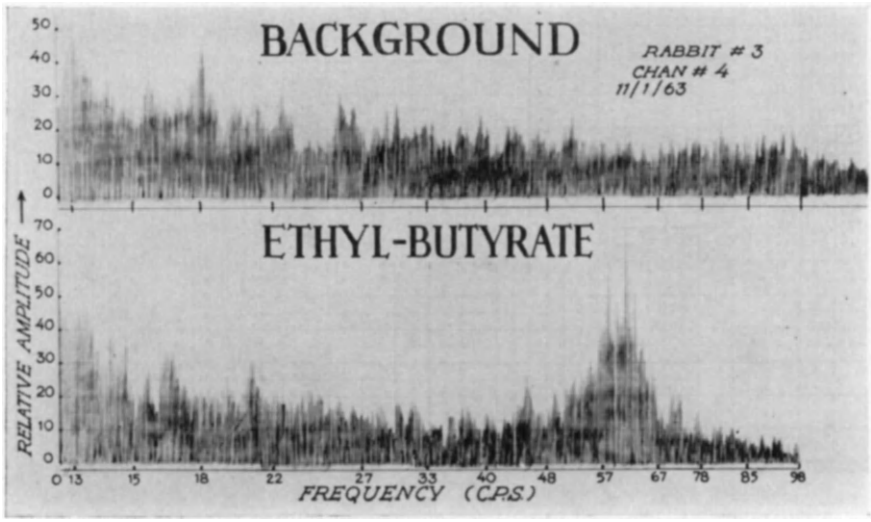


Fig. 5. Contrast between the analysis of background activity in the absence of any odor (top) and the response to an odor, ethyl-butyrate (bottom). Abscissa: frequency in cycles per second; ordinate: relative amplitude. Note the high amplitude peaks between 48–67/sec as the response to the odor and the absence of similar activity from the background profile.

The background activity of the olfactory bulb in an odorless environment consists of desynchronized activity of low amplitude without rhythmical bursts (Hughes and Mazurowski, 1962*a*). A frequency analysis of this type of activity is seen in Fig. 5, showing the absence of any prominent peak frequency over 20/sec. The latter profile contrasts with the ana-

lyzed response to an odor, ethyl-butyrate, which produces rhythmical bursts (similar to those seen in Fig. 3), appearing in the frequency analysis as the many peaks of activity between 48–67/sec. The peak of highest amplitude in the analyzed response, seen here as 61·5/sec, will be referred to in the remaining text as the *major* peak frequency; other peaks of lower amplitude will be called *minor* peaks.

Frequency analysis reveals marked differences in the responses to various odors from the same electrode site in the olfactory bulb. One of the most striking differences is seen in the major peak frequencies which appear distinctive for each odor, as seen in Fig. 6. This figure also shows the re-

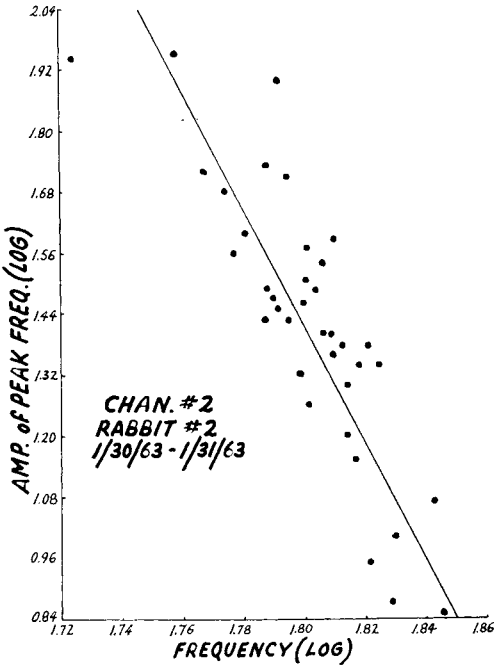


FIG. 6. Relationship between frequency and amplitude of the major (highest) peak in the analyzed responses to various odors. Abscissa: frequency (log) of major peak; ordinate: amplitude (log) of that peak. Each dot represents the response from one of the 37 different odors presented to Rabbit No. 2. Note the inverse relationship between frequency and amplitude of the major peak.

lationship between the frequency of the major peak and the amplitude of that component. On a log–log plot, an inverse relationship appears and permits a prediction that major peaks of relatively high frequency tend to be low in amplitude and major peaks of relatively low frequency tend to be high in amplitude.

In the search for other differences in the responses to different odors, the minor peaks of lower amplitude were studied, especially the peaks of highest and lowest frequency. Figure 7 shows that responses to different odors do not significantly vary according to the peak of highest frequency in the response, but vary with regard to the major peak and the peak of

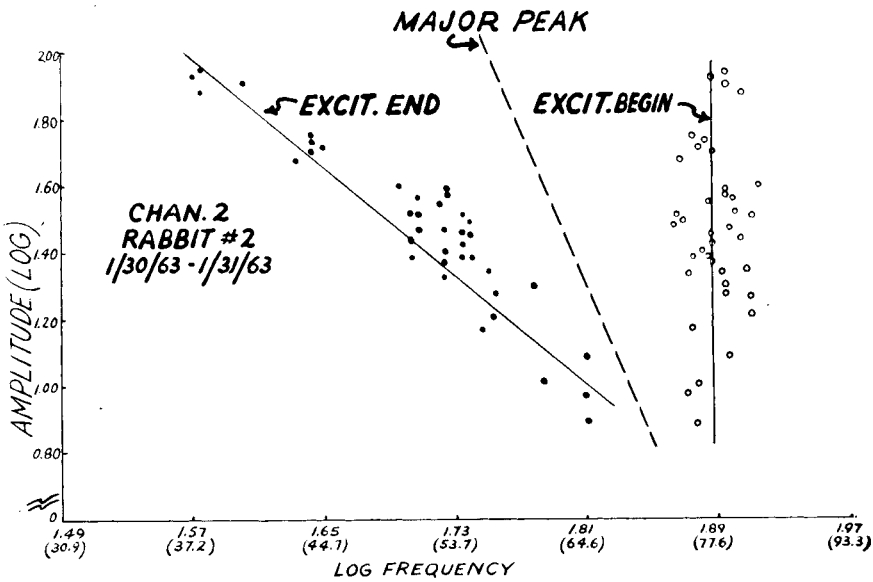


FIG. 7. Plot of components of highest and lowest frequency found in the responses to various odors. Abscissa: frequency (log) with actual cycles per second indicated in parentheses; ordinate: amplitude (log) of the frequency components. Each open circle represents the minor peak of highest frequency in the response to a given odor and each dot represents the minor peak of lowest frequency. The dotted line representing the major peaks (of highest amplitude) is the same line appearing in Fig. 6. Note that responses to different odors do not vary according to the peak of highest frequency, but do vary according to the major peaks and the peak of lowest frequency.

lowest frequency. Previous studies have shown that the unanalyzed response reveals the shortest period or highest frequency at the beginning of the burst-response (Hughes and Mazurowski, 1962*b*) and also, consistent with this finding, that the higher frequency components are maximal in amplitude at the beginning of the response with the lower frequencies peaking later in the burst (Hughes and Mazurowski, 1964). Since these components of high frequency appear at the beginning of each response and do not vary significantly according to the odor, our hypothesis is that they signal only the *presence* of an odor, as a general alerting mechanism.

Later in the burst, the major peak and the peaks of lower frequency, which do vary according to the odor, are maximal in amplitude; our hypothesis, is that these components signal the *identity* of the odor.

Other relationships to the major peak frequency have been investigated, especially the molecular weight of the odoriferous stimuli, as seen in Fig. 8.

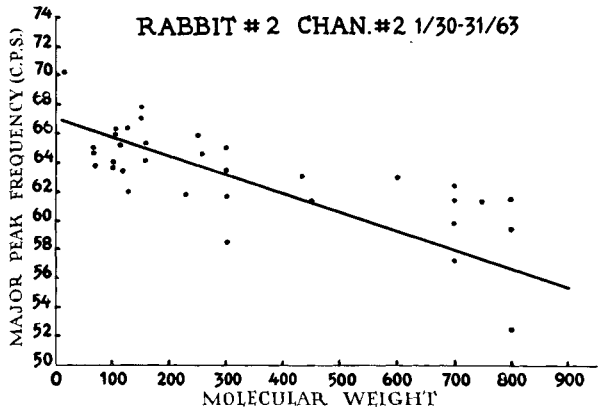


FIG. 8. Inverse relationship between the molecular weight of 34 different odoriferous substances (abscissa) and the major peak frequency (ordinate). The molecular weight of various smoke derivatives from condensation and fractionation of cigarette smoke were estimated as minimal values.

This figure shows an inverse relationship between these two variables and indicates that a tendency exists for compounds of low molecular weight to be associated with a relatively high major peak frequency and compounds of high molecular weight to be associated with a low peak frequency. The two figures, 6 and 8, are consistent with each other, since they show that heavy compounds tend to be associated with peaks of high amplitude and low frequency; the latter inverse relationship between amplitude and frequency is commonly noted throughout the nervous system and appears to be an important neurophysiological principle in macroelectrode recording of rhythmical activity, especially in clinical electroencephalography.

The frequency analysis of responses to various odoriferous stimuli have revealed both "excitatory" and "inhibitory" types of responses (Hughes and Mazurowski, 1962*b*). The term, "excitatory", is used to designate the increase in amplitude of activity above that seen in the background rhythm (in an odorless environment) and the term, "inhibitory", refers to the decrease of activity below the background level. Figure 9 shows that with

some stimuli both increased and decreased activity are noted but in different frequency ranges. A prominent excitatory response is seen between approximately 36–68/sec, while an "inhibitory" type of response is seen from 76–108/sec. As a contrast to this type of response from the same electrode (No. 1), but with another stimulus (Airkem), Fig. 10 fails to

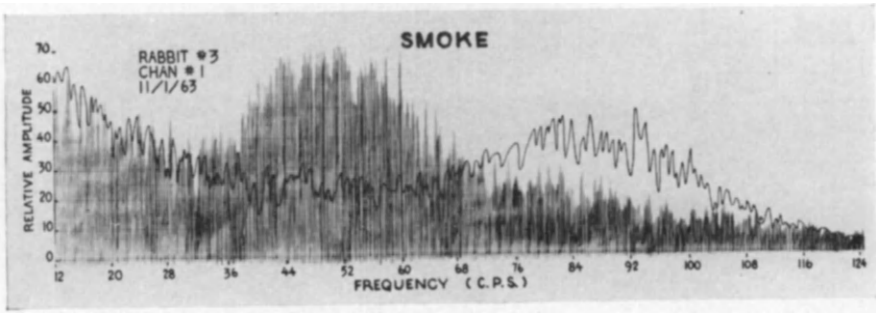


FIG. 9. Frequency analysis of the response to cigarette smoke (Chan. No. 1). Abscissa: frequency in cycles per second; ordinate: relative amplitude. Note the increased activity over the background level (solid black line) in the frequency range of 36–68/sec. Also, note the decreased activity between 76–108/sec.

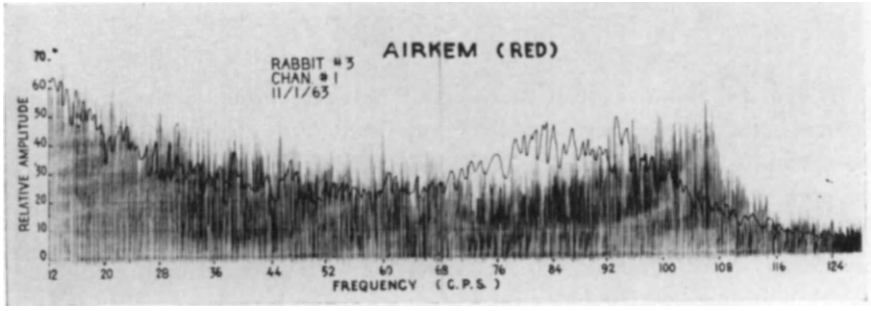


FIG. 10. Frequency analysis of the response of Airkem (Chan. No. 1). Abscissa: frequency in cycles per second; ordinate: relative amplitude. Note the increased activity over background activity (solid black line) between 98–114/sec and the decreased activity from frequencies just under that range. Also note a small peak at 60/sec, representing 60 cycle artifact that could often be identified and also used for the confirmation of the precise frequencies under analysis.

show any significant increased activity under 68/sec, but shows an excitatory type of response only at a higher frequency range (in this case between 98–114/sec). Often an "inhibitory" response is associated with increased activity and appears in a frequency range just below that showing

the excitation, as noted in Fig. 10. The latter 2 figures have presented contrasting types of response from the same electrode, but in response to different stimuli. Figures 10 and 11 show a similar contrast in response from different electrode sites, but with the same stimulus. Figure 11 indicates that the response to Airkem from Channel (electrode site) No. 4 shows an increased response at the lower frequency range, from 55–60/sec, and a decreased response at the higher frequency range, from approximately 76–108/sec. These last three figures (9–11) illustrate the points that both

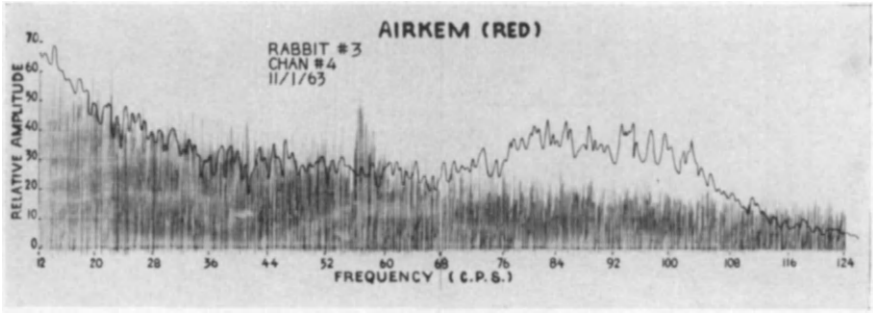


FIG. 11. Frequency analysis of the response to Airkem (Chan. No. 4). Abscissa: frequency in cycles per second; ordinate: relative amplitude. Note the increased response over background activity (solid black line) between 55–60/sec and the decreased response between 76–108/sec.

an increased response or a decreased response can appear from the olfactory bulb and that these responses can appear either within a low frequency range (37·5–75/sec) or within a high frequency range (75–125/sec.) All permutations and combinations of "excitatory" or "inhibitory" types of responses at the two different frequency ranges have been noted in the responses to many different odors from a given electrode. All such permutations and combinations have also been seen in the responses to a given odor from different electrode sites. The great variety of ways of responding which the many areas of the bulb have within their repertoire would seem to be consistent with the richness of information that the olfactory system presents to the higher centers so that fine and subtle olfactory discriminations can be made by the individual.

An attempt was made to relate the inhibitory type of response to other variables. Figure 12 shows various differences between the responses from compounds that showed inhibitory responses and those that did not show such responses. Excitatory responses that also included some decreased activity below the background level somewhere within the frequency range

of 30–125/sec were, on the average, 3 times more common than responses showing only excitation. Compared to responses only excitatory, those including inhibition tended to be associated with major peaks of *higher* amplitude and *higher* frequency (in the range of 37·5–75/sec) but between 75–125/sec inhibition was associated with major peaks of *lower* amplitude and *higher* frequency. Thus, inhibition tended to be associated with com-

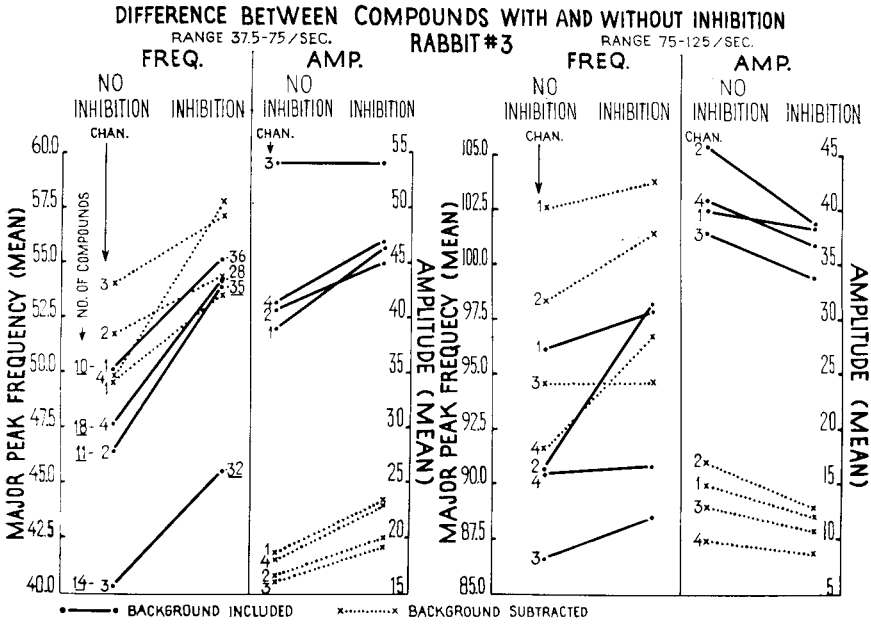


FIG. 12. Frequency and amplitude differences between compounds with and without inhibitory responses. Left half of the figure refers to data in the 37·5–75/sec frequency range; right half refers to the 75–125/sec range. Ordinate is major peak frequency (mean) on the left side of each half or amplitude (mean) on the right side of each half. Solid lines refer to data with background activity included; dotted lines to background activity subtracted. Note the comparison of values of responses with no inhibition to those with inhibition from all 4 channels (electrode sites). The slope of all 8 lines in the 4 different conditions shows that responses with inhibition are associated with higher peak frequencies in both frequency ranges, higher amplitudes in the 37·5–75/sec frequency range and lower amplitudes in the 75–125/sec range.

pounds which produce a prominent excitation within the main frequency range of response; the response of 13 of 40 compounds which included an especially *marked* inhibition (over 20 units) showed an average amplitude of excitatory responses within the highest quartile of a rank order of all

amplitudes. As further evidence that inhibition is usually associated with responses which also show marked excitation, the average number of peaks (another general measure of excitatory activity) for these 13 responses was within the highest third of a rank order with respect to this measure. However, these generalizations apply to the responses with excitation mainly in the 37·5–75/sec range, where the highest amplitudes were usually noted. On the other hand, for responses within the 75–125/sec range, inhibition was associated with lower amplitudes of excitation, mainly because within this frequency range the presence of the inhibitory type of response significantly overlapped or "cut into" the excitation. The *frequency* of the major peak was usually higher when associated with inhibition, probably because the inhibition usually appeared at a slightly lower frequency than the excitation and seemed to have the effect of "pushing" the major excitatory peak further out of the frequency range. Finally, electrode sites posterior and ventral within the bulb tended to show more inhibitory responses than other sites within the bulb.

The response to a given odoriferous stimulus varies significantly according to the electrode position within the olfactory bulb. Figure 13 (Rabbit No. 3) shows the different major peak frequencies associated with 44 different odors, arranged in rank order for electrode site No. 2.

The 44 compounds arranged from lowest to highest peak frequency were:

1. benzaldehyde
2. cherry oil
3. pinene
4. nutmeg oil
5. cinnamon oil
6. butyl alcohol
7. smoke deriv. (A_{12})
8. wintergreen oil
9. ethyl lactate
10. ethyl butyraldehyde
11. smoke deriv. (A_3)
12. sassafras oil
13. ammonia
14. benzyl alcohol
15. camphor
16. fenchone
17. cyclohexanol
18. terebene
19. ethanol
20. clove oil
21. paraldehyde
22. menthol
23. smoke (cigarette)
24. orange oil
25. juniper oil
26. eucalyptus oil
27. airkem
28. rose geranium oil
29. ethyl butyrate
30. methyl acetate
31. smoke deriv. (A_2)
32. smoke deriv. (A_{11})
33. lime oil
34. hemlock oil
35. octyl alcohol
36. cream sachet

37. amyl alcohol
38. rosemary oil
39. anise oil
40. ethyl bromo-iso-valerate
41. creosote beechwood
42. lemon oil
43. juniper tar
44. peppermint oil

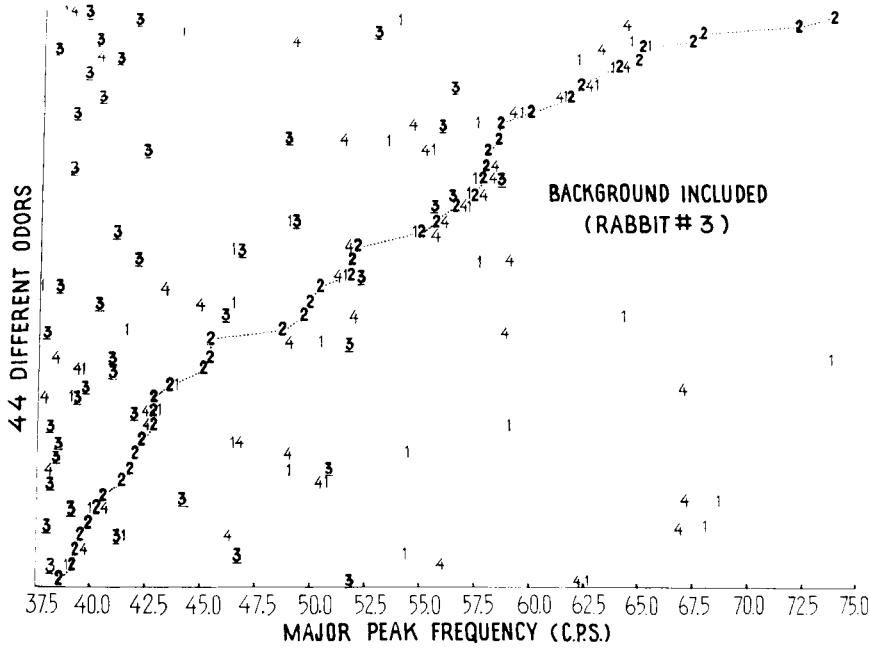


FIG. 13. Major peak frequencies of the responses from 44 different odors (background values included). Each of the 44 different rows represent the responses from one of the stimuli; these peak frequencies are represented by the position of the numbers 1, 2, 3 or 4 (electrode site) arranged along the abscissa. The different responses are arranged on the figure and connected by a dotted line according to the peak frequency seen on electrode site No. 2 from low (left) to high frequency (right). Note that values for electrode site No. 3 (underlined) tend to fall to the left side of all values, especially those representing electrode No. 2, indicating a relatively lower peak frequency for electrode site No. 3. Electrodes No. 1 (3 mm deep from dorsal surface) and No. 2 (2 mm deep) were 7 mm from the anterior tip of the bulb; electrode No. 3 (2 mm deep) and No. 4 (1 mm deep) were 6 mm from the anterior tip with all 4 electrodes 1 mm from the midline.

In Fig. 13 the scatter of No. 1, No. 3 and No. 4 from No. 2 in a given row represents the different peak frequencies for those different electrode sites in response to the same odor. In this figure, the values include the background rhythm and show that electrode site No. 3 tended to be distinctive in that the major peaks (37·5–75/sec range) recorded from this electrode

tended to be lower in frequency than the peaks from the other electrodes. Table 1 verifies this point and shows that the average (mean and median)

TABLE 1. COMPARISON OF AVERAGE MAJOR PEAK FREQUENCY AND AMPLITUDE IN 4 ELECTRODE SITES (37·5–75/sec) (BACKGROUND INCLUDED) RABBIT NO. 3, 44 STIMULI

	Freq.		Amp.	
	Mean	Median	Mean	Median
Elect. site No. 1	52·9/sec	54·4/sec	43·3	40
Elect. site No. 2	52·1	51·1	44·1	42
Elect. site No. 3	*43·9*	*41·1*	*53·8*	*54*
Elect. site No. 4	52·4	53·3	44·4	44

peak frequency was usually more than 10/sec lower than that from the other electrodes. Furthermore, electrode No. 3 recorded amplitudes usually more than 10 units above that from the other sites. As another way of representing the possible distinctiveness of electrode No. 3 when the background rhythm was included, the number of similar peak frequencies and also similar amplitudes were noted in comparing each electrode with the others. When electrode No. 3 was compared with the other electrode sites, less than 1/3 of the number of similar peaks were found than with the comparisons of any other two electrodes.

If the background rhythm is then subtracted from the responses, leaving the values representing the *changes* in the total activity when an odoriferous stimulus is presented, electrode site No. 3 lost its distinctiveness. Figure 14 shows a plot similar to Fig. 13, except that the values now represent only the *changes* above the background level; the values for No. 3 "fall into line" with the other values. Table 2 shows the average peak frequencies with their amplitudes and fails to show any distinctiveness for No. 3, after the background values have now been subtracted. Furthermore, electrode No. 3 no longer showed a significantly lower number of peaks, similar in peak frequency and amplitude, than those from the other electrode sites. Table 3 indicates that conclusions drawn from the responses in the 37·5–75/sec range need not apply to the responses noted in the range of 75–125/sec. This table shows that, regardless of whether the background values were included or subtracted, electrode No. 3 showed no distinctiveness either with regard to peak frequency or amplitude. A grouping is noted,

however, in that the average (mean) peak frequency tended to be similar in responses from electrodes Nos. 1 and 2 and also from Nos. 3 and 4, but the electrodes within each pair were only 1 mm away from each other. One important point in Tables 1–3 is that the two different frequency ran-

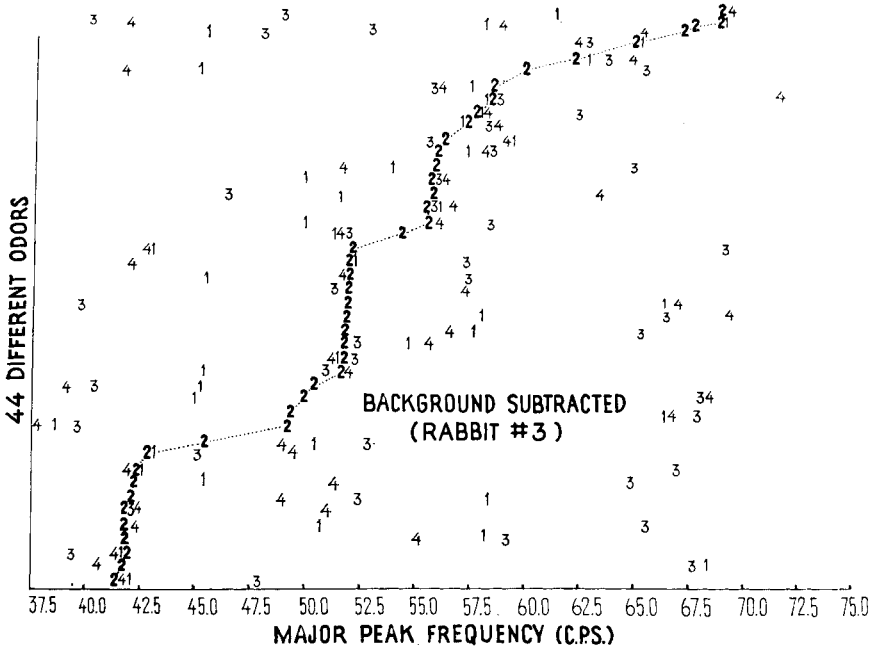


FIG. 14. Similar to Fig. 13, except that the values now have excluded the background activity. Note that the values from electrode site No. 3 show no clear tendency to be lower (or higher) than the other values.

TABLE 2. COMPARISON OF AVERAGE MAJOR PEAK FREQUENCY AND AMPLITUDE IN 4 ELECTRODE SITES (37·5–75/sec) (BACKGROUND SUBTRACTED) RABBIT NO. 3, 44 STIMULI

	Freq.		Amp.	
	Mean	Median	Mean	Median
Elect. site No. 1	53·0/sec	52·0/sec	22·1	20
Elect. site No. 2	54·3	53·4	19·3	16
Elect. site No. 3	55·9	55·9	18·4	18
Elect. site No. 4	54·0	55·4	20·6	20

ges, 37·5–75/sec and 75–125/sec, seem to represent independent ranges of response and not simply harmonically related modes of response which are dependent on each other.

TABLE 3. COMPARISON OF AVERAGE MAJOR PEAK FREQUENCY AND AMPLITUDE IN 4 ELECTRODE SITES RABBIT NO. 3 (75–125/sec)

	Background incl.		Background subtr.	
	Freq.	Amp.	Freq.	Amp.
Elect. site No. 1	97·7	39·2	102·8	13·5
Elect. site No. 2	95·8	40·5	100·6	14·3
Elect. site No. 3	87·9	35·2	95·3	11·9
Elect. site No. 4	90·7	38·0	94·5	9·8

The data from Figs. 13 and 14 and from Tables 1–2 provide evidence on one important question regarding the coding within the olfactory system, but also these data have reference to a question in general neurophysiology. This question relates to whether the significant information in the bulb regarding the nature of the olfactory environment includes or excludes the activity from the background level. In other words, does the bulb "look at" the total level of activity in the response or only the change in activity above background level that occurs when an odoriferous stimulus is presented. Conclusive evidence on this point will be difficult to gather, but presumptive evidence may be obtained in the form of stimulus–response relationships. In particular, the practical question can be asked as to whether more internally consistent stimulus–response relationships can be found in the activity that included or excluded the background level. According to the latter figures and tables, the responses from electrode site No. 3 in relation to the responses from the other electrodes have shown a special distinctiveness with regard to frequency, amplitude and similarity of peaks, when the background activity was included in the measurements. However, this electrode site lost all of its distinctiveness and became consistent with the activity from the other electrode sites when the background activity was subtracted. Two possibilities are, therefore, suggested: (1) electrode No. 3 truly lies in a distinctive area within the bulb and the areas represented by the other electrode sites are consistently unrepresentative of olfactory bulb activity, or (2) electrode No. 3 does not lie in an area that is any more distinctive than the other areas, but obeys the same rules

that apply to these other electrodes only after the background rhythm is subtracted from the total response. No data yet presented have suggested any special distinctiveness for electrode No. 3 and the remaining tables and figures will also provide evidence that the second possibility is likely correct. This emphasis on the *change* in the *response* from a background level is reminiscent of the early emphasis placed by Adrian (1947) on the *change* in the *stimulus* from a background level: "The sense organs, then, and the nerve cells to which they lead . . . are able to perform a process like differentiation in which absolute intensities become far less important than rates of change" (p. 85).

The previous tables and figures have indicated that from some electrode sites excitatory responses may be seen both in the frequency range of 37·5–75/sec and also between 75–125/sec. In the attempt to determine meaningful stimulus–response relationships we have investigated whether certain types of compounds show responses mainly within the lower frequency range, while other types of compounds show responses mainly at the higher frequencies. For a given electrode site (No. 1), the peak of highest amplitude was determined in the response to each stimulus and the responses were then divided into 2 categories depending on whether this peak was between 37·5–75/sec or 75–125/sec. Table 4 indicates that, among

TABLE 4. PEAK FREQUENCY OF HIGHEST AMPLITUDE (RABBIT NO. 3, ELECT. SITE NO. 1) (BACKGROUND SUBTRACTED)

Range	No. compounds	.... Mole. wt.		Amp.
		Mean	Median	Mean
37·5–75/sec	37	452·6(±240)	600	24·3
75–125/sec	6	118·0(±38)	125	20·0

37 different odoriferous compounds with a response mainly in the lower frequency range, the average (mean) molecular weight was over 400 (median = 600), in contrast to compounds whose major excitatory response was noted at the higher frequencies and whose average molecular weight was only 118 (median = 125). The mean amplitude was slightly higher for the compounds with the higher molecular weight, but the major point is that the main excitatory response for heavy compounds was under 75/sec and for light compounds was over 75/sec at this electrode location.

In the attempt to provide neurophysiological evidence for the Amoore Stereochemical Theory of Olfaction, compounds were placed into one of the 7 different categories, according to their major stereochemical configuration, as given by Amoore and his colleagues (Amoore, 1962*a*, Amoore, 1962*b*, Rubin *et al.*, 1962, Johnston and Sandoval, 1962, Saunders, 1962). Table 5 shows the different categories represented in the 2 ranges of res-

TABLE 5. ODOR CLASSIFICATIONS WITH MAJOR PEAK FREQUENCIES (RABBIT NO. 3, ELECT. SITE NO. 1)

Range	No. compounds	Abs. amp. Mean	Classifications
37·5–75/sec	24	49·9	18 mainly floral 3 Smoke compounds 2 mainly ethereal 1 mainly pepperminty
75–125/sec	19	45·3	8 mainly camphoraceous 4 mainly ethereal 2 mainly pepperminty 2 mainly pungent 2 Smoke compounds 1 mainly floral

ponse, in this case utilizing the amplitudes including the background rhythm (called absolute amplitude). This table shows that most of the odors with a main response under 75/sec were floral and over 75/sec many of the odors were categorized as camphoraceous, but with 4 other stereochemical categories also represented. However, all of the camphoraceous responses showed their highest amplitude in the high frequency range which included only one out of 19 floral responses. A certain degree of overlap in response categories would be expected, especially in view of an overlap in stimulus categories; i.e. most odors were not simply representative of one odor group alone, but were composed of more than one, although a given classification was assigned to each stimulus according to its most prominent characteristics. Smoke compounds derived from condensation of cigarette smoke with varying amounts of distillation, were usually grouped separately, since all stereochemical categories were likely represented within them. Further evidence that a relationship could be found between the stimulus and the response categories is seen in Table 6. This table shows the amplitudes (mean of highest values regardless of electrode site) from 8 camphoraceous,

7 ethereal and 18 floral compounds, within both the 37·5–75/sec and 75–125/sec ranges, both before and after subtraction of the background rhythm. Table 6 shows that, for both amplitude values, the highest means for the 37·5–75/sec range were found in the floral compounds, but for the 75–125/sec range were found in the camphoraceous compounds. The latter 2 tables are consistent with each other in that they show that the identification of a stimulus by its major stereochemical category helps to determine the main frequency range of response.

TABLE 6. AMPLITUDE FOR 3 DIFFERENT ODOR CATEGORIES (MEANS OF HIGHEST VALUES) RABBIT NO. 3

Categories	37·5–75/sec range Background		75–125/sec range Background	
	Incl.	Subtr.	Incl.	Subtr.
Camphoraceous (8)	55·3	21·1	*43·1*	*17·2*
Ethereal (7)	51·3	21·7	39·0	14·7
Floral (18)	*56·0*	*27·4*	40·9	15·2

After establishing a general specificity of response according to the classification of the stimulus, our next question was whether particular specificity existed according to the electrode site within the olfactory bulb. For the investigation of this problem, the measure of amplitude for each electrode site was a percentage, based on the highest amplitude (major peak) recorded from any one of the 4 electrodes in response to the given stimulus; these percentages were then averaged as a mean for 8 camphoraceous, 7 ethereal and 18 floral compounds. Table 7 shows the results within the

TABLE 7. MAJOR PEAKS: 37·5–75/sec-MEASURE OF AMPLITUDE FOR 3 DIFFERENT ODOR CATEGORIES IN 4 DIFFERENT ELECTRODE SITES (RABBIT NO. 3)

Categories	Amplitude (Mean % of max. for given resp.)							
	Background incl.				Background subtr.			
Elect. site	*1*	*2*	*3*	*4*	*1*	*2*	*3*	*4*
(C)amphoraceous (8)	76·4	80·0	96·8	*66·9**	87·5	78·5	73·9	*76·5*
(E)thereal (7)	80·6	84·0	98·6	*87·5*	85·9	79·6	83·0	*87·1†*
(F)loral (18)	85·1	83·7	96·4	83·9	*93·8‡*	84·2	*73·3‡*	85·2

*7 of 8 (C) responses-lowest amp. on Elect. Site 4; 1-next to lowest.
†4 of 7 (E) responses-highest amp. on Elect. Site 4.
‡10 of 18 (F) responses-highest amp. on Elect. Site 1; 11-lowest on Elect. Site 3.

37·5–75/sec frequency range and for values both including and excluding background activity. This table shows that the response to camphoraceous compounds was distinctively low in amplitude from electrode site No. 4. In particular, 7 of these 8 compounds had their lowest amplitude on this electrode and the eigth compound showed a response next to the lowest on this same site. With background activity included, the average amplitude of response from this electrode site was less than 2/3 of the maximal amplitude recorded from any electrode. This value (66 per cent) is relatively low, in view of the fact that, whenever a given area within the bulb responds to an odor, similar amplitudes are often seen throughout the bulb. No given electrode represented a high amplitude response to camphoraceous odors in this low frequency range. However, for ethereal odors, the same electrode No. 4 showed a distinctively high amplitude of response with 5 of the 7 responses appearing highest on that electrode. The 18 floral compounds showed a special high amplitude on electrode No. 1 with 10 of 18 responses highest on this site, which represented almost 94 per cent (background subtracted) of the highest amplitude from any location. This 94 per cent contrasts with 73 per cent from electrode No. 3, which recorded the lowest amplitude in 11 of the 18 responses. Thus, floral compounds were especially well represented on electrode No. 1, ethereal odors on No. 4, while camphoraceous and floral compounds were poorly represented on electrodes Nos. 4 and 3, respectively.

Interesting similarities and contrasts to the data in the lower frequency range are seen when the major peaks of 75–125/sec are considered. With the same type of analysis, Table 8 shows that electrode site No. 2 shows a high amplitude response for camphoraceous compounds with a 98 per cent

TABLE 8. MAJOR PEAKS: 75–125/sec-MEASURE OF AMPLITUDE FOR 3 DIFFERENT ODOR CATEGORIES IN 4 DIFFERENT ELECTRODE SITES (RABBIT NO. 3)

Categories	Amplitude (mean % of max. for given resp.)							
	Background incl.				Background subtr.			
Elect. site	*1*	*2*	*3*	*4*	*1*	*2*	*3*	*4*
(C)amphoraceous (8)	91·1	*97·9**	84·5	91·0	66·7	*88·8**	75·2	45·8†
(E)thereal	94·8	95·7	83·2	87·2	76·3	87·2	62·2	*42·8*‡
(F)loral (18)	86·5	88·9	87·3	85·2	*83·8*§	75·8	*67·5*§	72·2

*All except 2 (C) responses-highest amp. on Elect. Site 2.
†All except 3 (C) responses-lowest amp. on Elect. Site 4.
‡All except 1 (E) responses-lowest amp. on Elect. Site 4.
§All except 4 (F) responses-highest amp. on Elect. Site 1; lowest on Elect. Site 3.

representation of amplitude with background included and a 89 per cent with background activity excluded. The latter percentage contrasts with the 46 per cent value from electrode No. 4, which showed the lowest amplitude in all except 3 responses. Thus, for the camphoraceous compounds, no given electrode especially represented a prominent response in the lower frequency range, but electrode No. 2 showed a distinctively large amplitude in the higher frequency range. Electrode No. 4 recorded relatively small amplitudes in both frequency ranges.

Table 8 also shows that electrode site No. 4 poorly represented the ethereal odors. The low value of 43 per cent, with all except one ethereal response lowest on this electrode, contrasts with the data from Table 7 which shows the same electrode representing a high amplitude for the frequency range of 37·5–75/sec. Therefore, for some types of compounds (camphoraceous) a given electrode may show similar relative amplitudes for both the low and the high frequency ranges, but for other compounds (ethereal) opposite relationships may be seen in these two modes of response. Table 8 also shows that floral responses above 75/sec were well represented on electrode No. 1 and poorly represented on electrode No. 3, as in the case of the responses below 75/sec.

One other measure of the degree of activity recorded from a given electrode position is the number of peaks found in the response. This measure, as seen in Table 9, correlated well with the data from the previous table

TABLE 9. NO. OF PEAKS IN RANGE 75–125/sec (PERCENTAGE OF TOTAL IN GIVEN ELECTRODE SITE FOR GIVEN ODOR CATEGORY)

	Electrode site			
	1	2	3	4
Camphoraceous (8)	29·7	*41·8*	37·8	*25·2*
Ethereal (7)	27·8	33·5	37·0	24·2
Floral (18)	18·8	19·7	22·3	16·7

in that 42 per cent of all peaks from camphoraceous compounds were seen between 75–125/sec (58 per cent between 37·5–75/sec) on electrode No. 2, contrasted to 25 per cent on electrode No. 4. On the previous table electrode site No. 2 was shown to be associated with relatively high amplitudes for camphoraceous compounds and electrode No. 4 with low amplitudes. Table 9 also shows a relatively low percentage of peaks on electrode No. 4 for ethereal compounds, consistent with the low amplitude of response seen in the previous table. However, the measure of the number of peaks did not correlate well with the amplitude values for the floral compounds.

In the testing of the Frequency Component Hypothesis in relation to the essential coding mechanism of the bulb and in the testing of the Stereochemical Theory of Olfaction by neurophysiological data, different aspects of the analyzed response have been related to various aspects of the stimulus. As an example of the type of analysis that lends support to both the Frequency Component and Stereochemical Theories, data on camphoraceous compounds will be presented, with juniper tar as one example of such a compound. Figure 15 shows the frequency analysis of the response

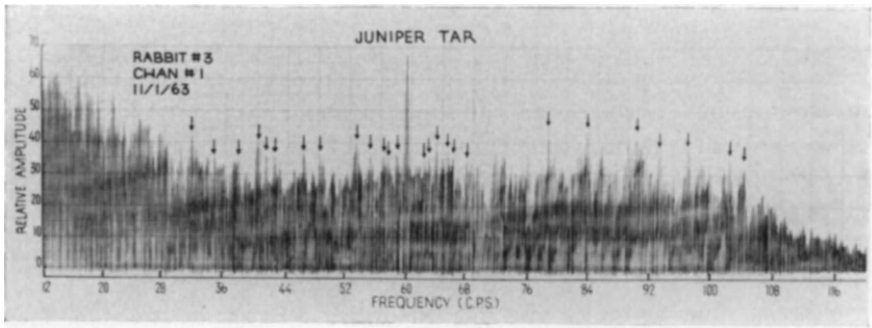


FIG. 15. Frequency analysis of the response to juniper tar. Abscissa: frequency in cycles per second; ordinate: relative amplitude. The arrows point to 26 distinct peaks with amplitudes more than 10 units above the background level; the prominent peak at 60/sec represents 60 cycle artifact.

(electrode site No. 1) to juniper tar, showing 26 different distinctive peaks. Table 10 shows that among these peaks the frequency components of

TABLE 10. JUNIPER TAR

Freq. components with amp > 10 units over background level:	26 peaks from 32·8 to 104·1/sec including			
Other camphoraceous compounds:	38·6/sec	41·6/sec	59·4/sec	66·5/sec
Camphor	×*	×		⊗
Fenchone		⊗	×	
Cyclohexanol	⊗*		⊗	⊗
Pinene		⊗	⊗	⊗
Benzyl alcohol	×	⊗	⊗	×
Benzaldehyde	⊗	⊗		⊗
Cherry oil		⊗	×	⊗

*Amp > 10 units over background.

38·6/sec, 41·6/sec, 59·4/sec and 66·5/sec are represented and also the extent to which these same frequency components are represented in the responses to the 7 other camphoraceous compounds. In all but one of these 7 compounds, the 41·6/sec and 66·5/sec components are seen, usually more than 10 units over the background level (noted circled *X*). The 38·6/sec component was seen in 4 responses and the 59·4/sec component was seen in 5 of the 7 responses. These data are presented as an example of one of the most prominent findings in our study; namely, many of the same frequency components found in the response to a given stimulus also are found in the response to compounds stereochemically related to that given stimulus.

An attempt was made to determine further the extent to which similarity in response could be correlated with similarity in the stimulus. As a measure of similarity of response, the number of peaks (more than 10 units above background level) with the same frequency ($\pm$ 0·1/sec) were counted for the 2 stimuli under comparison, juniper tar and each of the other 7 camphoraceous compounds. Table 11 shows the number of similar peaks on the

TABLE 11. COMPARISON OF JUNIPER TAR AND OTHER CAMPHORACEOUS COMPOUNDS (RABBIT NO. 3, ELECT. NO. 1)

Chemical data (most → least similar)	Physiol. data	No. similar peaks, amp > 10 over background
1. Cyclohexanol	1. Cyclohexanol	(8)
2. Fenchone	1. Fenchone	(8)
2. Pinene	1. Pinene	(8)
3. Camphor	2. Benzaldehyde	(7)
4. Benzaldehyde	3. Cherry oil	(6)
4. Cherry oil	3. Benzyl alcohol	(6)
4. Benzyl alcohol	4. Camphor	(4)

last column (right); the compounds are rank ordered from high to low with cyclohexanol, fenchone and pinene all showing 8 and both cherry oil and benzyl alcohol showing 6 similar peaks. A measure of the similarity of the stimuli to juniper tar was, of course, more difficult to assess. *Before* the analysis of responses, stimulus similarity was judged. Accordingly to known chemical analyses of juniper tar, cadinene, a bicyclical, unbridged 6-membered ring, is the major component, but camphene, pinene, juniper camphor and terpineol, an unbridged 6-membered ring, are also prominent components (Merck, 1960; Eliel, 1962). Cyclohexanol was judged most similar to juniper tar, because in contrast to the other compounds, it does

not have steric limitations imposed by a bridged molecular configuration. Fenchone and pinene were judged second because each is a 6-membered bridged compound and therefore with steric limitations. Camphor was considered third in the rank order since it is a 6-membered bridged ketone, but its steric configuration is relatively stable or invariant (see Amoore, 1962*a*, p. 2) and thus imposes more limitations or restrictions than fenchone or pinene. Benzaldehyde, cherry oil with its primary component as benzaldehyde, and benzyl alcohol were judged least similar because all three are 6-membered benzene rings with 3 double bonds, rather than 2, as found in juniper tar. Although Table 11 shows only very gross measures of similarity in stimulus and response, only one compound, camphor, appears out of order in the 2 columns. Therefore, a general tendency can be seen between similarity of stimulus from the stereochemical point of view and similarity of response from the frequency component point of view.

The next question was to determine if given frequency components were prominently represented in all 8 camphoraceous compounds for a given electrode site. Table 12 shows that for electrode No. 4 the frequency com-

TABLE 12. FREQUENCY COMPONENTS IN CAMPHORACEOUS COMPOUNDS ELECTRODE SITE NO. 4 (RABBIT NO. 3)

	38·6/sec	39·4/sec	41·6/sec	51·9/sec
Camphor	Highest (abs.)	—	×	Highest (rel.)
Benzaldehyde	—	—	×	Highest (rel.)
Fenchone	—	Highest (abs.)	Highest (rel.)	—
Pinene	—	Highest (abs.)	×	Highest (rel.)
Benzyl alcohol	×	×	Highest (rel.)	—
Cyclohexanol	Highest (abs.)	×	Highest (rel.)	⊗
Cherry	—	—	×	×
Juniper tar	×	—	⊗	Highest (rel.)

ponents of 38·6/sec, 39·4/sec, 41·6/sec and 51·9/sec showed an outstanding representation among these 8 compounds. The 41·6/sec component appeared in all 8, was the highest in amplitude in 3 after background was subtracted (rel. = relative) and was more than 10 units above background level in one (circled *X*). In 4 of the 8 compounds the 51·9/sec component was the highest peak (relative), while in 2 compounds each, the 38·6/sec and also the 39·4/sec component was the highest in amplitude, including background activity (abs. = absolute). Table 12 shows data from electrode No. 4 and these data can be compared to the data from electrode No. 1

seen in Table 10. The comparison shows that the prominent frequency components vary according to the electrode site, but similarities also appear, since two different components, 38·6/sec and 41·6/sec, were common to the responses from both electrode sites.

An attempt was, therefore, made to characterize the complete profile of "camphoraceousness" in frequency components from all 4 electrode sites within the same olfactory bulb. Table 13 shows that 6 different

TABLE 13. FREQUENCY COMPONENTS IN 8 CAMPHORACEOUS COMPOUNDS FROM DIFFERENT ELECTRODE SITES (RABBIT NO. 3)

	38·6/sec	39·4/sec	41·6/sec	51·9/sec	105·4/sec	107·3/sec
Elect. Site No. 1	6(1)	3(0)	6(1)	6(3)	4(1)	4(1)
No. 2	*2(0)*	3(2)	7(1)	5(3)	5(1)	5(3)
No. 3	4(1)	7(2)	*3(1)*	6(1)	4(2)	3(0)
No. 4	4(2)	4(2)	8(3)	6(4)	*2(1)*	2(1)
	No. compounds with freq. component	No. compounds with freq. component as highest peak			TOTAL = 110 (37)	

frequency components were chosen as the prominent representation of this quality of odor (in Rabbit No. 3) with 4 components in the low frequency range and 2 components in the higher range. For the 38·6/sec component, note the uniquely poor representation from electrode site No. 2 and also the poor representation from electrodes Nos. 3 and 4 for 41·6/sec and 105·4/sec, respectively. All electrodes showed a prominent 51·9/sec component, especially with regard to the total number (6) of the 8 compounds in which that frequency component appeared. There were 110 instances in which one of these components appeared in one of the electrode sites; 37 of these were major peaks or peaks of highest amplitude, either before or after the subtraction of the background activity.

One of the major points that Table 13 illustrates is that probabilistic theory must be added to the Frequency Component Hypothesis in order to adequately explain the stimulus–response relationships. According to our hypothesis, the identification of a given stimulus is not made by a certain few frequency components, which must *always* appear highest in amplitude in each response. The identification is likely made from a choice of a number of prominent frequency components, any 2 or 3 of which will provide the necessary conditions for identifying a given stimulus. The sufficient conditions probably include the information provided by the lower

amplitude minor components. This general point was previously made after the analysis of many different bursts from the same stimulus (cigarette smoke) recorded from the same electrode site (Hughes and Mazurowski, 1964). That previous study also showed that there was no frequency component which *always* appeared highest in amplitude in each burst, but there were 2 which were very prominently represented in all and, in addition, there were 3 other components which were often represented in the different bursts. Some variability of response from moment to moment from the same stimulus may be related to changes in respiration that vary the volume of air passing over the olfactory epithelium from time to time.

In order to investigate the effects of varying the amount of a given odor presented to the olfactory receptors, a study was done on an intensity series of stimuli (Rabbit No. 5). Three different intensities were used: the parent compound, a dilution of 10 times, and a dilution of 100 times by an odorless liquid (distilled water). In the case of indole, a solid, the parent compound was made with 100 grams/liter of distilled water. The analyzed responses to these 3 different intensities were compared on different electrode sites. Generally, a decreasing intensity of stimulus produced both a decreasing amplitude of response for the major peak and also a smaller number of peaks, a measure correlating well with amplitude. The changes seen on one electrode site usually paralleled the changes seen on the other electrodes. For an example, the response to ethyl butyrate on electrode site No. 3 showed amplitudes (relative) of 19, 19 and 15 with 55, 54 and 36 peaks at intensities of X, X^{-10} and X^{-100} respectively. On electrode No. 4, amplitudes of 20, 17 and 16 with 44, 33 and 32 peaks were seen for the same 3 intensities of stimuli. Another example of the same type of change was noted in the responses to quinoline. One of the most important points, however, in this intensity series was that a few given frequency components were always prominent in each of the responses to the 3 intensities. At times, a given component was the major peak in all 3 responses; an example is the 61·8/sec component seen as the highest amplitude peak both before and after subtraction of background rhythm for the responses to limonene at all 3 intensities (electrode No. 4). At other times, 2 different components would represent the major peak at the 3 different intensities; an example is the response to indole in which either the 38·3/sec or 45·3/sec component was highest in amplitude for the 3 responses. The point is again emphasized that the identification of a given stimulus likely does not require a given frequency component as the highest in amplitude in all of the responses to that same stimulus; the necessary conditions would seem to be that there is a choice of a few different components, which signal the main quality

of the stimulus. The other minor peaks help to identify the stimulus, as suggested by the relatively large number of common peaks seen within each intensity series for a given stimulus. On the average 35·8 per cent of peaks (268 out of a possible 749) were common on electrode No. 3 and 35·5 per cent (212 out of a possible 597) on electrode No. 4 for the intensity series of 6 different stimuli. These values, derived from comparing each response to the others within the intensity series for a given stimulus, contrast with the value of 26 per cent, representing the average number of common peaks in responses within the same stereochemical category.

In the analysis of responses to *decreasing* intensities of stimuli, examples were occasionally noted of *increasing* amplitudes of response. Again, the changes on one electrode usually paralleled the changes on the other electrodes. One example is the response to benzaldehyde with amplitudes of 17, 18 and 22 and 27, 26 (a slight reversal) and 46 peaks for intensities X, X^{-10} and X^{-100} respectively. The latter appeared on electrode No. 4 and on electrode No. 3, amplitudes of 12, 28 and 35 with 37, 54 and 69 peaks were noted for the same intensities. Another example of this type of change was seen in the responses to indole. The increase in response to decrease in stimulus is by no means unknown in neurophysiology and is reminiscent of a similar effect seen in the auditory system, in which an *increase* in stimulus strength at times evoked a *reduced* neural response with a longer latency for single cell discharges in the medial geniculate (Galambos *et al.*, 1952).

Table 13 had indicated that 6 different frequency components characterize the response from the camphoraceous odors at the 4 different electrode sites. The degree to which these components represent that quality of odor and only that quality needs further investigation, especially in view of the fact most of the odoriferous substances used as stimuli fell into more than one stereochemical category, but, for purposes of analysis, were assigned to the category which represented their main stimulus quality. A representation of the degree of distinctiveness and also overlap in stimulus–response relationships is seen in Table 14, which shows the 6 main frequency components for another stereochemical category, floral, and also the number of times that these frequency components were found in the responses to the 8 camphoraceous odors. A comparison between the last 2 tables shows that the frequency components are entirely different, except for the similarity between the 51·9/sec peak in the camphoraceous compounds and the 52·0/sec peak in the floral odors. The number of times in which each of these latter 2 components appeared in the 4 different electrode sites was similar, but not precisely the same since 51·8/sec, for exam-

TABLE 14. FREQUENCY COMPONENTS PROMINENT IN FLORAL COMPOUNDS APPEARING IN 8 CAMPHORACEOUS ODORS (RABBIT NO. 3)

	52·0/sec	56·0/sec	58·8/sec	77·5/sec	100·9/sec	104·8/sec
Elect. Site No. 1	6(2)	4(0)	6(0)	2(0)	4(0)	2(0)
No. 2	6(2)	7(0)	3(0)	3(0)	4(0)	4(0)
No. 3	5(1)	5(0)	3(0)	4(1)	5(1)	5(1)
No. 4	6(4)	4(1)	4(0)	3(1)	1(0)	2(1)

No. compounds with freq. component / No. compounds with freq. component as highest peak

TOTAL = 98 (15)

ple, would be counted as 51·9/sec in Table 13 but not as 52·0 in Table 14, in keeping with our predetermined rule of a $\pm$ 0·1/sec accuracy. The latter table shows that in 98 instances a "floral" frequency component was found in a camphoraceous response; this number is only slightly lower than 110 (noted in the previous table), representing the number of times that "camphoraceous" frequency components were found in their own responses. The main difference between the 2 tables is that relatively few (15) "floral" components appear as the highest peak in the camphoraceous responses; this low number contrasts with the corresponding number of 37, seen in Table 13. The important point, therefore, is that responses to odors in different stereochemical categories vary to some degree with regard to all of the frequency components seen in the responses, but mainly with regard to the major frequency components of highest amplitude. In other words, it appears that the main signature of a given quality of odor will be found in the peaks of response that are highest in amplitude, but also minor peaks of lower amplitude will likely help to specify the nature of the stimulus. It should be emphasized again that a certain degree of overlap or similarity in the components in the responses should be expected because of an overlap in the components in the stimuli.

A more detailed analysis was required to investigate whether all peaks, not just those of highest amplitude, were important in the identification of a given type of odor. A reasonable assumption would seem to be that, if all peaks helped to specify a given stereochemical category, a significantly larger number of common peaks should be found in the responses within a given stimulus category (for example, floral) than would be found between 2 different categories (floral and camphoraceous). Table 15 shows the results of this analysis, in which the number of common peaks was counted

TABLE 15. COMPARISON OF NO. OF COMMON PEAKS WITHIN AND BETWEEN ODOR GROUPS ELECT. SITE NO. 2, RABBIT NO. 3)

Within floral group (18) $= \frac{799}{3077 \text{ possible}}$ (26·0%)

Between floral (18) camphoraceous (8) groups $= \frac{797}{3713 \text{ possible}}$ (21·5%)

$\chi^2 = 15{\cdot}8$ ($P = < 0{\cdot}05$)

when each of 18 different floral responses was compared to the other 17 responses and also when each of 8 camphoraceous responses was compared to each of 18 floral responses. These 2 values were then based on the number of possible common peaks, given by the lower number of peaks found in one of the 2 responses under comparison. This table shows that, on the average, 26 per cent of the peaks found in 2 floral responses will be common, contrasted to 21·5 per cent of the total peaks found common when floral and camphoraceous responses are compared. The difference in these percentages may seem insignificant, but the Chi-Square test in this large sample shows a statistically significant difference between them. Therefore, this table indicates that when a large sample is used, a significantly larger number of common peaks are found within a given stimulus category than between two different categories. These data suggest that all of the peaks (not just those of highest amplitude) help to designate the quality of the odoriferous stimuli.

A detailed analysis of the responses to floral compounds is seen in the next 3 tables, as further evidence for the Frequency Component Hypothesis and Stereochemical Theory. Table 16 shows the 4 frequency components which were prominently represented in the responses to the 18 floral compounds recorded from electrode site No. 1. In only 18 instances out of a possible 72 did one of these 4 frequency components fail to appear in the responses to the floral compounds. In 19 instances in which the component was represented, the peak was highest in amplitude and in 16 of these 19, this peak was highest after subtraction of the background rhythm (relative values). These latter data emphasize a point made after a comparison of Figs. 13 and 14: more internal consistency is seen in these data after the background rhythm has been subtracted from amplitude values. Further evidence is, therefore, seen that the coding within the olfactory bulb deals more with the *change* from the level of intrinsic background activity to the height of the response than with the absolute level of the response including the background activity.

TABLE 16. COMPARISON OF FLORAL COMPOUNDS (RABBIT NO. 3, ELECT. SITE NO. 1)

Frequency component:	52·0/sec	54·5/sec	57·9/sec	58·8/sec
Juniper oil	Highest (rel.)	—	⊗	⊗
Rose geranium oil	⊗*	Highest (rel.)	⊗	⊗
Clove oil	⊗	⊗	Highest (rel.)	—
Lime oil	⊗	⊗	⊗	Highest (rel.)
Ethyl-bromo-iso-valerate	—	Highest (abs.)	×	Highest (rel.)
Cinnamon oil	×	—	×	—
Cream sachet	×	×	—	⊗
Airkem	⊗	—	⊗	—
Ethyl butyraldehyde	—	Highest (abs.)	×	Highest (rel.)
Nutmeg oil	⊗	—	Highest (rel.)	—
Lemon oil	—	×	Highest (abs.)	—
Ethyl butyrate	⊗	⊗	⊗	Highest (rel.)
Rosemary oil	⊗	⊗	⊗	Highest (rel.)
Sassafras oil	Highest (rel.)	—	—	Highest (rel.)
Hemlock oil	⊗	⊗	Highest (abs.)	Highest (rel.)
Anise oil	×	—	×	Highest (rel.)
Eucalyptus oil	⊗	Highest (abs. & rel.)	—	—
Orange oil	⊗	⊗	Highest (abs. & rel.)	⊗

* Amp > 10 units above background.

Table 17 shows the 6 different frequency components chosen as the profile of the floral quality for the 4 different electrode sites. These components, also appearing in Table 14, included 2 of the 4 which character-

TABLE 17. FREQUENCY COMPONENTS IN 18 FLORAL COMPOUNDS FROM DIFFERENT ELECTRODE SITES (RABBIT NO. 3)

	52·0/sec	56·0/sec	58·8/sec	77·5/sec	100·9/sec	104·8/sec
Elect. Site No. 1	15(2)	17(1)	12(8)	*1(0)*	*8(3)*	6(0)
No. 2	14(6)	15(5)	11(2)	4(0)	3(1)	8(3)
No. 3	13(3)	15(3)	10(3)	*6(4)*	6(2)	7(1)
No. 4	12(5)	16(4)	10(3)	5(2)	*1(0)*	3(2)

No. compounds with freq. component

No. compounds with freq. component as highest peak

TABLE 18. FLORAL COMPOUNDS (RABBIT NO. 3, ELECT. SITE NO. 1, 11/1/63)

"Unique" chemically within floral group	No. peaks Observed/Expected (Ave.)	"Similar" compounds within floral group	No. peaks Observed/Expected (Ave.)
1. Anise oil (anethole)	$\frac{73}{84} = 0{\cdot}87$	1. Clove oil	$\frac{92}{68} = 1{\cdot}35$
2. Cinnamon oil (cinnamaldehyde)	$\frac{96}{99} = 0{\cdot}97$	2. Rose geranium oil	$\frac{141}{124} = 1{\cdot}15$
3. Ethyl butyraldehyde	$\frac{149}{152} = 0{\cdot}97$	3. Lime oil	$\frac{113}{99} = 1{\cdot}14$
4. Ethyl butyrate	$\frac{126}{134} = 0{\cdot}95$	4. Nutmeg oil	$\frac{146}{134} = 1{\cdot}09$
5. Ethyl-bromo-isovalerate	$\frac{159}{148} = 1{\cdot}07$	5. Cream sachet	$\frac{141}{124} = 1{\cdot}14$
6. Hemlock oil (coniine)	$\frac{182}{148} = 1{\cdot}22$	6. Sassafras oil	$\frac{156}{148} = 1{\cdot}06$
		7. Eucalyptus oil	$\frac{119}{112} = 1{\cdot}06$
		8. Airkem	$\frac{147}{140} = 1{\cdot}05$
		9. Rosemary oil	$\frac{139}{134} = 1{\cdot}03$
		10. Juniper oil	$\frac{142}{145} = 0{\cdot}98$
		11. Orange oil	$\frac{140}{145} = 0{\cdot}96$
		12. Lemon oil	$\frac{119}{134} = 0{\cdot}90$

"Unique" — Chem. = 6; Physiol. = 4(0 < E)

(Reversals: 2 of 6)

"Similar" — Chem. = 12; Physiol. = 9(0 > E)

(Reversals: 3 of 12)

$\chi^2(z = 1{\cdot}70)$ $(P = 0{\cdot}045)$

ized electrode site No. 1 (see Table 16). In some cases (52·0/sec and 56·0/sec) a given frequency component was well represented on all 4 electrodes. In other cases (77·5/sec and 100·9/sec) a given component may appear frequently on one electrode, but not on another electrode site. These data

emphasize again the specificity of response according to location within the bulb and likely have reference to odor localization within this structure.

The 18 floral compounds seemed to include 2 different types of stimuli, those which were simple or "unique" in consistency and those which were complex, consisting of many similar chemical elements. Table 18 shows on the left 6 stimuli which were considered "unique" (with the main element noted in parentheses) and on the right 12 stimuli considered complex, but with similar chemical elements. In each case, the peaks for a given stimulus were compared to those from the other 17 responses and the number of similar peaks was counted; this number was then compared to the value representing the expected number, derived from the average number of similar peaks for all floral compounds. Our hypothesis was that the "unique" compounds, having fewer common chemical elements with the other floral compounds, would show relatively fewer common peaks and the "similar" compounds, having many similar chemical elements, would show relatively more common peaks. Table 18 shows that in only 2 of 6 instances did the "unique" group fail to show *fewer* common peaks than the average among all floral stimuli and in only 3 of 12 instances did the "similar" group fail to show *more* common peaks than the average. These differences

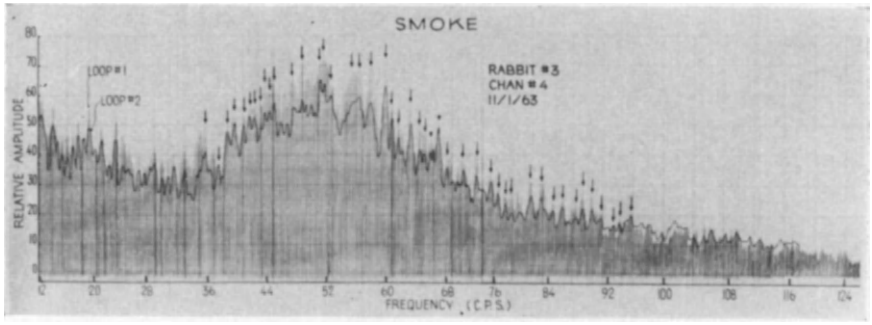


FIG. 16. Similarity of analyzed response during stable conditions with 2 different stimulus (smoke) presentations. Abscissa: frequency in cycles per second; ordinate: relative amplitude. Loop 2 refers to the analyzed response to the second stimulus presentation and is traced over the response to the first presentation (Loop 1). The arrows indicate distinctive frequency components which are common to both loops.

are shown to be significantly different on a Chi-Square Test. These data, therefore, provide further evidence that, even with a given stereochemical category, compounds with chemical similarities tend to show responses

with more similar frequency components than do compounds without such chemical similarities.

Considerable variability has been encountered in some of the analyzed responses in this study. This variability in response has usually been explicable in terms of changing environmental conditions, especially during varying states of alertness of the animal. Nevertheless, the Frequency Component Hypothesis would seem to require a demonstration of a relatively invariant response to a given stimulus, under stable conditions. Figures 16 and 17 provide examples of the great similarity of the analyzed responses, at either the low or the high frequency ranges, when stable conditions are achieved during 2 different presentations of a given stimulus.

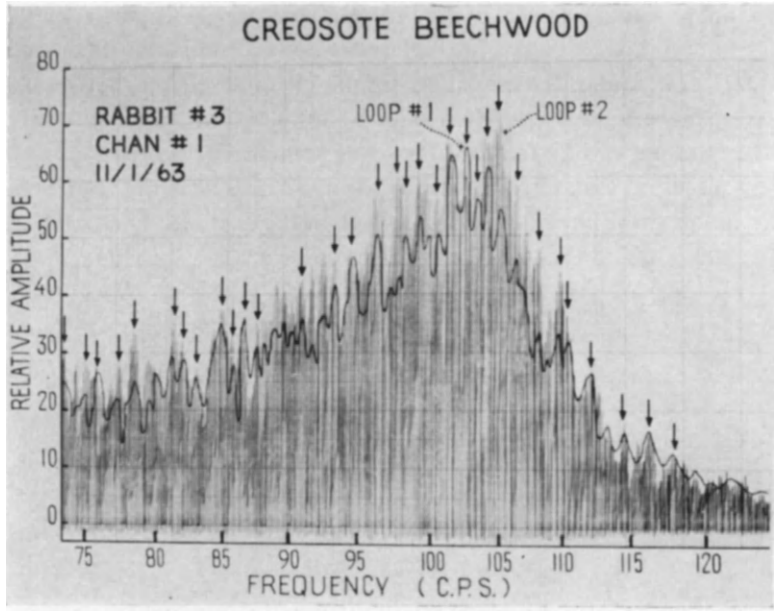


FIG. 17. Same as Fig. 16, except 2 different stimulus presentations of creosote beechwood which produced a response mainly in the high frequency range.

SUMMARY

1. This study dealt with the recording of rhythmical responses from the olfactory bulb of unanesthetized rabbits in response to many different odoriferous stimuli. The responses were analyzed into various frequency components which provided data for both the Frequency Component Hypothesis, relating to the essential coding mechanism within the bulb,

and also for a neurophysiological investigation of the Amoore Stereochemical Theory of Olfaction.

2. Low frequency components near 4–5/sec usually represent different respiration (sniffing) rates and other peaks of activity under 20/sec represent harmonics, difference and summation-frequencies of these rates.

3. An inverse relationship appears between the frequency of the major peak (of highest amplitude) and the amplitude of that component; an inverse relationship also appears between the frequency of the major peak and molecular weight.

4. Responses do not vary according to the components of highest frequency appearing at the beginning of the burst (likely signaling the *presence* of an odor), but vary according to the lower frequency components, including the peak of highest amplitude, appearing toward the middle or end of the burst (likely signaling the *identity* of the odor).

5. Both "excitatory" and "inhibitory" types of responses appear within 2 different frequency ranges 37·5–75/sec and 75–125/sec. All permutations and combinations of excitatory or inhibitory responses at the 2 different frequency ranges are possible in the responses to different odors from a given electrode site or in the responses to the same odor from different locations. This great variety of ways of responding may be related to the richness of the information in the olfactory system.

6. Excitatory responses that also included some inhibition were common and tended to be associated with responses of relatively high amplitude.

7. Evidence is noted that more internal consistency and more meaningful stimulus–response relationships appear after the values representing the intrinsic background activity have been subtracted from the total responses, leaving only the *change* in the activity that has occurred with the presentation of a stimulus.

8. Compounds of high molecular weight, especially floral odors, often show their main response within the 37·5–75/sec range. Compounds of low molecular weight, especially in the camphoraceous category, usually show their main response within the 75–125/sec range. Responses of relatively high amplitude tend to be seen on a given electrode site for all compounds within a certain stereochemical category; these amplitude relationships may or may not apply to both of the two different frequency ranges, which appear to be independent modes of response, rather than harmonically related and dependent on each other.

9. The number of peaks of the analyzed response, in addition to amplitude, appears to be a representation of the degree of activity.

10. Many of the same frequency components found in the response to

a given stimulus also are found in the response to compounds stereochemically related to that given stimulus. An intensity series for a given stimulus usually shows a decrease in amplitude with decrease in intensity, but an increase in amplitude may also be seen with some odors. However, the main finding was that certain frequency components were prominently represented at each intensity for a given stimulus. A general tendency can be seen between the similarity of stimulus from the stereochemical point of view and similarity of response from the frequency component point of view.

11. The prominent frequency components appearing for a given category of stimuli vary according to electrode site, providing further evidence for odor localization within the bulb.

12. Responses to odors in different stereochemical categories vary with regard to all of the frequency components seen in the response, but mainly with regard to the major frequency components of highest amplitude. Thus, the main signature of a given quality of odor will be found in the peaks of response that are highest in amplitude, but also minor peaks of lower amplitude will likely help to specify the nature of the stimulus.

13. Within a given stereochemical category a greater degree of chemical similarity between two different stimuli is associated with a larger number of common frequency components seen in the responses.

14. Responses to the same stimulus at two different presentations are remarkably similar from the point of view of the frequency components, if stable conditions exist.

15. These data provide evidence for the Frequency Component Hypothesis and the Stereochemical Theory of Olfaction.

REFERENCES

ADRIAN, E. D. 1947. *The Physical Background of Perception.* Oxford University Press, London.

AMOORE, J. E. 1962*a*. Identification of the seven primary odors. *Proc. Sci. Sect., Toilet Goods Ass'n.* Suppl. to No. 37, 1–12.

AMOORE, J. E. 1962*b*. Elucidation of the stereochemical properties of the olfactory receptor sites. *Proc. Sci. Sect., Toilet Goods Ass'n.* Supp. to No. 37, 13–23.

AMOORE, J. E. 1963. Stereochemical theory of olfaction. *Nature*, **198**, 271–272.

ELIEL, E. L. 1962. *Stereochemistry of Carbon Compounds.* McGraw-Hill Book Co., New York.

GALAMBOS, R., ROSE, J. E., BROMILEY, R. B. and HUGHES, J. R. 1952. Microelectrode studies on medial geniculate body of cat. II. Response to clicks. *J. Neurophysiol.* **15**, 359–380.

HUGHES, J. R. and MAZUROWSKI, J. A. 1962*a*. Studies on the supracallosal mesial cortex of unanesthetized, conscious mammals. II. Monkey B. Responses from the olfactory bulb. *Electroenceph. clin. Neurophysiol.* **14**, 635–645.

HUGHES, J. R. and MAZUROWSKI, J. A. 1962*b*. Studies on the supracallosal mesial cortex of unanesthetized, conscious mammals. II. Monkey C. Frequency analysis of responses from the olfactory bulb. *Electroenceph. clin. Neurophysiol.* **14,** 646–653.

HUGHES, J. R. and MAZUROWSKI, J. A. 1963. Frequency analysis of responses from the olfactory bulb of unanesthetized mammals. *Proceedings of the Conference on Data Acquisition and Processing in Biology and Medicine.* (1962). Pergamon Press, N.Y.

HUGHES, J. R. and MAZUROWSKI, J. A. 1964. Comparative studies on the frequency analysis of responses from the olfactory bulb of unanesthetized monkeys and rabbits. *Proceedings of the Conference on Data Acquisition and Processing in Biology and Medicine* (1963). Pergamon Press, New York.

JOHNSTON, J. W. JR., and SANDOVAL, A. 1962. The validity of muskiness as a primary odor. *Proc. Sci. Sect., Toilet Goods Ass'n.* Suppl. to No. 37, 34–45.

Merck Index (7th Edition) 1960. RAHWAY, N. J.

OTTOSON, D. 1959. Studies on slow potentials evoked in the rabbit's olfactory bulb and nasal mucosa. *Acta Physiol. Scand.* **47,** 136–148.

OTTOSON, D. 1963. Generation and transmission of signals in the olfactory system. *Proceedings of the First International Symposium on Olfaction and Taste,* **1,** ed. Y. Zotterman. Macmillan Co., New York

RUBIN, M., APOTHEKER, D. and LUTMER, R. 1962. Structure and odor: 1, 44-cyclohexane lactones and related compounds. *Proc. Sci. Sect., Toilet Goods Ass'n.* Suppl. to No. 37, 24–33.

SAUNDERS, H. C. 1962 Some odor observations in terms of the Amoore theory. *Proc. Sci. Sect., Toilet Goods Ass'n.* Suppl. to No. 37, 46–47.

STEVENS, S. S. and DAVIS, H. 1938. *Hearing.* John Wiley & Sons, New York.

PROPERTIES OF RECEPTORS THROUGH MOLECULAR PARAMETERS OF ODORIVECTORS*

ANDREW DRAVNIEKS

IIT Research Institute
Chicago, Illinois

ABSTRACT

Olfactory characteristics, primarily thresholds, of selected groups of odorivectors permit insight into intermolecular interactions that occur between the odorivectors and the olfactory system. Thus they indirectly describe the properties of the olfactory system. Interactions of the electron donor-acceptor (charge transfer or molecular complex) type appear to play an important role. Hence Mullins' olfaction theory, which considers only the cases in which the activity coefficient for the odorivector in the receptor membrane is unity or larger, should be expanded to the region of fractional coefficients. The olfactory receptor system may consist of barrier, storage phase, and receptor matrices.

1.0 INTRODUCTION

The search for correlations between the odor and the molecular or bulk parameters of odorivectors has occupied many researchers. Two classes of studies have been conducted. In one, odor similarities are compared with similarities in some characteristics of the odorivector without attempting to postulate the properties of the receptor or the mechanism of olfaction. Beets' (1957, 1961, 1962) theory of profile functional groups, Wright and Michels' (1964) analysis of correlations to infrared spectra based on a hypothesis advanced by Wright (1954, 1957, 1964), and Amoore's (1965) recently modified stereochemical theory are examples of this class. In the other, assumptions are made about properties of the receptors and some aspect of the olfaction mechanism. Thus, Amoore (1952, 1962, 1964), in the earlier form of his theory, postulated shaped receptor sites, Mullins (1955) proposed classification of receptor membranes through solubility

* Based in part on work supported by the National Institutes of Health (Neurological Diseases and Blindness, Grant 5-R01-NB05310-02). A travel grant from the National Science Foundation is also gratefully acknowledged.

parameters, and Davies (1955, 1962, 1965) advanced correlations between odor and adsorption at the oil–water interface. This interface served as a model of the boundary between the receptor membrane and aqueous fluids.

Dravnieks (1965) pointed out that basically most of the existing theories are not in conflict. They emphasize different aspects of the same effect. Discrimination of odor is possible only because differences exist in the interaction of odorivectors with some discriminating phase. Molecular shape, size, flexibility, and the functional group factors participate in determining these interactions.

Olfactive discrimination can be based either on differences in the concentration of odorivector molecules in the sensor (Mullins, 1955; Davies, 1962, 1965) or on temporal and spatial effects (Adrian, 1950; Beidler, 1954; Stuiver, 1958). In the second case, kinetics of the molecular migration processes, diffusion or desorption, most likely provide for the discrimination. Even if it is only nominally kinetic, the migration process still depends on the concentration of odorivector molecules in some hypothetical barrier matrix.

In both cases, the partition coefficient k is a significant parameter:

$$k = \frac{n_c}{n_g}. \tag{1}$$

Here n_g and n_c are the concentrations of the odorivector molecules, in molecules cm^{-3}, in gas (air), and in the captive state, respectively. The captive state may consist of dissolution in a receptor membrane, in a receptor–mucus interface, or in the outer layer of a diffusion barrier. In (Mullins') theories that propose that the significant factor is merely the presence of the odorivector molecules in or at the sensor matter, k is the principal factor. If, on the other hand, the rate of migration is the important factor, k is a codetermining parameter.

For instance, the rate of diffusion through a barrier matrix is approximated by:

$$J = \frac{RT}{6\pi\eta d} \cdot \frac{1}{r} (kn_g - n_e). \tag{2}$$

This is Fick's diffusion equation with substitution of the Stokes–Einstein value, $RT/6\pi\eta dr$, for the diffusion constant. Here n_e is the concentration of the odorivector at the exit from the barrier. For a mechanism in which the rate of migration is the significant discriminative step, $kn_g \gg n_e$. The other terms are: R, gas constant; T, absolute temperature; η, viscosity; d, thickness of the barrier; and r, effective radius of the odorivector molecule.

Davies' (1965) odor quality theory, based on the aftereffect (puncturing or healing) in the receptor membrane following the desorption of an odorivector molecule, employs similar terms: the free energy of adsorption is ΔG, which is proportional to $-\log k$ when n_c in Equation (1) is applied to the membrane–mucus interface; the molecular size factor in terms of the cross section of the odorivector molecule; and a parameter describing the viscosity of the membrane. Davies' theory has the advantage of proposing the mechanism of ionic puncture of the membrane as the signal-generating step. Purely formally, the same parameters can be used in any theory based on assumption that the rate of diffusion of molecules through a discriminating barrier is the factor that determines the olfactory qualities.

Thus, the intermolecular interactions of odorivector with receptors or prereceptor barriers must be significant olfactory parameters, regardless of the mechanism. Mechanism based exclusively on chemical conversion (Ruzicka, 1957; Rosano and Scheps, 1964) seem unlikely because of odor similarities among substances with completely different functional groups and stabilities.

The purpose of this paper is to explore whether it is possible to outline the properties of receptors by analyzing the olfactory characteristics, principally thresholds, of odorivectors.

2.0 THERMODYNAMICS RELEVANT TO OLFACTION

A case can be made for broadening of Mullins' (1955) and perhaps Davies' (1965) approach, keeping in mind also the possibility of barrier mechanisms in addition to the solubility-in-the-receptor-wall mechanisms. As pointed out, in either case the partition coefficient is significant and is determined by the interactions of the odorivector molecules with the condensed phases in bulk or at their boundary. For simplicity, consideration of all intermediate phases is omitted and the discussion is limited to the partition coefficient between the condensed (sensing or barrier) phase and air.

The following textbook equations, transcribed into form convenient for consideration in olfaction, are applicable.

$$k = \frac{n_c}{n_g} = B \cdot \frac{Z_c Z_{ic}}{Z_r Z_{ig}} \cdot e^{\frac{\Delta U}{RT}} \tag{3}$$

$$n_c = 6 \cdot 10^{23} \cdot \frac{d_1}{M_1} \cdot \frac{1}{\gamma_2} \cdot \frac{n_g}{n_{\text{sat}}} \tag{4}$$

$$k = \frac{n_c}{n_g} = 6 \cdot 10^{23} \cdot \frac{d_1}{M_1} \cdot \frac{1}{\gamma_2} \cdot \frac{1}{n_{\text{sat}}} \tag{4a}$$

$$\frac{n_g}{n_{\text{sat}}} = A_2, \quad \text{or} \quad \frac{(n_g)_{\text{thr}}}{n_{\text{sat}}} = A_0 \tag{5}$$

(at olfactory threshold)

$$\gamma_2 = \exp\left(\frac{\Delta F_E}{RT}\right). \tag{6}$$

Equation (3) is from statistical thermodynamics. Here the Z's are the partition functions for the various forms of kinetic energy. The translation partition function, Z_{tr}, of the molecule in gas phase is not shown, but is equivalent to $B \cdot n_g$, where B is constant for the same molecular weight at constant temperature; Z_r is for the rotation of the whole molecule in the gas phase; Z_{ig} is for various internal (intramolecular) vibrations, rotations around a bond, bendings, etc., in the gas phase; Z_c is for the movements of the molecule as a whole in the captive state and includes, in essence, bouncing and wobbling in the intermolecular spacings of the matrix; Z_{ic} is for the intramolecular motions in captive state.

The values of Z_{ig} and Z_{ic} may differ, since the matrix can interfere with the intramolecular deformations of the odorivector molecules. The exponential term is the partition function for the potential energy, with ΔU describing the potential energy increment arising from the interaction of the molecule with the matrix. The physical meaning of ΔU is that of the heat of solution or adsorption at 0°K, when the molecule is in the same position as at T. When a molecule is captured, translational and rotational freedoms are replaced by a limited freedom of movement in the matrix. The $Z_c/Z_{tr}Z_r$ ratio is small and depends then significantly on the mass of the molecule, and through rotational momenta, on the distribution of the mass within the molecule.

Equation (3) is the most fundamental and informative among Equations (3) through (6), but its application is very limited, since the values of Z_r and Z_{ig} cannot be accurately calculated for most of the molecules significant for olfaction, and other terms are even less well known. However, the equation should serve as a guide for assessing the significance of shape and molecular size factors.

Equation (4), from thermodynamics of nonideal solutions, relates n_c to the ratio n_g/n_{sat}. This ratio,* denoted A_2 in Equation (5), is the relative

* Mullins (1955) uses $A_0 = P_{th}/P_0$, where P_{th} is the partial vapor pressure of the odorivector at the olfactory threshold and P_0 is the vapor pressure of the same odorivector in pure state at 37°C.

vapor pressure of the odorivector; n_{sat} is the concentration of the odorivector, molecules cm^{-3}, in air at equilibrium with the pure odorivector, namely, at 37°C for the purpose of olfaction. The matrix ("solvent") of a receptor or a barrier is described through its density, d, and its molecular weight, M_1. The parameter γ_2 is the activity coefficient for a particular odorivector in a particular matrix and is defined by Equation (6). Here ΔF_E is the excess free energy of evaporation from a particular nonideal solution as compared with an ideal solution.

For an ideal solution, $\Delta F_E = 0$ and $\gamma_2 = 1$. In the absence of specific interactions between the odorivector and the matrix, this case represents approximately the highest odorivector concentration that can be reached in the matrix from the threshold concentration in air. When molecules of the odorivector and the matrix differ in size, the concentration of the odorivector is low, the intermolecular interactions are primarily dispersion forces, and the excess energy is always positive:

$$\Delta F_E \approx (\delta_1 - \delta_2)^2 \tag{7}$$

δ_1 and δ_2 are the solubility parameters (Hildebrand and Scott, 1962), or the cohesive energy densities of the matrix and the odorivector, respectively. These were the concepts used by Mullins (1955), and they can provide only for negative deviations from the solubility with $\gamma_2 > 1$ (Equation (4)). In his theory, the receptor membrane is most sensitive when $\delta_1 = \delta_2$. For an odorivector with $\delta_2 \neq \delta_1$, the solubility in the same receptor will be less, since $\gamma_2 > 1$ and n_c is correspondingly slower regardless of whether $\delta_2 > \delta_1$ or $\delta_2 < \delta_1$.

Equations (3)–(6), however, can provide a more comprehensive viewpoint than the case selected by Mullins. Intermolecular complexes, known also as electron donor–acceptor or charge transfer complexes (Mulliken, 1952; Briegleb, 1961; Andrews and Keefer, 1964), are a class of compounds formed through interactions of the electronic structures of the molecules. Association through hydrogen bonding is sometimes considered a case within this class. Significance of electron donor–acceptor interactions in biochemical mechanisms gains increasing acceptance (Pullman and Pullman, 1961).

The formation of donor–acceptor complexes is rapid. The bond formed is not very strong, and the complexes dissociate to form the original molecules when concentrations are adjusted accordingly. The result of the interaction is an offsetting of the positive values of ΔF_E arising from size mismatch and permitting negative ΔF_E. In such cases, $\gamma_2 < 1$, and the so-

lubility of the odorivector in matrix at the same A_2 can be higher than in the ideal case.

The same interactions increase the exponential term in Equation (3), but the available heats of formation, H_f, for various charge-transfer complexes describe the values of ΔU very imperfectly, since the latter apply to 0°K and would have to be obtained by extrapolation of H_f to this temperature.

Equation (3) through (6) provide a framework for considering the variations in the receptor or barrier properties that could account for differences in olfactory thresholds. As mentioned, in the case of a barrier mechanism, the viscosity barrier and its thickness become additional significant parameters. They decrease the arrival rate of the odorivector to the receptor to a level not distinguishable from general chemical noise.

3.0 COMPARISON OF THRESHOLDS

Although the enrichment of the receptor or barrier matrix by odorivector molecules is, in principle, fully describable through Equation (3), molecular parameter data for applying this equation fully are not available. The values of various Z's in the numerator and of Δu are interdependent and cannot be isolated on the basis of present knowledge. For example, a higher Δu can mean less freedom in the captive state; hence Z_c is smaller and possibly Z_{ic} is smaller.

However, following approaches remain open. One, from the entropy data, it is possible to rank the odorivectors by the sum of their rotational and vibrational entropies and consequently by the magnitude of the denominator in Equation (3). Two, Equation (4) can be used, by taking values from the literature on donor–acceptor complexes and using expressions that relate γ_2 to molecular sizes, or by using purely empirical observations on the relative retention volumes in gas chromatography on selective stationary phases. The ratio of the retention volumes for two odorivectors, a and b, are $k_a : k_b$, where k is the partition coefficient (see Equation (4a)). In this approach, various stationary phases serve as sets of matrices, each representing certain combinations of molecular weights, densities, intermolecular spacings, and interaction forces that vary with the chemical nature of the phase.

Thresholds to be compared present another difficulty. The literature data on olfactory thresholds are inconsistent, especially when work by different authors is compared. The thresholds derived from sniffing dilute solutions of odorivectors in nonodorous solvents are useless; using Equation (4) in reverse, where n_c now is the concentration of an odorivector in the solvent,

one sees that n_g depends on density and molecular weight of the solvent and the activity coefficient of the particular odorivector in this solvent (see Jellinek, 1964). The thresholds or other olfactory parameters obtained by mixed airstream techniques depend on the technique used (Pangborn *et al.*, 1964), panel (see ethylmercaptan data, Katz and Talbert, 1930), and frequently are unreliable because of the unknown purity of the odorivectors. Hence, the selection of data is best restricted to sets obtained by the same author or cases in which thresholds differ by orders of magnitude.

3.1 Thresholds of C_4 Hydrocarbons

Mullins' (1955) data on olfactory thresholds of saturated and unsaturated hydrocarbons with 4 carbon atoms present a case of molecules with approximately the same size. Table 1 gives some physicochemical constants for these compounds. Low and high thresholds occur at similar mole volumes, solubility parameters, ionization energies, polarizabilities, parachors, and dipoles. Similarly, molecules with one double bond occur on both ends of the series. None of these indices give any indication for the differences in thresholds. Thus, Van der Waals and dipole forces cannot explain the order of thresholds, and especially the difference between the *cis*- and *trans*-butenes.

Partial ordering can be achieved through use of Equation (3) by assuming that the principal function of the receptor or barrier phase is to restrict translational and rotational freedoms of the odorivector molecule. In lieu of sufficient knowledge, it is assumed tentatively that ΔU is the same for all C_4 molecules and $Z_{ig}=Z_{ic}$. The rotational partition function Z_{rot}, is related to the rotational entropy, S_{rot}, as seen from the following simplified relations (cf. Janz, 1958; Moelwyn-Hughes, 1961).

$$Z_{\text{rot}} = C_1 \frac{(I_a I_b I_c)^{1/2}}{s}, \tag{8}$$

$$\frac{S_{\text{rot}}}{R} = C_2 + \ln \frac{(I_a I_b I_c)^{1/2}}{s}. \tag{9}$$

Here I_a, I_b, and I_c are the rotational momenta of the molecule around its principal axes, s is the symmetry factor for the molecule, and C_1 and C_2 are constants consisting of several coefficients and are dependent on temperature. Hence, Z_{rot} is proportional to exp S/R, and, from Equation (3), if the concentration in the captive state at threshold would be constant, log n_g should vary linearly with S/R.

Rotational entropy was estimated by subtracting the calculable translational entropy from the standard total entropies of the compounds. Vib-

TABLE 1. THRESHOLDS AND PHYSICOCHEMICAL PARAMETERS FOR C_4 HYDROCARBONS

Carbon skeleton of the hydrocarbon	n_g, Mole cm^{-3} at threshold in 10^{14} units	$A_0 \times 10^6$ (Mullins)	Mole volume cm^3 $mole^{-1}$ at 25°C	Solubility parameter, δ $cal^{\frac{1}{2}}$ $cm^{-\frac{3}{2}}$	Ionization energy, eV	Total polarizability cm^3 $mole^{-1}$	Parachor	Dipole, debye units
C=CH | | (*cis*) C C	2·9	4	91	7·1	9·31	21·4	178	0
C–C–C=C	3·1	4	95·5	6·7	9·76	21·4	178	0·4
C=C–C=C	21	20	87		9·07		202	
C–C≡C–C	76	230	79				168	0

C–C–C
  |
  C
	140	120	105	6·1	10·8	20·9	180	0

C
|
C=C (*trans*)
  |
  C
	264	350	93·5	6·8	9·29	21·4	178	

C–C=C
  |
  C
	490	500	95	6·7	8·9	20·9	178	0·49

C–C–C–C	550	700	101	6·5	10·8	20·6	180	0

rational entropy was not subtracted, as it is a small fraction of the total and cannot be accurately estimated on the basis of available data. Figure 1 shows a plot of S/R versus log n_g at threshold. Only *cis*-2-butene and 1-butene have thresholds much lower than expected from their approximate rotational entropies. Probably the interaction energy, ΔU (Equation (3)), and other terms have been unjustly ignored. The difference in thresholds between both 2-butenes is not explainable on this basis.

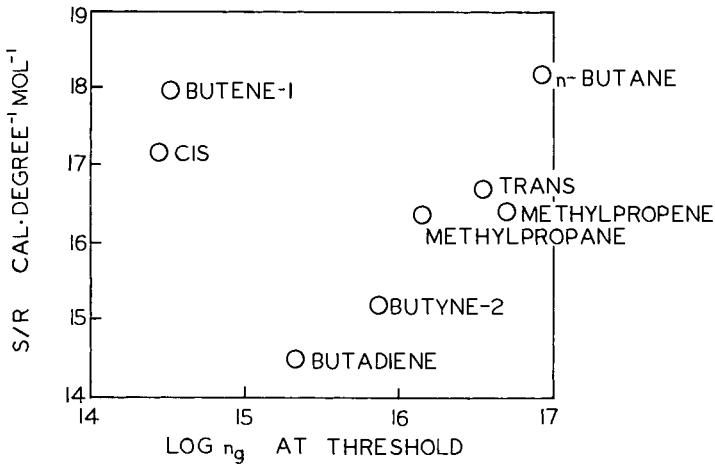


FIG. 1. Thresholds for C_4 hydrocarbons vs non-translational entropy.

Another possibility for threshold differences may be in the interactions originating from π electrons of the double and triple bonds. These electronic structures act as electron donors. In the electron donor–acceptor complexes steric hindrances that govern the approach between the π structure of the donor and the acceptor are important, and the amount of interaction, which makes γ_2 (Equation (4a)) smaller, depends on the selection of the appropriate acceptor. In comparison with the behavior of C_4 hydrocarbons toward various acceptor matrices, indications of correlations were found with respect to a strong acceptor phase, benzyl cyanide–silver nitrate, used in gas chromatography.

Data on retention volumes on this acceptor phase (Gluekauf, 1958) are plotted versus olfactory thresholds in Fig. 2. The retention volumes are proportional to k (Equation (4)) and reflect the distribution of the odorivector between the gas and the stationary phase. The point for 2-butyne is not shown, since it falls far outside the graph. Data for methylpropane are not available. However, the *cis*- and *trans*-2-butene threshold difference is paralleled by the larger interaction of the *cis*-2-butene with the selected

electron acceptor matrix, as evidenced by the larger retention volume. A similar difference between the *cis* and *trans* form is demonstrated by alkene argentation constants, (Muhs and Weiss, 1962).

In forming the molecular complex in the condensed phase, rotational freedoms are drastically changed, so that some parallelism between Figs. 1 and 2 is not surprising. However, Fig. 2 includes empirical values reflecting interaction energy constants excluded in Fig. 1.

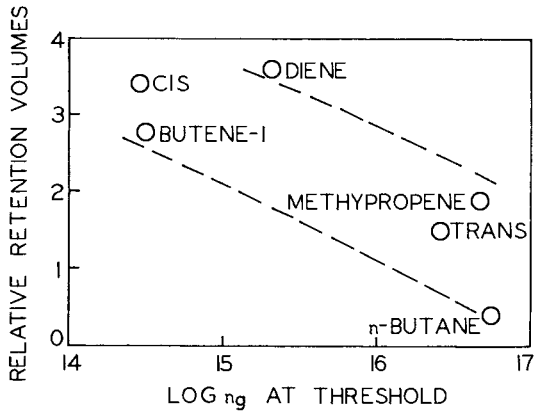


FIG. 2. Thresholds for C_4 hydrocarbons vs retention volumes on benzyl cyanide–silver nitrate column.

Thus, although threshold differences in C_4 hydrocarbon series are not fully explained, indications exist that the receptor or barrier phase acts as a rotation restrictor and an electron acceptor. Little is known about electron acceptors in the olfactory area, but hydroxylic substances capable of donating a hydrogen atom and forming a hydrogen bond can interact with a π-electron donor (Baker and Shulgin, 1958; Tamres, 1952).

3.2 Thresholds of Selected Organic Compounds

To explore further what properties the receptor or the barrier phase must have, data for odorivectors with different chemical structures were examined. Selection of the odorivectors was narrowed to those for which reliable data exist on olfactory thresholds, physicochemical constants, and thermodynamic parameters of charge-transfer complex formation with well-known electron acceptors.

Table 2 lists the selected odorivectors, their olfactory thresholds, A_0, and some physicochemical constants. The diverse functionalities cover a

TABLE 2. THRESHOLD AND PHYSICOCHEMICAL PARAMETERS FOR SEVERAL ODORIVECTORS

Odorivector	A_0*	Mole volume $cm^3\ mole^{-1}$	Dipole, debye units	Molecular refraction, R_D, cm^3
CH_3OH	$4{\cdot}5\times10^{-2}$	40	1·68	8·2
C_2H_5OH	$3{\cdot}1\times10^{-2}$	58	1·68	12·8
$C_2H_5OC_2H_5$	$1{\cdot}0\times10^{-3}$	104	1·15	22·3
$CH_3COOC_2H_5$	$1{\cdot}0\times10^{-3}$	98	1·81	22·2
C_4H_9OH	$7{\cdot}0\times10^{-4}$	92	1·72	22·1
Methyl-propene	$5{\cdot}0\times10^{-4}$	95	0·49	21·4
Trans-butene-2	$3{\cdot}5\times10^{-4}$	93·5		21·4
C_6H_6	$1{\cdot}0\times10^{-5}$	89	0	26·1
Pyridine C_5H_5N	$4{\cdot}3\times10^{-6}$	80	2·23	24·1
Cis-butene-2	$4{\cdot}0\times10^{-6}$	91	0	21·4
$C_2H_5SC_2H_5$	$2{\cdot}1\times10^{-8}$	107	1·58	29·6
$C_2H_5SeC_2H_5$	$1{\cdot}8\times10^{-8}$	112		

* From Mullins, Alison and Katz, and Katz and Talbert.

large range of A_0 values within a relatively narrow range of the molecular-volumes. Assume that in Equation (4) the receptor or the barrier matrix is the same for all compounds in the table and that at threshold the same concentration of the odorivector must be reached in this matrix. Since at the threshold $n_g : n_{sat}$ is A_0, lower A_0 would require lower γ_2. The values of A_0 characterize the strength of interaction of the odorivector molecules with the matrix as compared with the interaction with the odorivector's own molecules in condensed state. The lower the A_0, the stronger the interaction with the matrix. If the only reason for the variations of γ_2 is in the molecular size (cf. Littlewood, 1962),

$$\gamma_2 = \frac{V_2}{V_1} \exp\left(1 - \frac{V_2}{V_1}\right), \tag{10}$$

where V_2 and V_1, for the present application, are the molecular volumes of the odorivector and the matrix, respectively. Variations in the V_2 for the compounds in the table cannot produce sufficient variation in γ_2 even when $V_1 = 18$ (water). Most likely $V_1 > V_2$, and the range of γ_2 with odorivector molecular volumes from Equation (10) alone should not exceed a factor of 2.

Differences in dipole moments and polarizabilities as indicated by the molecular refraction values also cannot explain the wide range of A_0. Hence, neither dipole interactions nor Van der Waals–London dispersion forces are the threshold-determining parameters of the matrix.

A more general expression for γ_2 is:

$$\gamma_2 = e^{+\Delta F'/RT} \qquad (11)^*$$

where $\Delta F'$ is the excess free energy for evaporation of the vapor (odorivector) from a nonideal solution in a matrix as compared with evaporation from an ideal solution. From Equations (4) and (11), log A_0 should vary

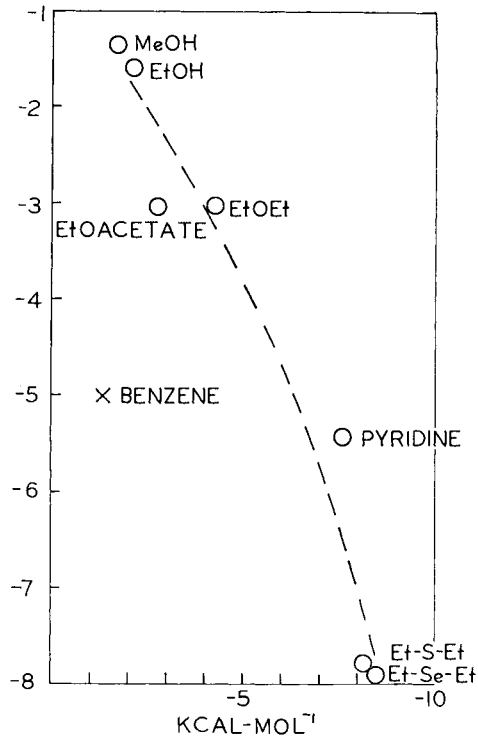


FIG. 3. Heats of formation for complexes with iodine and the olfactory thresholds.

linearly with $\Delta F'$ if the substance chosen as a model for the discriminator matrix interacts with the odorivectors through a similar set of intermolecular forces.

Iodine is the most thoroughly investigated σ-acceptor, since its complexes are easily studied from shifts and intensities of light absorption bands. It can interact with π-donors such as benzene; n-donors such as oxygen, sulfur, or nitrogen-containing organic molecules; and donors that combine both characteristics, such as perhaps pyridine.

* Littlewood, 1962, p. 63.

In Fig. 3, heats of formation, ΔH, of complexes between iodine and some other odorivectors are plotted versus A_0. Briegleb's (1961) tabulations were used. The data for sulfur compounds are from more recent work (Tsubomura and Lang, 1961). Heats of formation are numerically larger than ΔF by $T\Delta S$, but since for the iodine complexes $T\Delta S$ increases approximately linearly with ΔH, this substitution, necessitated by the data sources, should modify only the slope but not the nature of the correlation. The only exception is benzene, which is also the only typical π-donor in the set of compounds.

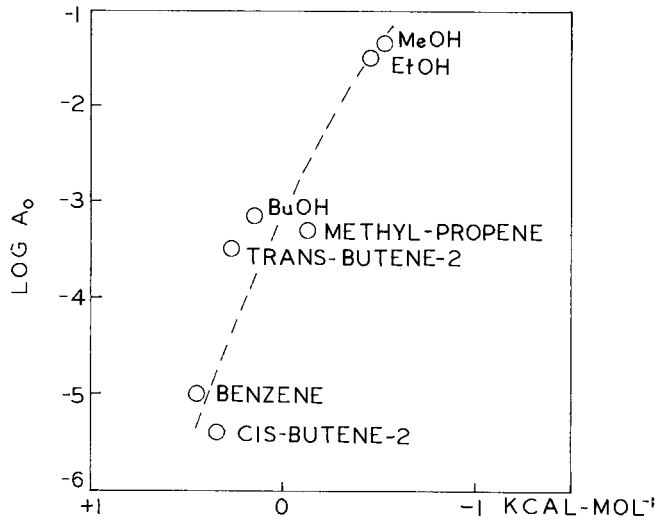


FIG. 4. Free energies of formation for complexes of odorivectors with sulfur dioxide and the olfactory thresholds.

Figure 4 shows correlations between A_0 and ΔF for formation of the charge-transfer complex with sulfur dioxide, O=S=O, which is a π-electron acceptor with the electronaccepting ability not localized on some selected atom of its molecule. The sulfur dioxide complex parameters are from Briegleb's (1961) compilation of other authors' data. The odorivectors that interact more strongly (right) have higher A_0 values. This correlation cannot be explained by a π-acceptor in a receptor or a barrier, since then the correlation should be in reverse. The mechanism for explaining Fig. 4 cannot be based on a steady-state diffusion flux leading toward near-equilibrium between the odorivector concentration in the receptor and the air. The simplest mechanism to qualitatively explain Fig. 4 can be based on the concept of concentration wave. It requires a diffusion path consisting

of two consecutive barriers and would operate by producing a transient response, with the time delay acting as the discriminator parameter. The response time at depth x is approximately:

$$t = \frac{x^2}{2D} = Cx^2\eta \cdot r, \tag{12}$$

where C is a coefficient, and r is the radius of the diffusing molecule. Thus the response time depends primarily on the thickness and the viscosity of the barrier.

The first barrier should act as high diffusion resistance with fast response time and hence low k (Equation (2)) and η. The second barrier can act as a temporary storage phase with the capacity dependent on k and the response time dependent on η. For the same viscosity, the delay in reaching the receptor will be larger if the interaction (activation energy of diffusion) with sites along the diffusion path through the second barrier's matrix is stronger. Receptor sites with differentiated barrier layers will respond in a temporal pattern. This highly speculative hypothesis, coupled with cross-inhibition of responses from adjacent receptors or receptor sites, offers a blueprint for an olfactory mechanism that may be in agreement with the broad, poorly differentiated response observed in electrophysiological studies.

Thus, the correlations between the threshold values and the interactions of the odorivectors with σ- or π-electron acceptors strongly suggest that the receptor or barrier phase acts as an electron acceptor. Hydrogen-donor materials can act as acceptors. However, since the oxygen atom in alcohols is a stronger hydrogen-bonding site than the double bond in alkenes, it appears that the hypothetical electron acceptor responsible for the trend in the Fig. 4 receptor or barrier matrix is not a hydrogen donor.

3.3 Variation of Threshold with Molecular Size

Figure 5 compiles A_0 values for several sets of homologs arranged by the molecular weights of the odorivectors.* The compounds, with the exception of the paraffin series, each have only one functional group and only nonbranched carbon atom chains. The molecules are essentially rodlike. From the left to the right, the contribution of the molecule's functional

* Data from International Critical Tables were not used, since these seem to be in conflict with more recent and more carefully obtained data (even after correction for the wrong order of magnitude).

group to the total interaction energy with a function-specific matrix is increasingly supplemented by interactions supplied by the —CH_2— group chains. The question then is: Why do the A_0 values first decrease and then begin to increase with growing molecular weight?

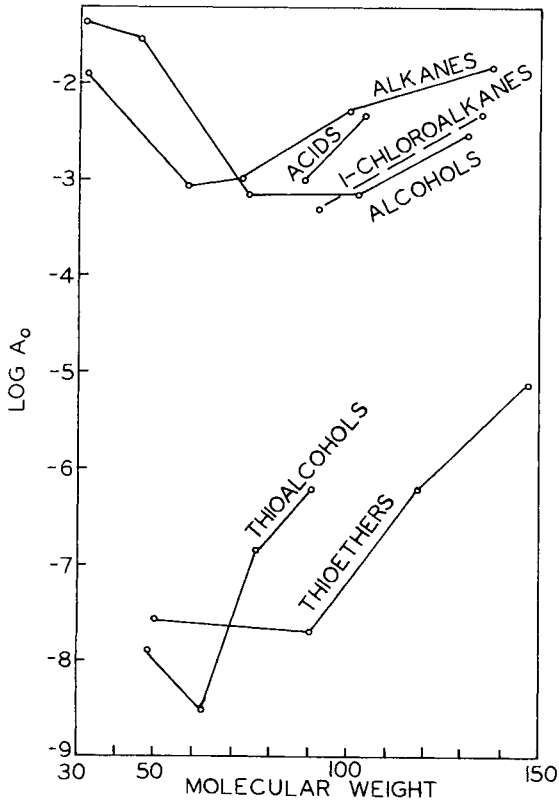


FIG. 5. Relation of A_0 to molecular weight for non-branched chains.

At lower molecular weights, addition of —CH_2— groups enhances intermolecular interaction with the matrix without interfering with the interaction of the functional group with the matrix. With further increase in the number of —CH_2— groups, which coil up somewhat, the functional group becomes less accessible and the compound behaves more and more like a hydrocarbon. This tendency is seen in Fig. 5.

The occurrence of a minimum in A_0 in the *n*-paraffin series needs a separate explanation. Mullins' proposal would require a receptor with a solubility parameter of 6·5 to 7, since δ is 6·5 for butane and 7·1 for pentane.

In addition to *n*-butane and *n*-pentane, δ values in this range are found among branched hydrocarbons with up to 8 or 9 carbons and in fluorocarbons. None of these appear to be likely materials for receptors or barriers in living creatures. However, the increase in A_0 with increasing molecular weight of *n*-alkenes can be explained on the basis of Equations (10) and (4) for any reasonable selection of molecular volume for the receptor or barrier phase.

TABLE 3. ACTIVITY COEFFICIENTS OF *n*-ALKANES IN A HYPOTHETICAL PHASE AND OLFACTORY THRESHOLDS

Number of C atoms	log γ_2 in water	Triethylene glycol	X = average hypothetical log γ_2*	Y log A_0†	$Y-X$ (proportional to log n_c)
5	3·1	1·4	2·25	—3	—5·25
7	4·2	1·65	2·92	—2·3	—5·22
9	5·2	1·9	3·55	—1·8	—5·35
11	6·2	2·1	4·15	—1·4	—5·55

* Log γ_2 values from Pierotti *et al.* (1956).
† Log A_0 from Mullins (1955).

The decrease from C_2 to C_4 may be speculatively explainable by an aqueous matrix. From the solubility of ethane and butane in water at 20°C and 1 atm pressure, k for these gases in water is 0·05 and 0·15, respectively. Thus, to produce, for example, the same flux through an aqueous barrier, the concentration of butane can be smaller than that of ethane. For higher alkanes in aqueous solution the activity coefficient increases rapidly by a factor of approximately 3 per additional carbon atom (Pierotti *et al.*, 1956). This increase is too fast to maintain a constant n_c in Equation (4), since A_0 increases more slowly. However, by assuming a hypothetical phase in which the activity coefficient is a geometrical mean between that in water and that in the less polar triethylene glycol, the data in Table 3 are obtained. Here the last column purports to indicate the constancy of n_c is Equation (4). This exercise illustrates that, by assigning to the receptor or barrier matrix empirically feasible characteristics, the change in thresholds for *n*-alkanes between C_5 and C_{11} can be approximately explained.

3.4 Electron Donor Characteristics of Matrix

Under Sections 3.1 and 3.2 evidence was cited that some threshold effects possibly relate to the electron donor characteristics of the odorivectors. This suggests the presence of electron acceptors in the matrix of a barrier or a receptor phase. In reverse, if striking differences in the olfactory parameters could be associated with the electron acceptor characteristics of odorivectors, this would suggest that the matrix contains discriminatory donors.

TABLE 4. DONORS AND ACCEPTORS REPORTED TO FORM MOLECULAR COMPLEXES

π-Donors:	π-Acceptors
Alkenes	π-donors with halogens, nitro groups, —CN as substituents
Alkynes	SO_2
Aromatics and their Alkyls	Some acid anhydrides
Polyaromatics, their Alkyls	Chloranil
	Quinone
	Trinitrobenzene
	Tetracyanoethylene
σ-Donors:	σ-Acceptors:
Alkylhalides	Halogens
Cyclohexane	
n-Donors:	
Alcohols	
Ethers	
Ketones	
Amines	
Thioalcohols	
Thioethers	
Pyridine (also π)	
Aromatic derivatives of n-donors (also π)	

Table 4 lists classes of donors and acceptors, with some examples of compounds for which the donor or acceptor strengths have been reported. Examination of the table indicates the presence of strongly irritating odorants among smaller molecules in the acceptor class. The acceptors with larger molecules are more pleasant, e.g. nitrobenzene. The A_0 value for nitrobenzene is 2×10^{-3}, not exceptionally low. Larger acceptor molecules do not seem to excel by odor.

Thus, electron donor characteristics may have to be assigned to the barrier or receptor matrix to explain the olfactory activity of the odorivectors that are pronounced electron acceptors. "Olfactory" in the preceeding text does not intend to differentiate trigeminal and olfactory parameters.

4.0 ODOR QUALITY

With the indication that electron donor and acceptor characteristics of the odorivectors and, conversely, of the receptors or barriers are significant factors in determining the olfactory threshold, similar concepts probably can be extended to classification of odors. Amoore (1952) has anticipated the significance of electrophilic and nucleophilic olfactory receptor sites long ago, and Johnston (1965) has recently proposed that these characteristics superimpose over the shape- and size-relevant types.

The mechanism through which threshold effects could convert into odor-type discrimination effects is no doubt very complex. This author sees some possibility in the mechanism discussed in connection with the propagation of concentration waves. The propagation of the concentration waves of, say, odorivectors *A* and *B* through barriers or storage phases arranged in patches on the same sensor or on a neurally related sensor begins as soon as *A* and *B* reach the outer barriers. The solubilities of the odorivector in the patches are arranged so that *A* always reaches the receptor layer first unless the concentration of *B* in the air is very high. Odorivector *A* triggers the neural impulse, perhaps by Davies' (1965) mechanism, and the later arrival of *B* at some other point of the same or related receptor encounters the inhibited neuron. This mechanism should be able to assure identification of *A* through a temporal excitation–inhibition sequence, in the presence of a wide range of *B* concentrations.

5.0 CONCLUSIONS

A review of possible interactions of odorivectors with condensed matrices indicates that electron donor–acceptor types of interactions may be important in determining thresholds and perhaps other olfactory parameters. Mullins' concept of the solubility parameter match or mismatch is not able to explain adequately the odorivector–matrix interactions for smaller molecules, in which the nature of the functional groups dominates the olfactory characteristics. Similarly, the shape and the dipoles of smaller molecules cannot explain the olfactory differences. Donor–acceptor complex formation with the matrix can explain at least a part of the threshold

variations. Furthermore, indications exist that the interaction of the odorivector with an olfactory system may include, in addition to stimulating the sensing structure, selective diffusion barriers and storage phases that introduce temporal patterns.

REFERENCES

ADRIAN, A. D. 1950. *Electroencel. Clin. Neurophysics*, **2,** 377.

ALISON, V. C. and KATZ, S. H. 1919. *Ind. Eng. Chem.* **11,** 336.

AMOORE, J. E. 1952. *Perfum. Essent. Oil Rec.* **43,** 321.

AMOORE, J. E. 1962. *Proc. Sci. Sect. Toilet Goods Assoc.*, Suppl. to No. 37, 1.

AMOORE, J. E. 1965. Psychophysics of Odor. XXX. *Cold Spring Harbor Symposium on Quantitative Biology*. Sensory Receptors. (in press)

ANDREWS, L. J. and KEEFER, R. M. 1964. *Molecular Complexes in Organic Chemistry*. Holden-Day Inc., San Francisco.

BAKER, A. W. and SHULGIN, A. T. 1958. *JACS* **80,** 5358.

BEETS, M. G. J. 1957. Molecular Structure and Organoleptic Quality, *Soc. Chem. Ind. Monograph* No. 1, p. 54, London.

BEETS, M. G. J. 1961. *Amer. Perfumer Aromat.* **76,** 54.

BEETS, M. G. J. 1962. *Perfumer Cosm. Savons*, **5,** 1.

BEIDLER, L. M. 1954. *Ann. N. Y. Acad. Sci.* **58,** 52.

BRIEGLEB, G. 1961. *Elektronen-Donator-Acceptor Komplexe*. Springer Verlag, Berlin.

DAVIES, J. T. and TAYLOR, F. H. 1955. *Perf. Essent. Oil Rec.* **46,** No. 1.

DAVIES, J. T. 1962. *Symp. Soc. Exper. Biol.* **16,** 170.

DAVIES, J. T. 1965. *J. Theor. Biol.* **8,** 1.

DRAVNIEKS, A. 1964. *Ann. N. Y. Acad. Sci.* **116,** 429.

DRAVNIEKS, A. 1965. Current Status of Odor Theories, in press, in *Advances in Chemistry*, Flavors.

GESTELAND, R. C. 1964. Initial Events in the Electro-Olfactogram. *Ann. N. Y. Acad. Sci.* **116,** 440.

GLUECKAUF, E. 1958. *In Gas Chromatography*, Desty, Editor, p. 69, Academic Press, N. Y.

HILDEBRAND, J. H. and SCOTT, R. L. 1962. *Regular Solutions*. Prentice Hall, Inc.

JANZ, G. J. 1958. *Estimation of Thermodynamic Properties of Organic Compounds*. Academic Press, Inc., New York.

JELLINEK, J. S. 1964. The Effect of Intermolecular Forces on Perceived Odor. *Ann. N. Y. Acad. Sci.* **116,** 725.

JOHNSTON, J. W. 1965. Personal Communication.

KATZ, S. H. and TALBERT, E. J. 1930. Intensities of Odors and Irritating Effects of Warning Agents for Inflammable and Poisonous Gases. U.S. Dept. of Commerce, Bureau of Mines, Technical paper, 480.

LITTLEWOOD, 1962. *Gas Chromatography*. Academic Press, New York.

MOELWYN-HUGHES, E. A. 1961. *Physical Chemistry*. Pergamon Press, New York.

MONCRIEFF, R. W. 1957. *Proceedings 2nd International Congress of Surface Activity*, **4,** 321.

MOULTON, D. G. and TUCKER, D. 1964. *Ann. N. Y. Acad. Sci.* **116,** 380.

MUHS, M. A. and WEISS, F. T. 1962. *JACS* **84,** 4697.

MULLIKEN, R. S. 1952. *J. Phys. Chem.* **56,** 801.

MULLINS, L. J. 1955. *Ann. N. Y. Acad. Sci.* **62,** 249.

PANGBORN, R. M., BERG, H. W. ROESSLER, E. B., and WEBB, A. D. 1964. *Motor Skills* **18,** 91. Southern Universities Press, California.

PIEROTTI, G. J., DEAL, C. H., DERR, E. L. and PORTER, P. E. 1956. *J. Am. Chem. Soc.* **78**, 2989.

PITZER, K. S. and BREWER, L. 1961. *Lewis-Randall Thermodynamics*, p. 283–294. McGraw-Hill Book Company, Inc., New York.

PULLMAN, B. and PULLMAN, A. 1961. *Quantum Biochemistry*, Interscience Publishers, New York.

ROSANO, H. L. and SCHEPS, S. Q. 1964. *Ann. N. Y. Acad. Sci.* **116**, 590.

RUZICKA, L. 1957. *Fundamentals of Odour Chemistry: A Summary in Molecular Structure and Organoleptic Quality*, Soc. Chem. Ind. Monograph No. 1, London.

SCHUTZ, H. G. 1964. *Ann. N. Y. Acad. Sci.*, **116**, 217.

STUIVER, M. 1958. *Biophysics of the Sense of Smell.* Doctoral Thesis, Groningen, Holland.

TAMRES, M. 1952. *JACS*, **74**, 3375.

TSUBOMURA, M. and LANG, R. P. 1961. *J. Am. Chem. Soc.* **83**, 2085.

WRIGHT, R. HAMILTON. 1954. *J. Appl. Chem.* **4**, 611.

WRIGHT, R. HAMILTON. 1957. *Odour and Molecular Vibration, in Molecular Structure and Organoleptic Quality*, p. 91, Soc. Chem. Ind. Monograph No. 1, London.

WRIGHT, R. HAMILTON. 1964. *Ann. N. Y. Acad. Sci.* **116**, 652.

WRIGHT, R. HAMILTON. 1964. *The Science of Smell.* Basic Books, Inc., New York, N. Y.

WRIGHT, R. HUEY, and MICHELS, K. M. 1964. *Ann. N. Y. Acad. Sci.* **116**, 535.

SPATIO-TEMPORAL PATTERNING OF RESPONSE IN THE OLFACTORY SYSTEM

D. G. MOULTON

Department of Biology, Clark University, Worcester, Mass.

INTRODUCTION

The electrophysiological approach has so far failed to demonstrate, with any clarity, the basis for odor quality in vertebrates. It may be that there exists—as in insects—receptors having a relatively high degree of specificity for a given odor or group of odors. If so, they have not been convincingly displayed. It may also be that the mode of dispersal and binding of odorant molecules across the receptors sheet, alone permits every odor to be distinguished from most others. If so, it is not yet obvious.

On the other hand, not all odors stimulate in the same way. For example, they can, to some extent, be segregated by the spatio-temporal patterns of excitation which they elicit in the olfactory bulb. Part of the evidence for this derives from studies of alert rabbits having electrodes chronically implanted in, or close to, the mitral cell layer of the bulb (Moulton, 1963; 1965). These leads pick up spike discharges of neurones, each separated by one synapse from the receptors. Thus activity from a number of bulbar points or "sites" can be studied simultaneously. In this report such excitation patterns are examined further. But first, there follows a summary of some previous findings.

When an alert rabbit is breathing filtered air, all bulbar sites show a tonic multispike discharge. Introduction of an odorant into the air stream generally produces a burst of increased activity with each inspiration, although if the concentration is high the tonic discharge may be partially suppressed. However, if we restrict attention to the median segments of the stimulus–response curves, certain consistent distinctions in the spatial excitation patterns for different odorants are sometimes evident.

For example, in a group of 15 selected test odorants, many could be identified by a peculiarity in the pattern—some feature which persisted from trial-to-trial, from day-to-day, and—within the dynamic range of sensitivity—from concentration-to-concentration. But the features most

useful in characterizing an odorant were seldom related in any obvious way to the gross regional distribution of electrodes in the bulb (although such differences exist). Rather they involved, for example, the behaviour at a particular site; even the contrast in responses to the same odorants at neighboring sites. (However, sites far apart may also show striking differences in sensitivity.)

Because of the difficulty in placing electrodes in precisely the same site in any two rabbits, it is not surprising that a given odor elicited different excitation patterns in different animals. An exception is the tendency of some odorants—such as butyl ether to be more nearly equally effective in stimulating all sites than are the majority of odorants (Moulton, 1965).

Since such odor-specific patterns of excitation may give some insight into the basis for odor quality it is important to examine them further.

MATERIALS AND METHODS

The techniques of implanting electrodes and recording multispike activity from the olfactory bulb of the alert rabbit have been outlined previously (Moulton, 1963; 1965; Moulton and Tucker, 1964), and the essential points of the stimulating and recording procedures are summarized in Figs. 1 and 2. Figure 1 shows in simplified form the olfactometer and test chamber. Filtered air is split into three streams, one of which is saturated with a selected test odorant while a second is used to dilute the first. The third stream supplies the rabbit with filtered air between trials but can also be used to give a second stage of dilution. The air and odor streams reach the

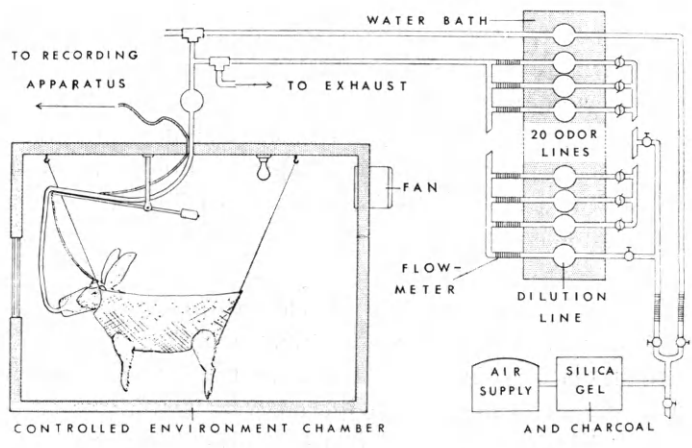


FIG. 1. Method of stimulating rabbit with odors.

animal by way of a teflon face mask, and are exhausted through vents at the rear of the mask. All parts in contact with the air or odor streams (including solenoid valves) are of glass or teflon. The rabbit is suspended from a nylon hammock and can be observed through a one-way glass screen.

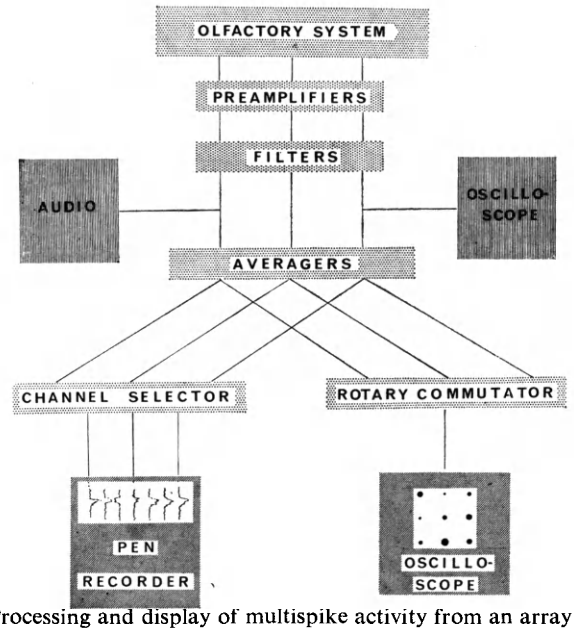


FIG. 2. Processing and display of multispike activity from an array of electrodes chronically implanted in the olfactory system.

A separate odor line, not shown in Fig. 1, provides another means of stimulating the animal. It consists of a line of filtered air into which air saturated with a given test odorant can be injected. This is done simply by inserting a hypodermic needle into the line and delivering 30 cc of vapor at a known, controlled rate. The technique is useful for screening a large number of odorants. A few of them can then be selected and incorporated into the olfactometer which lacks this flexibility, but provides closer control of the stimulus.

The animals are first habituated to this test environment until the heart rate declines to a relatively stable plateau. Thereafter only data obtained from animals that are alert but inactive, are analyzed.

As is indicated in Fig. 2, the multichannel output from the array of implanted electrodes is first fed through amplifying circuits. At this point the spike discharge from any channel (for simplicity, only three are shown) can be monitored on the oscilloscope or loud speaker. In each channel the

impulses are then processed to a smooth curve by means of an "averager" (short time averaging circuit or "integrator"), and can be displayed either as a series of pen traces, or as a mosaic of spots of light on the oscilloscope face. In the mosaic, the intensity of each spot is modulated by the activity occurring at the corresponding pair of electrodes in the olfactory bulb. Thus the array of spots can be positioned to provide a two-dimensional map of the electrode array.

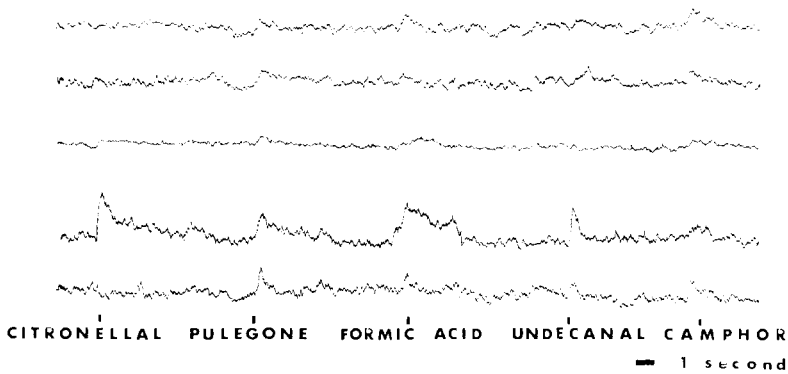


FIG. 3. Integrated multiunit responses of the olfactory bulb to odor stimulation. Stimulus duration: 3 sec. Responses were obtained at least 10 min apart but have been placed together for comparison.

Display of the signals as a series of pen traces has the advantage that it is easier to quantify the responses. Also, as can be seen in Fig. 3, the temporal evolution of response at a particular site is more immediately evident.

RESULTS AND DISCUSSION

In Fig. 3 each horizontal trace is a pen recording of averaged activity occurring at one site in the olfactory bulb. Responses of 5 sites to 5 odorants are shown (all concentrations are—at most—10^{-2} of saturation at 22°). The responses to each odorant were derived simultaneously. Thus each vertical row of deflections represents the responses of 5 sites to one odorant.

It is clear from this figure that the odorants differ markedly in their relative effectiveness at the 5 sites. Citronellal, for example, elicits little response at the bottom trace but a vigorous response at the one above. Camphor has the reverse effect. There are also differences in the temporal evolution of response to a given odorant. Thus the maximum amplitude of response is reached within the first half second at most sites but at some

there may be a more gradual increase (as in the response, seen in the second trace, to undecanal). While there is much variation in the detailed pattern of such responses, both from trial-to-trial and from concentration-to-concentration, the more salient features often persist. As was previously shown these may be sufficiently characteristic to distinguish an odor from other members of a test group (Moulton, 1965). However, at the upper segment of the stimulus–response curve, where high concentrations are involved, the distinguishing features may no longer be present (due, in part, to suppression of the interburst tonic discharge at some sites but not others). Similarly at the lower end of the stimulus-response curve, where low concentrations are involved, there is usually too much variation in the excitation pattern, to allow identification of a given odor.

An alternative method of displaying responses is shown in Fig. 4. To obtain these photographs, the averaged tonic activity from each of 9 pairs

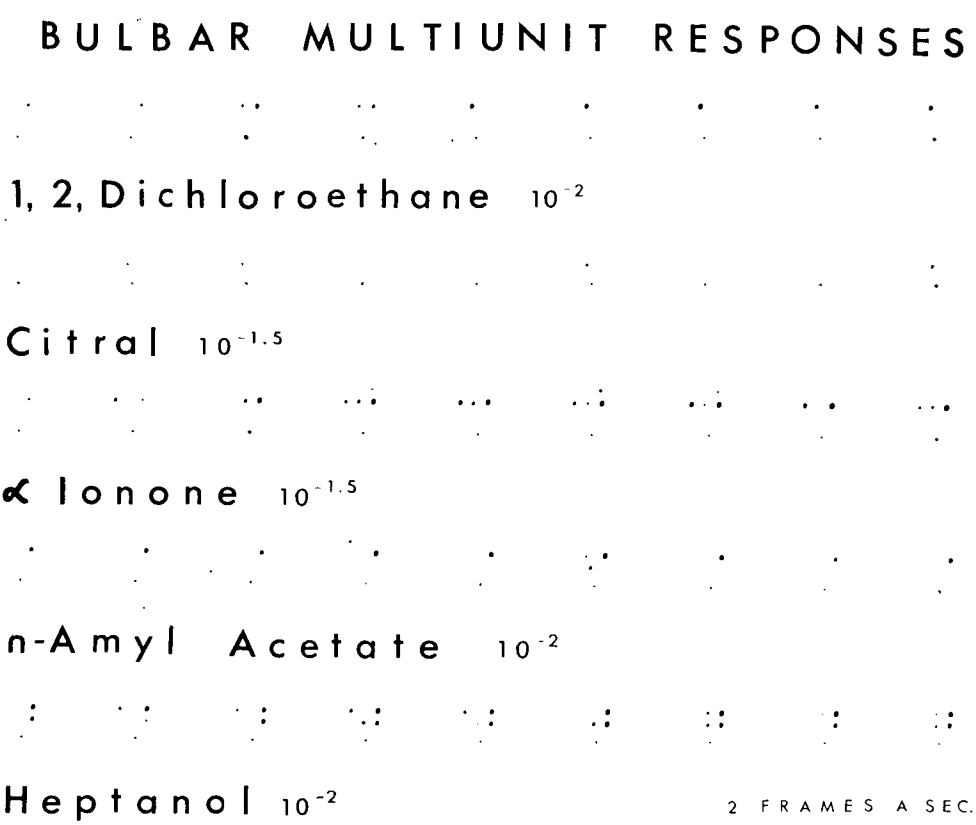


FIG. 4. Integrated bulbar multiunit responses displayed as a mosaic of dots. (For explanation see text.)

of electrodes was displayed as a mosaic of spots on the face of the oscilloscope (according to the system outlined in Fig. 2). The intensity of the spots were first adjusted so that they were about equal and then slowly decreased until they were no longer visible. Thus an increase in activity at a given site induced the appearance of a spot at a fixed position on the oscilloscope face—the greater the activity the larger and denser the spot. This method is particularly effective in picking out the sites most responsive to a given odorant. The oscilloscope face was filmed twice a second. The first 9 frames following the arrival of the stimulus at the face mask are shown here for each of five odorants. A frame can be located, and the position of any induced spot identified, by the marker dot at the bottom of each frame. The presence of the dot also indicates that an odor was being presented. Notice in Fig. 4, that in this print none of the odors tested induced the appearance of more than four spots out of a possible nine. However, each odor induced a different combination of spots. (Unfortunately it was difficult—with the photographic techniques available—to reproduce the full gradiations in intensity with accuracy, and the fainter spots are not evident here.)

Figure 5 shows a similar set of comparisons derived by the same methods except that in this case the spots were not dimmed to the same extent before testing the animal with odors. Thus instead of being entirely blank the prestimulus frames show some scattered spots, reflecting varying levels of tonic activity. Here one such prestimulus frame begins each set of 8 frames. Butyl ether was the first and last stimulus presented. (Figures 5 and 6 were derived from different animals.)

To compare excitation patterns for a larger number of odors it is convenient to express result as bar graphs. Those in Fig. 6 are derived from one animal. In each block of 6 bars, each bar represents the aggregate of 5 successive responses at one bulbar site to the same odor. The index of response is the maximum deflection of the pen recorder during the first 3 seconds of stimulus presentation. Responses at the 6 sites were recorded simultaneously.

Clearly, some of these profiles show close similarities. There are, in fact, highly significant correlations between patterns for such odorants as benzothiazole and anisole, *l*-menthol and *d*-limonene. On the other hand, there are pairs of patterns which show no statistically significant correlation—for example amyl acetate and α-ionone, 2-butanone and citronellal. Furthermore, odorants which appear to elicit similar spatial patterns of excitation, can sometimes be distinguished on the basis of temporal differences in the development of responses.

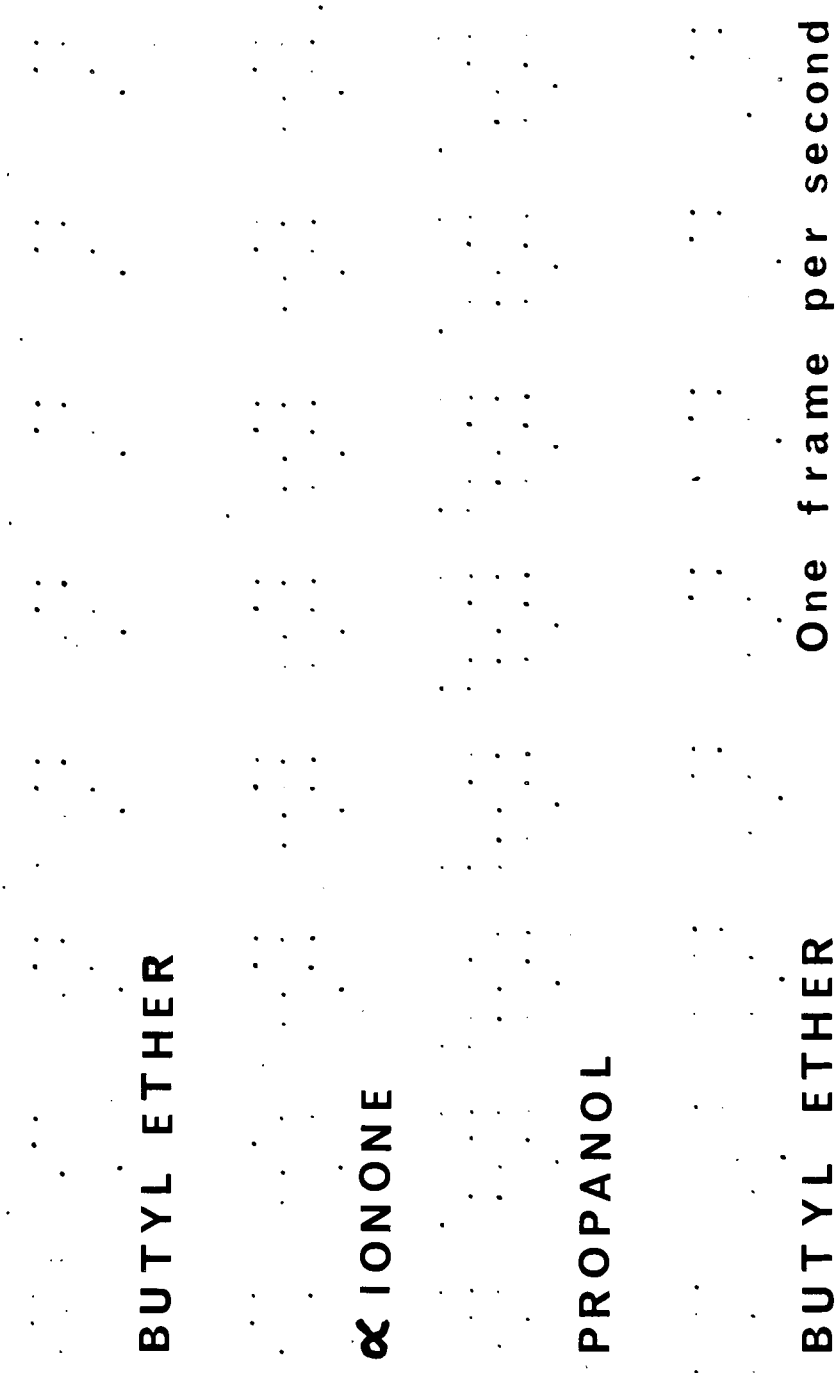


FIG. 5. Integrated bulbar multiunit responses displayed as a mosaic of dots.

No doubt each odor elicits a far richer spatio-temporal pattern of excitation in the olfactory bulb than can be demonstrated by available techniques. But even the limited evidence reported here suggests that such a patterning could play an important role in odor quality discrimination.

(This work was done in the Department of Biological Sciences, Florida State University, Tallahassee, Florida.)

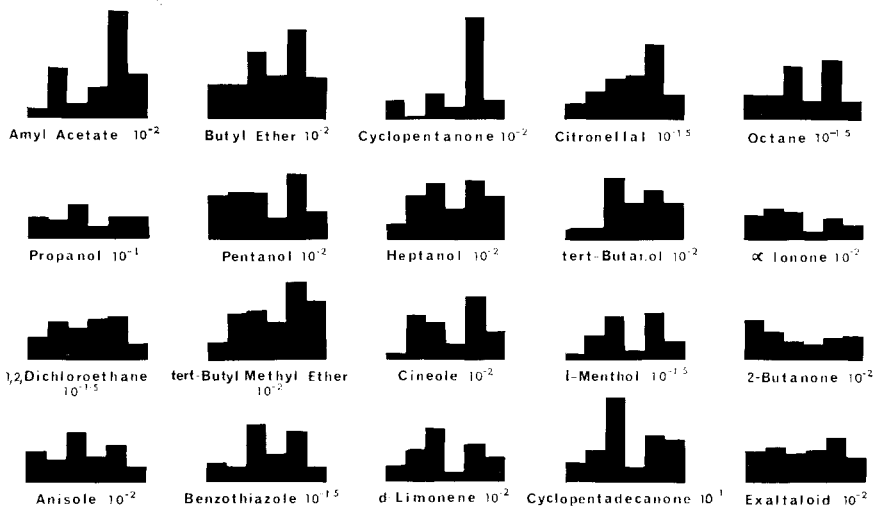


FIG. 6. Integrated multispike responses from 6 sites in the olfactory bulb to 20 odors. Concentrations relate to the saturated vapor at 20°C.

REFERENCES

MOULTON, D. G. (1963). Electrical activity in the olfactory system of rabbits with indwelling electrodes. In *Olfaction and Taste*. Y. Zotterman, editor: 71–84. Pergamon Press, Oxford, England.

MOULTON, D. G. (1965). Differential sensitivity to odors. In *Sensory Receptors, Cold Spring Harbor Symposia in Quantitative Biology* (in press).

MOULTON, D. G., and TUCKER, D. (1964). Electrophysiology of the olfactory system. *Ann. N. Y. Acad. Sci.* **116**, 380–428.

THE EFFECT OF CONCENTRATION UPON THE SPATIOTEMPORAL CODING OF ODORANTS*

MAXWELL MARK MOZELL

Physiology Department, State University of New York
Upstate Medical Center, Syracuse, New York

ONE of the major problems in olfaction is to determine the mechanisms which, at the mucosal level, play a role in olfactory discrimination. Single unit recordings from the olfactory bulb (Adrian, 1954) and olfactory mucosa (Gesteland *et al.*, 1963) indicate that olfactory discrimination may depend, at least partially, upon a selective sensitivity of individual receptors for different groups of chemicals. An additional mechanism, supposedly operating in conjunction with this selective sensitivity, was proposed by Adrian (1950, 1954) who extrapolated to the olfactory mucosa conclusions based upon multi-unit recordings from the olfactory bulb. He suggested that the molecules of different chemicals distributed themselves differently across the mucosa. In addition, he found evidence of a temporal analysis at the bulbar level which he likewise extrapolated to the mucosa. However, direct evidence of a spatiotemporal analysis at the level of the mucosa was still lacking. In an attempt to collect such data Mozell (1964a, 1964b) made use of the distribution of the olfactory nerve branches to different mucosal regions (Fig. 1). By recording the antidromically conducted discharges elicited by electrical stimulation of different olfactory nerve branches, it was determined that these nerve branches subserved different regions of the mucosa. Thus, the magnitude of the activity in different regions of the mucosa could be sampled by recording from the appropriate nerve branches. It was found that the magnitude of the summated neural discharge recorded from the most lateral nerve branch and that recorded from the most medial nerve branch were in a ratio (i.e. lateral nerve branch discharge/medial nerve branch discharge) which was dependent upon the chemical presented. This suggested a spatial analysis of odorants. Likewise,

* This work was supported by NIH Grant NB 03904.

the latency difference between the lateral nerve branch response and the medial nerve branch response depended upon the chemical used, thus suggesting a temporal analysis. However, the chemical vapors presented to the animal were prepared by simply bubbling purified air through the odorant so that the air became saturated at room temperature. This would result in a different concentration for each of the chemicals used (geraniol, citral,

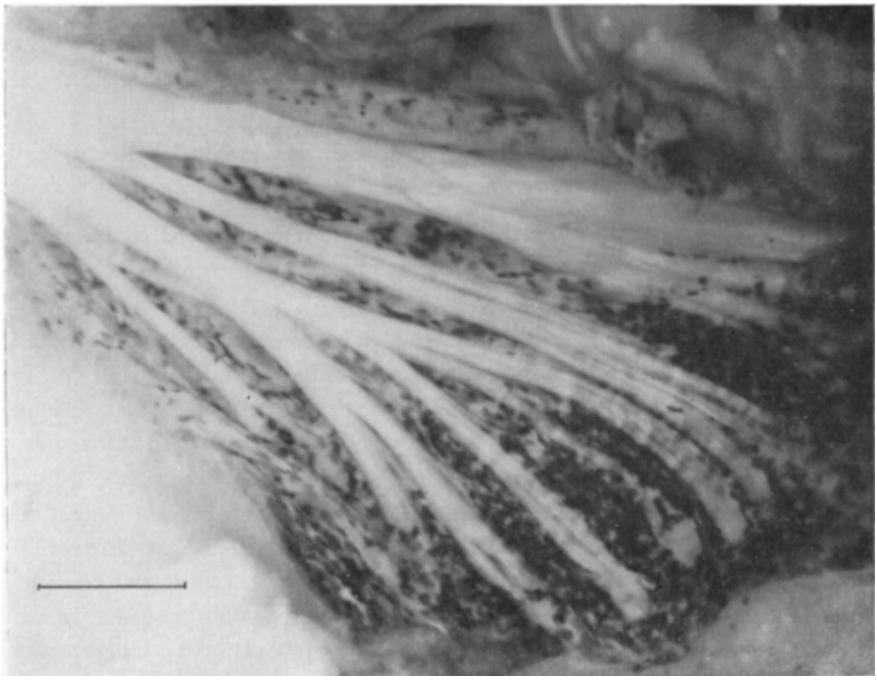


FIG. 1. Photograph of the frog olfactory nerve as it branches over the dorsal aspect of the olfactory sac. The calibration line represents 1 mm.

d-limonene, and octane) since they all have different vapor pressures at room temperature. Thus, the apparent spatiotemporal code could be dependent either upon molecular concentration or molecular species.

In order to distinguish between these two possible dependencies it was necessary to present the chemical vapors at equal partial pressures. This was accomplished in the present experiment by means of a flow dilution olfactometer. This olfactometer could be set to dilute the saturated, odorized air stream with the appropriate quantity of purified air needed to obtain a given partial pressure of the odorant. The resultant concentration,

as specified at the naris, was then presented to the frog *(Rana catesbeiana)* in the form of an artificially produced sniff of known volume and flow rate. This was accomplished by closing off one naris and connecting the other naris to a side arm coming from the main channel of the olfactometer. A teflon tube connected at one end to a Harvard withdrawal pump was passed into the buccal cavity through an appropriately drilled hole. The esophagus and trachea were closed off and the mouth was shut so as to be air tight. Thus, the withdrawal pump could provide an artificially produced sniff by sucking a sample of air from the olfactometer into the frog's olfactory sac. Deodorized, humidified air was constantly passed through the olfactory sac between stimulations.

The recording apparatus and treatment of these data have been described in detail previously (Mozell, 1964a). In summary, one of the measures used is the ratio of the amplitudes of the two summated neutral discharges as recorded on a Honeywell Visicorder (i.e. lateral nerve branch discharge/ medial nerve branch discharge). This ratio can be interpreted as a measure of the spatial coding of different chemicals. The other measure, the temporal measure, is the latency difference or time interval between the summated lateral and the summated medial nerve branch discharges.

Photographs of visicorder records showing the summated neural discharges of one animal for one presentation of each chemical at each partial pressure are shown in Fig. 2. The expected general increase in the discharges on both the lateral and medial nerve branches as the partial pressure is increased can easily be observed by reading across the rows. Of more importance to the present discussion is that even when the different chemicals are at the same partial pressure (reading down the columns) they still produce different lateral-to-medial-nerve-branch ratios. Somewhat less obvious by simple visual inspection is the fact that the latency differences also differ characteristically between chemicals even when their vapors are held at equal partial pressures.

It is, of course, necessary to test the consistency of these results within each animal and across several animals. Consequently, each chemical at each partial pressure was presented at least three times to a given animal and the median amplitude ratio and latency difference for each concentration of each chemical was computed. Then within each concentration the chemicals were ranked in order of the magnitude of their median ratios with "1" representing the smallest ratio and "4" representing the largest ratio. This was also done according to the magnitude of the latency differences. This procedure was followed in ten animals. Finally the percentage of the total number of animals in which each chemical fell into a given rank

within each concentration was determined. These data are presented in Table 1 (ratios) and Table 2 (latency differences).

Table 1 shows that although no chemical yielded ratios confined only to a single rank in all animals at any given partial pressure, there is nevertheless a strong tendency toward a consistent orderliness. In addition, the order appears to be the same at all concentrations. For instance, at all partial pressures octane yielded the largest ratios (highest ranks) most

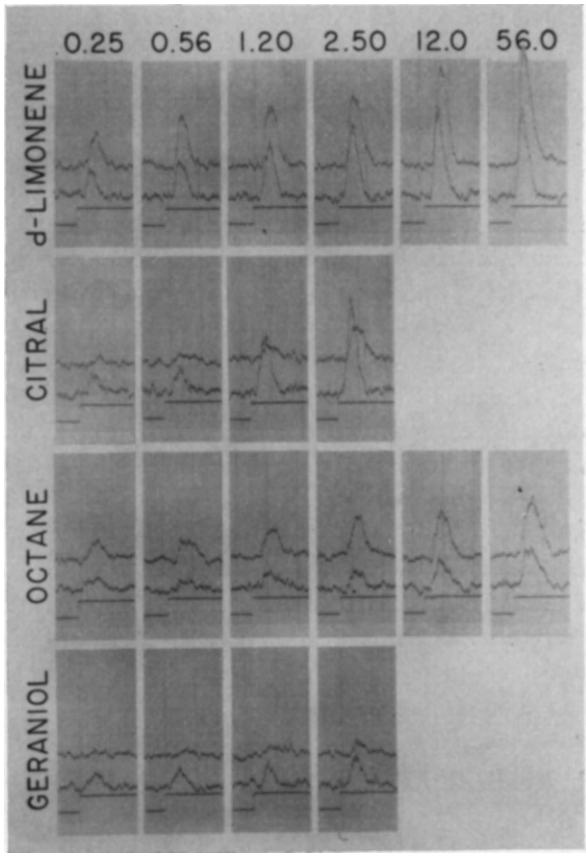


FIG. 2. Photographs of visicorder records showing the summated discharges for one animal. The upper response in each pair is from the lateral nerve branch and the lower response is from the medial nerve branch. The partial pressures noted along the top are in terms of mm $Hg \times 10^{-2}$. At room temperature (23°C) the partial pressures of 12×10^{-2} and 56×10^{-2} mmHg cannot be reached for citral and geraniol. The signal marker shows only the onset of the stimulation. The distance between the vertical chart lines signifies 10 seconds.

TABLE 1. THE PERCENTAGE OF THE TOTAL NUMBER OF ANIMALS ($N = 10$) IN WHICH THE RATIO PRODUCED BY EACH CHEMICAL FALLS INTO A GIVEN RANK AT EACH PARTIAL PRESSURE

Chemical		Octane				*d*-Limonene				Geraniol				Citral				W	P
Rank		1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4		
Partial pressure ($\times 10^{-2}$ mm Hg)	0·25	0	0	20	80	0	0	80	20	50	50	0	0	50	50	0	0	·84	<·01
	0·56	0	0	30	70	0	0	70	30	30	70	0	0	70	30	0	0	·83	<·01
	1·2	0	0	15	85	0	0	85	15	50	50	0	0	50	50	0	0	·85	<·01
	2·5	0	0	0	100	0	0	100	0	65	35	0	0	35	65	0	0	·91	<·01
	12·0			10	90			90	10										
	56·0			15	85			85	15										

W = Kendall's coefficient of concordance.
P = probability of W.
Note: At room temperature (23°C) the partial pressures of 12×10^{-2} and 56×10^{-2} mmHg cannot be reached for citral and geraniol.

TABLE 2. THE PERCENTAGE OF THE TOTAL NUMBER OF ANIMALS IN WHICH THE LATENCY DIFFERENCE PRODUCED BY EACH CHEMICAL FALLS INTO A GIVEN RANK AT EACH PARTIAL PRESSURE

Chemical		Octane				*d*-Limonene				Geraniol				Citral				*W*	*P*
Rank		1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4		
Partial pressure ($\times 10^{-2}$ mmHg)	0·56	43·7	18·8	25	12·5	43·7	56·3	0	0	0	12·5	50	37·5	12·5	12·5	25	50	·40	·05
	1·20	60	25	10	5	40	60	0	0	0	15	35	50	0	0	55	45	·65	·01
	2·5	55	45	0	0	45	55	0	0	0	0	40	60	0	0	60	40	·80	·01
	12·0	65	35			35	65												
	56·0	75	25			25	75												

W = Kendall's coefficient of concordance.
P = probability of W.

often and *d*-limonene yielded the next to largest. Neither *d*-limonene nor octane ever yielded ratios which ranked first or second. Citral and geraniol were always ranked first or second and never third or fourth. The high values for Kendall's coefficient of concordance (W) at each partial pressure and their high statistical significance (P) also testify to the great degree of orderliness across animals at each partial pressure. Consequently, it would appear that the ratios produced by different chemicals at equal concentrations do differ in a characteristic order and that this same order is maintained as concentration is varied. This strongly suggests that the spatial code is dependent upon molecular species rather than upon molecular concentration.

Similar conclusions concerning the order of the latency differences can be drawn from Table 2. However, this can be done with somewhat less certainty than for the ratios since these data are more variable. These data are especially variable at the lower partial pressures, but this may be due more to the difficulty in measuring the latencies of small summated responses (see Fig. 2) than due to some underlying biological factor. Indeed, at the lowest concentration ($0{\cdot}25 \times 10^{-2}$ mmHg) the lateral nerve responses were so small that no attempt was made to report the latency differences. As the concentration is increased there is more internal consistency in the data. A definite tendency for the different chemicals to yield latency differences that fall into the same ranks at equal partial pressures in different animals is apparent. This is most clear at the $2{\cdot}5 \times 10^{-2}$ mmHg level and a little less clear at the $1{\cdot}2 \times 10^{-2}$ mmHg level. The W and P values again attest to the statistical significance of the orderliness although to a lesser degree than they did for the ratios. It should also be noted that the order tends to be the same for different concentrations. Thus, at equal concentrations the latency differences produced by different chemicals differed in a characteristic way and this did not change as the concentration was varied. Consequently, there is a strong suggestion that the temporal code noted previously is dependent upon the molecular species rather than the molecular concentration.

The above results can be taken as support for the concept of a spatio-temporal analysis of incoming vapors at the level of the olfactory mucosa which may play a role in the discrimination of different odorants.

REFERENCES

ADRIAN, E. D., 1950. Sensory discrimination with some recent evidence from the olfactory organ, *Brit. Med. Bull.*, **6**, 330.

ADRIAN, E. D., 1954. The basis of sensation—some recent studies of olfaction, *Brit. Med. J.*, **1**, 287.

GESTELAND, R., LETTVIN, J. Y., PITTS, W. H. and ROJAS, A., 1963. In *Olfaction and Taste*, edit. by Zotterman, Y., 9, (New York, Pergamon Press).

MOZELL, M. M., 1964a. Olfactory discrimination: electrophysiological spatiotemporal basis, *Science*, **143**, 1336.

MOZELL, M. M., 1964b. Evidence for sorption as a mechanism of the olfactory analysis of vapors, *Nature*, **203**, 1181.

LOW FREQUENCY MOLECULAR VIBRATION IN RELATION TO ODOR

A. DEMERDACHE and R. H. WRIGHT

British Columbia Research Council
Vancouver 8, Canada

INTRODUCTION

When certain air-borne vapors enter the nose, sensations are perceived which may be odors or trigeminal sensations, or combinations of both. We are here concerned with odors only, and if there is any reason to think that a substance, such as phenol, is giving a mainly trigeminal response we will exclude it from present consideration.

We will use the adjective "osmic" to indicate relevant properties of odorous substances, and the adjective "olfactory" to indicate the relevant qualities of odor sensations.

The physical chemistry of the process by which osmic properties initiate olfactory sensations is unknown as long as the specifically osmic properties of molecules are not identified. Non-specific osmic properties are well known—the best example is volatility—but they throw no light on the olfactory process.

The osmically specific properties might be identified in either of two ways.

We could construct a matrix comprising all the measurable physical and chemical properties and all the observable olfactory qualities of a great many odorous substances, and look for certain qualities to regress on certain properties. This has been tried, using a computer, without much success apart from obvious non-specific correlations like vapor pressure and molecular weight (Schutz, 1961).

The alternative is to guess the answer and to look for the kinds of correlation that would exist if the guess is correct. This is the procedure to be followed here, and our guess will be the hypothesis that molecular vibrations of some sort constitute the *specifically* osmic properties of odorous substances.

PART 1

(A. DEMERDACHE)

SPECTROSCOPIC BACKGROUND AND NORMAL MODE CORRELATION

To test the vibrational hypothesis properly, two conditions are necessary, namely, complete vibrational assignments and reliable odor evaluations. The simple monosubstituted derivatives of benzene come closer than almost any other group towards complying with the first requirement, and at the same time, a number of them have a distinctive "bitter almond" odor (Wright, 1954; Klouwen, 1963).

Previous papers by Wright (1954) and by R. Huey Wright (1963) depended on Raman data. Only in the last few years have any appreciable numbers of substituted benzene compounds been systematically studied, and there is still no complete agreement. To make complete and accurate vibrational assignments (of both symmetry class and mode) calls for a many-sided attack.

For example, Jakobsen (1965), in establishing the vibrational assignments of benzonitrile, worked with the deuterated as well as the normal compound, and made measurements with a Cary Model 81 Raman spectrometer and a photographic instrument, and supplemented these with infrared measurements on Perkin–Elmer spectrophotometers Models 221G, 102 and 301, using observations of the vapor as well as the liquid, and of the solid at temperatures down to —190°C. The interpretation also called for analogies with observations on phenyl acetylene (Evans, 1960), C_6D_5F (Steele, 1960), and C_6H_5F (Scott, 1956).

This illustrates the monumental effort in completely interpreting the vibrational modes of this relatively simple compound, and explains our heavy dependence on the literature. Our own experiments with a Perkin–Elmer Model 301 instrument were mainly to repeat the spectra on the newer instrument and to check band intensities. A critical bibliography of the monosubstituted benzenes is included as Appendix 2 to this paper.

The first complete discussion of the assignment of characteristic vibration frequencies of substituted benzenes is that by Randle and Whiffen (1955) later extended by Bogomolov (1960). Of the 30 fundamental frequencies, or normal modes, of C_6H_5X (where for simplicity X is regarded as a point mass although it may have up to six atoms other than hydrogen), five are of the type C—H stretching with frequencies around 3050 cm^{-1}, 19 others are insensitive to the nature of X, and the remaining six vibrations are all sensitive to the nature of X. Randle and Whiffen treated the

TABLE 1. CHARACTER TABLE C_{2v} GROUP

Symmetry species	Elements of symmetry					
C_{2v}	E	C	$\sigma(xy)$	$\sigma(yz)$		
A_1	1	1	1	1	$\tilde{z}$	x^2, y^2, z^2
A_2	1	1	−1	−1	Rz	xy
B_1	1	−1	+1	−1	$\tilde{x}, Ry$	xz
B_2	1	−1	−1	+1	$\tilde{y}, Rx$	yz

1. A and B are not degenerate.
2. A sym. with respect to C_2-axis, B anti-sym.
3. Subscript 1 & 2 attached to A's and B's means sym. and anti-sym to xz plane.
4. $\tilde{z}$ coordinate transforms as A_1.
5. A_1, B_1 and B_2 infrared active, A_2 is inactive.
6. A_1, B_1, B_2 and A_2 all Raman active.

monosubstituted benzenes as if they belonged to the symmetry group C_{2v} for which Table 1 is the spectroscopist's "character table". It summarizes all the symmetry properties of this group, and indicates infrared and Raman activities of the different symmetry species.

Table 2, Stephenson (1960), gives average values for the normal mode frequencies of the monosubstituted benzenes (column 3). Column 4 shows the corresponding values for the unsubstituted benzene molecule. The last column illustrates the point that only six vibrations are affected by the substituent.

Figures 1 and 2 illustrate approximately the nature of the oscillations, represented in Table 1. The diagrams are deduced from Ingold's (1946) studies of monodeuterated benzene. The forms were largely determined by simple geometrical reasoning based on the symmetry and orthogonality of the normal coordinate, without any prior knowledge of the frequencies or the force system. Recently Scherer (1963) using powerful methods of calculation has drawn the in-plane vibrations of 16 chlorinated benzenes, but Ingold's simpler presentation is more than adequate for our purpose.

The method of representation in Fig. 1 is not conventional. In each diagram, the benzene ring with the six C—H bonds is drawn to scale and the equilibrium position is indicated by thin lines. The bold lines show the shape the benzene ring acquires at one extreme of the vibrational movement. The thick line extending out from one carbon atom represents a substituent group. The thin arrows indicate the direction of motion. The figure is divided into sections in which modes of vibration having common features are grouped. For example, the upper right rectangle in

TABLE 2. AVERAGE VALUES FOR THE FREQUENCIES OF MONOSUBSTITUTED BENZENES

Vibration No. (Herzberg notation)	C_{2v} symmetry species	Frequency cm^{-1}	Benzene	% change
1	A_1	*ca.* 3062	3062	0
2	A_1	650–800	992	−(20–34)
3	B_2	1270	1340	−5
4	B_1	751	673	+11·0
5	A_1	*ca.* 3060	3060	0
6	A_1	1001	1010	−1
7	B_1	982	995	−1·3
8	B_1	697	703	−1·0
9	B_2	1324	1310	−1·0
10	B_2	1072	1152	−7·4
11	A_2	837	850	−1·5
11′	B_1	908	850	+6·8
12	A_1	1050–1220	3080	−(60·5–66·8)
12′	B_2	*ca.* 3080	3080	0
13	A_1	1499	1485	+1·0
13′	B_2	1451	1485	−2·3
14	A_1	1029	1037	−1·0
14′	B_2	200–400	1037	−(60–80)
15	B_2	*ca.* 3047	3047	0
15′	A_1	*ca.* 3047	3047	0
16	B_2	1588	1596	−1
16′	A_1	1606	1596	−1
17	B_2	1156	1178	−2
17′	A_1	1177	1178	0
18	B_2	620	606	+2·0
18′	A_1	250–525	606	−(13–60)
19	A_2	962	975	−1·0
19′	B_1	450–500	975	−(49–55)
20	A_2	406	405	0
20′	B_1	150–250	405	−(38–62)

Fig. 1 shows two normal modes which are mainly described as ring deformations, though this is clearly a very approximate way to picture an oscillation that also involves some C—C stretching.

The four hatched diagrams represent X-sensitive oscillations. Two of them involve substantial motion of the bond joining the substituent to the ring, and the other two are the ring "breathing" and skeletal bending modes which involve substantial motions of the entire ring.

Figure 2 gives isometric, three-dimensional views of the nine out-of-plane modes of the monosubstituted molecules. The equilibrium configurations are shown by dots. The closely hatched areas fall below the benzene plane and the lightly hatched areas above it. The unhatched areas do not move.

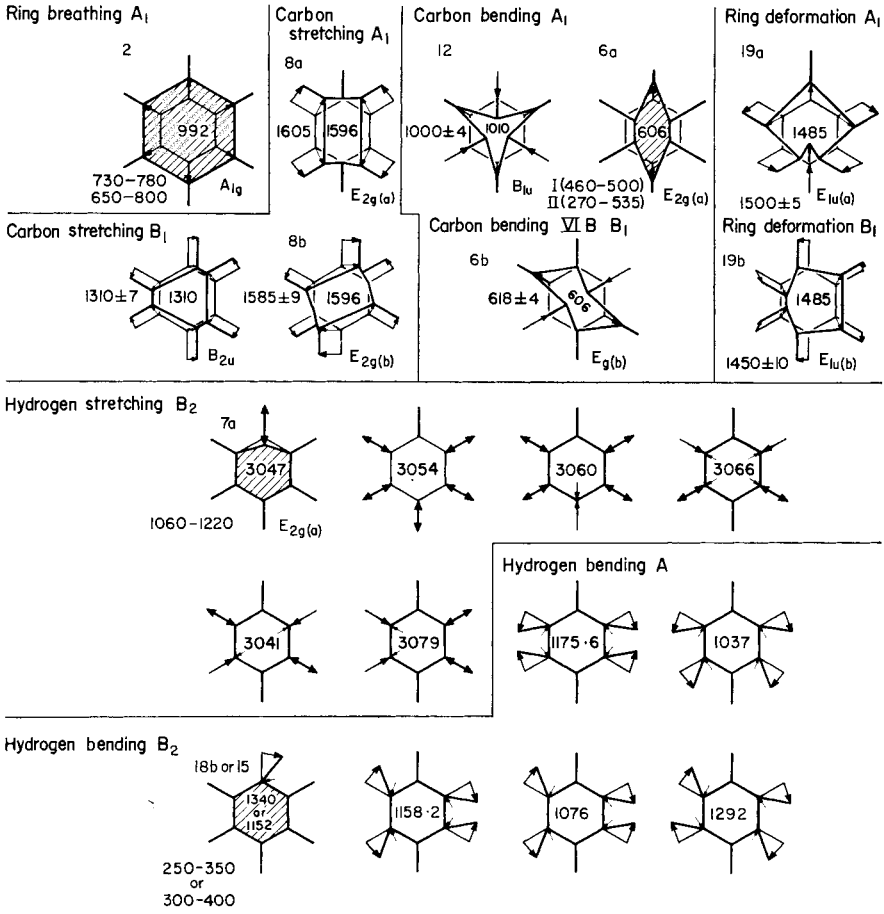
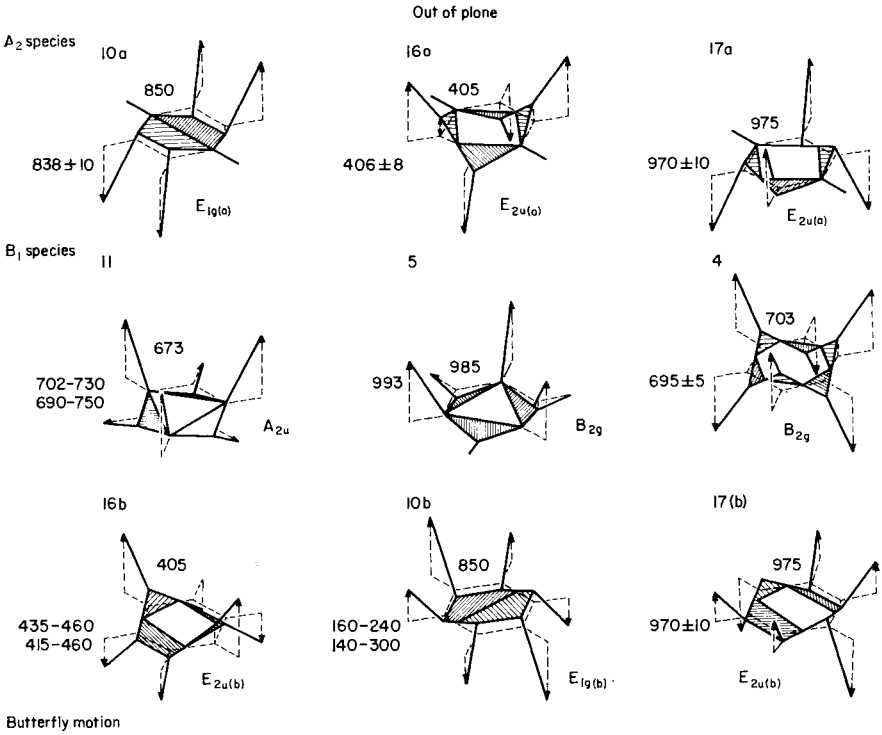


FIG. 1.

Only two of the out-of-plane deformations are "X-sensitive". They involve motions of the entire ring as well as the bond joining it to the substituent.

From Table 2, of the six X-sensitive vibrations, only four fall below 650 cm^{-1} (Nos. 14′, 18′, 19′, and 20′). Two more modes (Nos. 18 and 20) fall in this region. In both of them, the substituent remains almost at rest during the oscillation.

For convenience, the five normal oscillations that usually fall below 500 cm^{-1} and which might be significant in the correlation of vibration and smell, are collected in Fig. 3. Wilson's notation is used in the second column rather than Herzberg's because it is more commonly found in the current literature. The first two are in-plane ring deformations, one being



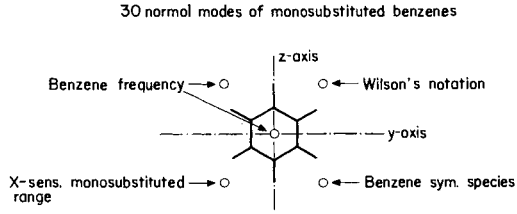


FIG. 2.

totally symmetric (a_1) while the other (b_2) may take either form according to different authorities.

The out-of-plane, X-insensitive mode (16*a*) is generally agreed upon. The two remaining out-of-plane motions may be described by any two of the four modes (10*b*, 11, 16*b*, and 17*b*).

The approximate nature of the pictorial representations and of the specific assignments, must be emphasized lest too much significance be attached to them, but the distinction between in-plane and out-of-plane oscillations is clear.

Because of the many difficulties involved, authorities sometimes differ in their interpretation of the spectra and the assignment of the normal modes. The literature has been searched diligently and the information is collected in Appendix 1 so as not to complicate the discussion at this point.

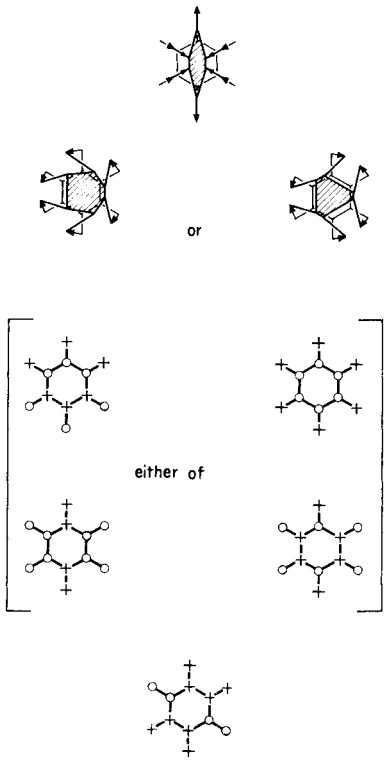


FIG. 3.

Table 3 shows the "best values" we have derived in this way, and which form the basis for a search for osmic correlations.

One important question which must be considered is whether these frequencies and assignments are valid for adsorbed molecules.

This question can only be answered inferentially at present. Rowe (1963), in discussing the spectra of adsorbed substances, distinguishes between cases of physical adsorption and chemisorption. Chemisorption involves surface bonds strong enough to act like valence bonds and produce complexes for which we do not have analogs among the usual chemical compounds. Physical adsorption, on the other hand, can perturb the motions enough to cause small shifts in some group frequencies, and surface forces

TABLE 3. NORMAL MODES OF COMPOUNDS CONSIDERED
(frequencies in cm^{-1})

C_6H_5F	C_6H_5Cl	C_6H_5Br	C_6H_5I	$C_6H_5CH_3$	$C_6H_5C_2H_5$
496	467	457	447	465	486
407	418	404	394	405	455
240	392	314	268	345	404
	296	251	218	217	347
	195	180	170		314
					158

$C_6H_5OCH_3$	C_6H_5NCS	$C_6H_5-COCH_3$	C_6H_5NO	C_6H_5CN	C_6H_5CHO
443	491	464	420	462	450
414	437	406	397	401	437
390	396	366	255	381	402
263	350	225	176	172	237
209	245	163	139	162	225
113	180				130

TABLE 3A. NORMAL MODES OF MONOSUBSTITUTED COMPOUNDS WITH "BITTER ALMOND" SMELL

C_6H_5CN		C_6H_5NO		C_6H_5CHO	
Frequency cm^{-1}	Assignment	Frequency cm^{-1}	Assignment	Frequency cm^{-1}	Assignment
462	a_1	420	b_1 and b_2	450	b_1
401	a_2	397	a_2	437	a_1
381	b_2	255	b_2	402	a_2
172	b_1	176	b_1	237	b_2
162	b_1	139	a_2	225	b_1
				130	a_2

may lower the symmetry of the adsorbed molecules so that new bands can appear. Since olfaction probably involves physical adsorption in most cases, and since the molecules under consideration are of low symmetry already, the most that is likely to happen is a frequency shift perhaps comparable to that observed in going from the vapor to the liquid state.

However, Miller *et al.* (1965), using a 7·5 meter gas cell heated up to 60°C to increase vapor pressure, state that the torsional frequency of benzaldehyde in the far infrared provides an extreme example of frequency shift on condensation. In the gas the torsion is 111 cm^{-1}. For the liquid the frequency is 133 ± 2 cm^{-1}, i.e. a shift of 22 cm^{-1}, a 17 per cent change. This cannot be considered as minor. Sirkar *et al.* (1964) have studied the infrared spectra (650–3000) cm^{-1} of fluorobenzene, chlorobenzene, bromobenzene and iodobenzene in the vapor state by using a Perkin–Elmer one meter gas cell and compared the spectra of the pure liquids and their solutions in carbon tetrachloride and chloroform. They observed significant changes with the change of state. They claim that from the spectra of fluorobenzene and chlorobenzene in the different states it has been concluded that in both these cases the vapor consists of both monomeric and dimeric molecules and the liquid consists almost wholly of dimers. In the case of bromobenzene and iodobenzene the vapor consists predominantly of monomeric molecules and in the liquid state there is a smaller percentage of monomeric molecules and a greater proportion of dimeric molecules. Whether the spectra of the halogenated benzene between 650–80 cm^{-1} undergo significant changes can only be answered by carrying out the experiment. If there is any reason to suspect chemisorption, the best course is to postpone consideration until the spectra of the adsorbed materials have been studied.

To our best knowledge, the spectra of the absorbed molecule has not been examined in the far infrared. *The values in Table 3 can be accepted as being nearly correct for the absorbed molecules only if we assume that the molecule preserves its identity in the physically adsorbed film.*

Finally, the uncertainty regarding the spectra of the adsorbed molecules, the relatively small number of molecules with completely assigned spectra and the large number of molecules whose far infrared spectra show no well-defined peaks (Wright, Demerdache, Rayner, 1964) seriously limit the effectiveness with which this hypothesis can be studied at present.

CORRELATION BETWEEN NORMAL MODES OF VIBRATION AND SMELL

Table 3A shows the normal mode frequencies for benzonitrile, nitrobenzene and benzaldehyde whose odors are generally agreed to be very similar, but not identical. Figure 4 is the correlation diagram of these three compounds. Thick lines represent fundamental vibrations and related modes are connected by thin lines. At the top of the figure, related oscilla-

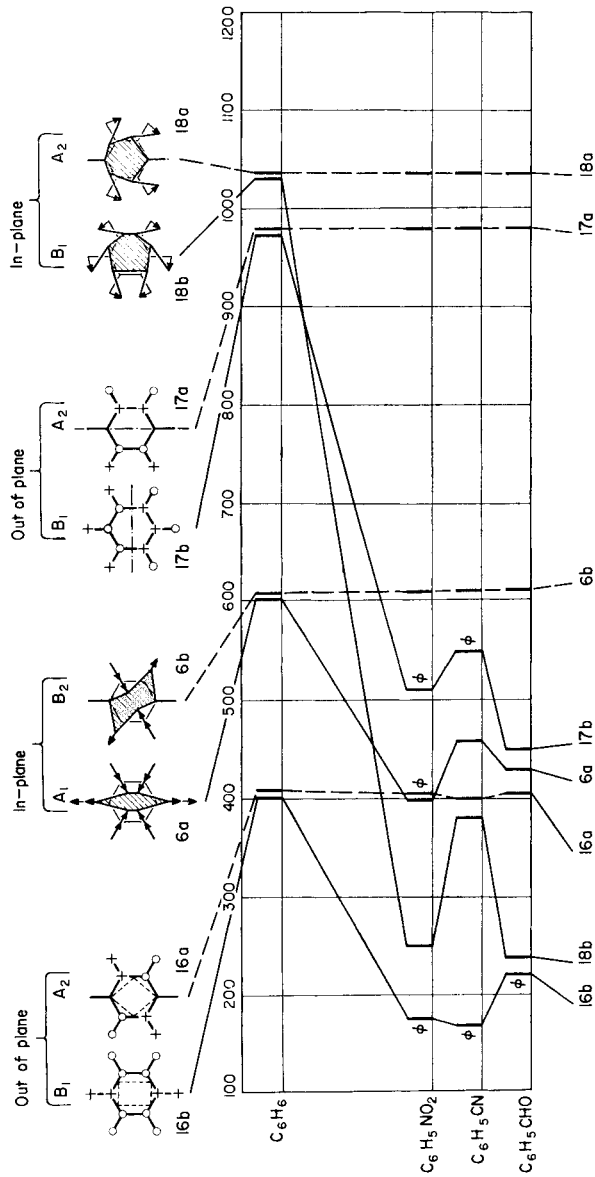

In-plane
A2
B1
18a
18b
Out of plane
A2
B1
17a
17b
In-plane
B2
A1
6b
6a
Out of plane
A2
B1
16a
16b
1200
1100
1000
900
800
700
600
500
400
300
200
100
C6H6
C6H5NO2
C6H5CN
C6H5CHO
18a
17a
6b
17b
6a
16a
18b
16b

FIG. 4.

tions are sketched. Modes which are degenerate (i.e. have the same frequency but different shapes) in benzene are denoted by two adjacent-vertical lines and by braces.

Figure 4 and Table 3A fail to reveal a systematic pattern or correlation between the almond odor and the normal mode frequencies, apart from the X-insensitive mode near 400 cm^{-1} which is common to all monosubstituted derivatives of benzene.

Wright (1964) with less complete data, but noting such correspondences as the 172 and 176 cm^{-1} modes of benzonitrile and nitrobenzene, thought that there were indications of a correlation, but remarked that it was "by no means convincing". The more complete normal mode data of Table 3A does not improve the picture. A preliminary examination of the observed far infrared spectra of nine disubstituted benzene derivatives with a bitter almond odor (Klouwen, 1963) also failed to provide a correlation. Since, however, their spectra are incomplete and also lack complete assignments, it was decided not to consider them further at this time.

An alternative test of the vibrational hypothesis is to compare the smells of normal and deuterated compounds. Far infrared data on deuterated aromatic compounds are scarce, but recently Gebbie (1964) has studied and tentatively assigned the low frequency modes of $C_{10}H_8$ and $C_{10}D_8$. For solutions in benzene, the strong 183 and 363 cm^{-1} modes of naphthalene become 169 and 331 cm^{-1} in the fully deuterated material. These shifts are large enough to suggest a perceptible difference in smell if the vibrational hypothesis is correct.

Dr. R. S. Stuart (1965) has kindly arranged a preliminary, triple-blind test of the two substances. Using 10 gram samples and six observers he reports that, "Four were able to pick out the D^8 as having a different odor which they described as 'weaker'; one person detected no difference; and one other suggested that one of the D^0 samples had a different odor." He adds the comment that "because of the small quantity of samples, there is a considerable difficulty determining whether the material is smelled immediately after the bottle is opened or a minute or so later." A further test will be arranged when larger samples become available.

If this observation is confirmed by more extensive and thorough tests, and especially if the difference is one of quality as well as strength, it will establish a fact that only the vibrational hypothesis can explain.

PART 2

(R. H. WRIGHT)

MOLECULAR VIBRATION

Let us begin with the assumption that a molecule is basically a system of mass points and elastic forces which can be made to vibrate in various ways by suitable additions of energy. The energy can come from various sources, but in the absence of chemical reaction it normally comes either from collisions with other molecules or from the absorption of radiation.

For there to be a predominantly one-way transfer of radiant energy into a molecule, the source of the radiation must be at a higher temperature than the molecule. If they are at the same temperature, the transfer of energy is necessarily the same in both directions. This is a consequence of the Second Law of Thermodynamics, and it explains why the theory which Beck and Miles put forward some years ago (1947) was called a "hot nose" theory, and could only work in the presence of a temperature gradient. While such gradients might just conceivably exist in warm-blooded land animals, they are almost inconceivable in fish or insects whose olfactory capabilities are nevertheless well established.

From this it follows that one-way transfers of radiant energy are not involved in the process of olfactory stimulation. It is necessary to register this fact very clearly because quantum mechanical principles which are relevant to radiative energy transfers have no *direct* relevance to olfaction. They can, however, have an indirect relevance from what they tell us about the vibrations of molecules.

The basic quantum principle relating to the question is expressed in the equation,

$$E_2 - E_1 = nh\nu$$

where E_2 and E_1 are the energies of the molecule in two states of vibrational excitation, and ν is the frequency of the radiation that is absorbed or emitted when the molecule passes from one "level" to the other. Quantum mechanics is concerned with giving an account of the various possible energy levels and with establishing rules to define which transitions have a high probability and which a lower probability. Radiative transitions can take place between various levels but not all with the same probability, and the relative strengths of the optical intensities depend on the numbers of molecules that make the various possible transitions.

Two things determine the number of radiative transitions and the absorption intensities. One is the number of molecules in the given state of initial excitation, and the other is the intrinsic probability of a transition

from that state to some other state. This probability depends partly on the symmetry of the vibrations in the initial and final states and so is not the same for all transitions. The symmetry rules are well understood, but their application to particular cases is often complicated. Their main value to us is in sometimes suggesting mechanical models whose movements approximately reproduce the relative movements of the atoms in the vibrating molecule.

The fact that radiative transitions depend on two basically independent factors (namely, the original population of a level and the intrinsic transition probability) means that a line may be weak or absent in the optical spectra for two quite distinct reasons. In any event, symmetry considerations and anharmonicities that operate in radiative excitation are meaningless in collisional excitation because the impacts momentarily deform the molecules so as to destroy all their symmetry.

From all this, it follows that optical measurements cannot tell us directly anything about the populations of the various levels when the excitation is due to collisions. Furthermore, the relative numbers of molecules in each vibrational level—that is, their populations—are uniquely determined only under conditions of thermodynamic equilibrium. This means conditions of uniform temperature, and as already explained, this is not the condition that exists during the optical measurements. Uniform, or near-uniform temperature is, however, the condition under which odors are regularly perceived, and therefore the populations are important because a level that is completely unoccupied can have no osmic properties or olfactory significance.

When the temperature is uniform, vibrational excitation takes place only through collisions between molecules whose kinetic energy depends on the prevailing temperature, and calculations show that it is only the rather low frequency vibrations that can be so excited. The frequency of the oscillatory movement is taken as the same as the frequency of the radiation absorbed or emitted in transitions between that vibrational level and the level of minimum vibrational energy. Physically, the vibration frequency is the number of times per second that the oscillating molecule passes through its equilibrium configuration, and the period (which is the reciprocal of the frequency) is the interval between transits. In practice, it is convenient to divide the frequency so defined by the velocity of light to give a quantity called the wave number (but commonly called the frequency also). The wave number is derived directly from optical measurements, being simply the reciprocal of the wave length. Frequencies mentioned in what follows will be wave number frequencies.

Thermal excitation by collision may excite a single vibrational mode in a molecule, but it is also possible that two normal modes can be excited by collision in the same molecule at the same time, provided their combined energies are not too high. This is important because, for example, given molecules with 150, 200 and 400 cm^{-1} modes, the population of molecules simultaneously excited in the 150 and 200 cm^{-1} modes would be greater than the population of the 400 cm^{-1} mode.

When a single mode is excited, the period of the vibration is the interval between successive passages through the equilibrium configuration. When two normal modes are excited simultaneously, the period of the complex oscillation is defined in exactly the same way. However, if one of the normal modes has a frequency a and the other a frequency b, and if they both pass through the equilibrium configuration together at a certain instant, they will pass through it together again when the faster oscillation has gained one complete cycle on the slower one. If this interval—which is the new period—is T, the faster mode will make aT oscillations while the slower one makes bT oscillations, and,

$$aT = bT+1$$

or

$$a-b = 1/T = \text{frequency}.$$

Thus a molecule in which two modes of vibration with frequencies a and b are excited simultaneously, must exhibit a new frequency $(a-b)$, and if the population of molecules in this state of complex excitation is appreciable, this frequency should be as osmically significant as pure normal modes of comparable population.

This fact has not been taken into account in previous searches for "osmic frequencies". They therefore omitted a possibly important part of the evidence and so failed to produce substantial correlations.

FORMULATING THE PROBLEM

The search for a correlation between olfactory sensation and molecular vibration presupposes that the vibrations are at levels which are appreciably populated at the ordinary temperature. Application of the Planck formula shows that, at 300°K, modes with frequencies higher than 650 cm^{-1} will be populated less than 5 per cent of the time. Modes below about 80 cm^{-1} do not appear in any but the largest molecules or as lattice vibrations in solid crystals. Therefore, if osmic vibrations exist, they must have frequencies between about 80 and about 650 cm^{-1}.

To account for the high informational content of an odor, we must suppose that the nose can perceive about 25 primary odors, and that most and perhaps all of our olfactory sensations are due to the simultaneous stimulation of several of these primaries. The vibration theory suggests that a given primary stimulus is somehow related to molecular vibrations whose frequencies fall in a relatively narrow range, so that our primary stimuli are essentially intervals in a scale of frequencies. The "band widths" that characterize various osmic frequencies can only be conjectured at this stage. Dividing the 80–650 cm^{-1} range into 25 intervals gives a constant band width of about 21 cm^{-1}. Dividing it logarithmically allows band widths to vary between about 8 and about 50 cm^{-1}. These estimates are intended to give no more than an approximate order of magnitude, and while it is possible that bands may overlap it is simpler to assume that they do not. Also, some bands may be osmically inactive for physiological or biochemical reasons.

For our experiments we have in the monosubstituted benzenes a group of compounds with a distinctive odor and with well-defined molecular structures and well-understood normal modes of vibration. What we have to look for is a correlation between this almond odor and a particular combination of frequencies, remembering that no single frequency is likely to characterize any commonly recognized odor, and that each primary osmic frequency can take part in generating many different smells, depending on what others are participating in the blend.

PROCEDURE

Our point of departure will be benzonitrile. The first step is to list the known normal mode frequencies below 500 cm^{-1}. The "difference frequencies" obtained by subtracting two normal mode frequencies are then worked out, omitting, however, differences below 100 cm^{-1} and differences between modes whose combined frequency is over 650 cm^{-1}. This means that while vibrational levels may be appreciably populated up to 650 cm^{-1}, we are confining our search for osmic frequencies to the 100 to 500 cm^{-1} range. If a correlation exists, it will certainly be apparent between these limits, and it can be left for further work to define the actual limits more precisely.

The results of this tabulation are shown in Table 4. If the vibrational theory is correct, the bitter almond odor must be due to the combination of some, but not necessarily all, of these frequencies. To identify the osmically active ones, we must collate the frequencies for benzonitrile with the

TABLE 4. NORMAL MODES AND POPULATED COMBINATIONS
100—500 cm^{-1}

C_6H_5—CN	C_6H_5—NO_2	C_6H_5—CHO
		450
462	420	437
401	397	402
381	281 (420–139)	320 (450–130)
300 (462–162)	258 (397–139)	307 (437–130)
290 (462–172)	255	272 (402–130)
239 (401–162)	244 (420–176)	237
229 (401–172)	221 (397-176)	226
219 (381–162)		
209 (381–172)		
172	176	176 (402–226)
162	139	165 (402–237)
	116 (255–139)	130
		107 (237–130)

similarly-derived frequencies for nitrobenzene and benzaldehyde which also have bond-defined structures and very similar almond odors.

Table 4 shows the result. The frequencies that are common to all three compounds fall at 174 ± 2, 225 ± 4, and 400 ± 3 cm^{-1}, which is an agreement within about 1 per cent, but in all three compounds there are other frequencies which do not conform to any pattern and which we must therefore suppose are osmically inactive for some reason. This is an arbitrary thing to do and it may properly be asked why we are justified in doing it.

Our object is to see whether there is any correlation between low frequency vibrations and odor. If such a correlation can be found—no matter how empirically—it can be studied. If no such correlation can be found despite our best efforts, then the vibrational hypothesis must be abandoned in favor of some other approach to the understanding of odor. The important thing is not to abandon any line until it has been explored fully.

No vibrational correlation could be established as long as only the spectroscopists' normal mode frequencies were considered, but when we bring in the comparably populated states of double excitation (difference frequencies) *as is done here for the first time*, a certain amount of regularity begins to appear, but is overlaid with extra frequencies which we have to suppose are osmically inactive for some reason. Although this designation is arbitrary, it is consistent to the extent that when we label a normal mode "inactive" we can label all the difference-terms derived from it as inactive also without destroying the basic regularity.

TABLE 5.

Mode	Assignment	Osmic quality
C_6H_5—CN		
462	Ring deformation, in-plane	Inactive
401	Ring deformation, out-of-plane	Active
381	Ring deformation, in-plane	Inactive
172	Ring deformation, out-of-plane	Active
162	Side-chain, C—N flexing	Inactive
C_6H_5—NO_2		
420	Side-chain, out-of-plane bending	Inactive?
397	Ring deformation, out-of-plane	Active
255	Ring deformation, in-plane	Inactive
176	Ring deformation, out-of-plane	Active
139	Side-chain, torsion	Inactive
C_6H_5—CHO		
450	Ring deformation, out-of-plane	Inactive?
437	Ring deformation, in-plane	Inactive
402	Ring deformation, out-of-plane	Active
237	Ring deformation, in-plane	Inactive
226	Ring deformation, out-of-plane	Active
130	Side-chain, torsion	Inactive

When we go on to examine the vibrational assignments that competent authorities have made to the various normal modes, some interesting consistencies begin to appear as can be seen from Table 5. Thus, without exception, the modes which appear to be osmically active in Table 4 are out-of-plane deformations of the benzene ring, while the ones that appear to be osmically inactive are mainly in-plane ring deformations or localized oscillations of the side-chain. If, following up this hint, we associate osmic inactivity with in-plane or localized oscillations, we must put the 420 mode of nitrobenzene and the 450 cm^{-1} mode of benzaldehyde in a doubtful category, but even if we class them as osmically active, the basic pattern derived from Table 4 is not seriously affected, as can be seen from Table 6. The addition of the 450 cm^{-1} mode to the benzaldehyde pattern would

TABLE 6. OSMIC FREQUENCIES OF THE ALMOND ODOR

C_6H_5-CN	$C_6H_5-NO_2$	C_6H_5-CHO
	420	450
401	397	402
	244 (420–176)	
229 (401–172)	221 (397–176)	226
172	176	176 (402–226)

account for the slight differences between its odor and those of the other two. Including the 420 cm^{-1} mode in the nitrobenzene pattern makes a more substantial difference, but its osmic activity might well be weak if it is as localized a deformation as, for example, the out-of-plane torsion of the side chain.

If it is a coincidence, it is certainly an interesting one that with so few exceptions the frequencies we have termed "osmically inactive" turn out to be either localized motions of the side-chain or in-plane oscillations of the whole molecule of the kinds that are approximately represented in Fig. 1. By contrast, the frequencies that do appear to correlate with the odor are in all three cases the out-of-plane modes that involve substantial deformations of the whole molecule, somewhat in the manner shown in Fig. 2. If knowing the vibrational assignments makes us reconsider our preliminary "osmic assignment" of the 420 mode of nitrobenzene and the 450 cm^{-1} mode of benzaldehyde, no harm is done, for at this stage we are looking for a general trend rather than for perfection in every detail.

The apparent association of osmic activity with out-of-plane oscillations but not with in-plane deformations of the benzene ring is not intrinsically improbable. The benzene rings might well be expected to lie flat on the olfactory receptor, and in this position, in-plane movements would be parallel to its surface. From this we may perhaps deduce an additional criterion of an osmically active vibration as one with a substantial component of movement perpendicular to the receptor surface on which the odorous molecule is adsorbed.

On this basis, then, the inference to be drawn from Table 6 is that the almond odor may correlate with out-of-plane deformations of the benzene ring having frequencies near 175, 225, and 400 cm^{-1}. If the available frequency spectrum is divided logarithmically into about 25 non-overlapping intervals, the bitter almond odor would then be due to the simultaneous stimulation of olfactory receptors sensitive to vibrations normal to their

surfaces and falling in the following approximate intervals:

170–182 cm^{-1}

218–232

380–405

OTHER COMPOUNDS

The next step is to examine other monosubstitution products of benzene to see whether these indications are at all general.

There is a distinct almond-like note in the odors of acetophenone and phenyl isothiocyanate, although other notes make them readily distinguishable from benzonitrile. Table 7 shows the frequencies for these two compounds tabulated according to the same rules used in compiling Table 4.

TABLE 7. NORMAL MODES AND POPULATED COMBINATIONS

100—500 cm^{-1}

C_6H_5-NCS	$C_6H_5-COCH_3$
491	464
437	406
396	366
355	301 (464–163)
257 (437–180)	243 (406–163)
245	225
216 (396–180)	203 (366–163)
180	181 (406–225)
175 (355–180)	163
151 (396–245)	141 (366–225)
110 (355–245)	

Phenyl isothiocyanate has all the "almond frequencies" together with several others. The 355 and 245 cm^{-1} frequencies are due to in-plane oscillations, and if they and the difference terms that depend on them are taken out, what is left is a close approximation to the almond pattern, but with enough additional frequencies to modify but not submerge the almond character (see Table 8). The 491 cm^{-1} mode is an out-of-plane ring deformation, and 437 cm^{-1} is an out-of-plane side-chain bending like the 420 cm^{-1} mode of nitrobenzene, whose osmic activity was doubtful.

TABLE 8. OUT-OF-PLANE FREQUENCIES

C_6H_5-NCS	$C_6H_5-COCH_3$	
	Lebas	Delorme
491	464	464
437	406	406
396	301 (464–163)	366
311 (491–180)	243 (406–163)	301 (464–163)
257 (437–180)	225	243 (406–163)
216 (396–180)	181 (406–225)	203 (366–163)
180	163	163

With acetophenone the situation is confused because the conflicting vibrational assignments have been made by Lebas (1962) and Delorme (1964). They agree that the 464, 406, and 163 frequencies represent out-of-plane modes, but Delorme says the 225 cm^{-1} mode is in-plane where Lebas calls it out-of-plane. Delorme did not assign the 366 cm^{-1} frequency, and Lebas considered it in-plane. If we accept Delorme's assignments, the almond pattern disappears (Table 8), but if the in-plane deformations identified by Lebas are discarded as osmically inactive, the almond pattern is clearly present and not excessively overlaid by additional frequencies. For what it is worth, the olfactory evidence therefore favors the Lebas assignments.

SUBSTANCES WITHOUT THE ALMOND ODOR

So far we have examined the frequencies of substances whose odors all have a certain distinctive note, and because all the populated compound vibrational levels have been included, there has sometimes been a considerable number of figures to choose from. Some sort of concordance might therefore be due simply to chance. Table 9 shows a compilation made in the same way as before for seven monosubstitution products of benzene which are not generally considered to be almond-like, but which might be expected to vibrate in the same general way as the compounds we have been considering. Table 10 shows the residue after taking out (as before) the frequencies assigned to in-plane oscillations or side-chain torsions.

The almond pattern is absent from fluorobenzene, toluene, ethyl benzene, and anisole, but it is complete in bromo- and iodobenzene. Several professional perfumers were therefore asked for an expert appraisal of the odors of toluene, and of chloro-, bromo- and iodobenzene, in relation to

TABLE 9. NORMAL MODES AND POPULATED COMBINATIONS
100–500 cm^{-1}

C_6H_5F	C_6H_5Cl	C_6H_5Br	C_6H_5I	$C_6H_5CH_3$	$C_6H_5C_2H_5$	$C_6H_5OCH_3$
496	467	457	447	465	486	443
407	418	404	394	405	455	414
240	392	314	277–	345	404	390
167–*	296	277–	268	217	347	330–
	223–	251	224–	188–	328–	301–
	197–	224–	218	128–	314	277–
	195	180	170		297–	263
		134–			246–	209
					189–	205–
					158	181–
					156–	150–
						113

* –= difference term

TABLE 10. OUT-OF-PLANE FREQUENCIES

C_6H_5F	C_6H_5Cl	C_6H_5Br	C_6H_5I	$C_6H_5CH_3$	$C_6H_5C_2H_5$	$C_6H_5OCH_3$
496	467	457	447	465	404	414
407	392	404	394	405	246	390
240	197	277	277	217	158	209
167	195	224	224	188		205
		180	170			181

nitrobenzene. The response was unanimous that toluene does not have the almond note, but varied as to the halogenated benzenes:

Observer group	Opinion
A	"C_6H_5Cl has almond-like character, others not available."
B	"Almond-like character in all three."
C-1	"No almond-like character."
C-2	"C_6H_5—Cl and —Br, no; but C_6H_5—I is 'chemical almond'."
C-3	"Have a note similar to nitrobenzene."
D	"If C_6H_5CHO is 100, $-NO_2$ is 60, and —Cl, —Br, —I about 5."

Table 10 does not indicate enough additional frequencies to modify the almond odor as much as it appears to be masked in bromo- and iodoben-

zenes, but if the large halogen atom causes the molecules to lie at an angle to the receptor surface, the in-plane modes shown in Table 9 might not all be inactive osmically. The reported almond note in chlorobenzene remains unexplained.

DISCUSSION

If olfaction is connected with the fact that a molecule *is vibrating* where spectroscopy is concerned with *transitions between vibrational levels*, then complex oscillations due to the excitation of two normal modes can have a relevance to olfaction that they do not have in spectroscopy. What we are concerned with is the vibrational states that are populated at the ordinary temperature, and if transitions involving some of them do not appear in the spectra it does not mean that they have no real existence. On the other hand, their absence from the spectra notwithstanding their apparent relevance to olfaction may tell us something rather important about the way olfactory sensations are initiated.

What it suggests is that the process of olfactory stimulation *does not depend on a radiative exchange of energy* between an oscillating dipole in the odorous molecule and another oscillating dipole in the substrate. Instead, it suggests that when the odorous molecule becomes adsorbed onto a site that "throbs" with roughly the same frequency, the synchronous movements of the two permit electronic (Van der Waals) synchronization to become established and to be substantially greater than the forces between randomly moving sites and olfactor molecules. If this is so, the small size of the vibrational energy quantum is unimportant because the vibrational *energy* is not involved directly in stimulating a nervous discharge.

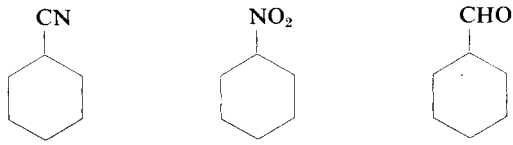
Further discussion of possible "triggering mechanisms" would be premature.

Aniline, which has little or no odor, and phenol, whose "odor" is mainly trigeminal, have substitutent groups that would probably give point-attachment or chemisorption on the olfactory receptor. If their molecules do not have an appreciable area of contact with the surface there could well be less vibrational interaction, and *ex hypothesi* less odor, which is what we actually observe.

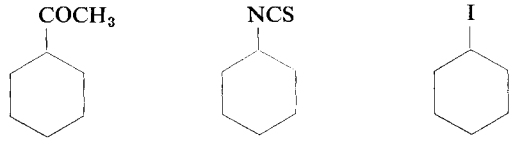
CONCLUSION

This has been an attempt to answer three very precise questions:

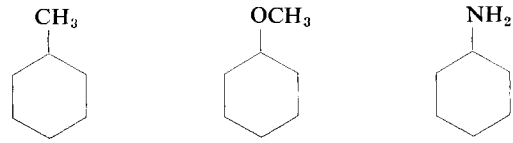
First: What is the specific osmic property that gives these three compounds a very similar, almond-like, olfactory quality?



Second: What specific osmic properties give these compounds an olfactory quality similar to the first three but modified by additional "notes"?



Third: What specific osmic properties do these compounds lack so that their olfactory qualities are entirely different from the first three?



The chemical characters of these compounds are so diverse and their physical conformations are so similar, that these questions are very searching ones.

As long as we considered only the frequencies of the independently excitable normal modes of vibration, no correlations appeared, but a pattern did begin to emerge when consideration was given to the additional frequencies that must appear when two normal modes are excited simultaneously. The governing requirement is that the vibrational levels—whether normal modes or combinations—shall be populated by an appreciable number (say 5 per cent or more) of molecules at the ordinary temperature. Unpopulated levels can have no osmic significance.

The low frequency vibrations of simple monosubstituted derivatives of benzene are of three kinds:

1. Out-of-plane ring deformations;
2. In-plane ring deformations;
3. Torsional or other oscillations mainly localized in the side-chain.

Not all the observed vibration frequencies appear to correlate with the almond odor, and the rest must be arbitrarily assumed to be osmically inactive. It turns out that in the compounds considered here, the frequencies which are classed as inactive in this *ad hoc* fashion are nearly all in-plane

ring deformations or side-chain oscillations; and the osmically active frequencies are without exception due to out-of-plane deformations of the ring.

Since these molecules would all be expected to lie flat (or nearly so) on the receptor surface, it is not surprising to find that in-plane and out-of-plane movements have a different osmic significance.

If it is permissible to generalize on such limited data, we may provisionally conclude that osmic vibrations have the following general properties:

1. Low Frequency: ?80 to ?500 cm^{-1};
2. Whole-molecule character;
3. Substantial components of motion perpendicular to the surface of the receptor organ when they are adsorbed on it.

With these postulates we can, with one exception (C_6H_5Cl), account for the presence or absence of an almond quality in compounds C_6H_5X, where X is: —CN, —NO_2, —CHO, —$COCH_3$, —NCS, —F, —Cl, —Br, —I, —CH_3, —C_2H_5, —OCH_3, —NH_2, —OH.

The idea that the orientation of the molecules in the olfactory membrane can be important is already explicit in Beets' (1957) "Profile-Functional-Group" theory, and implicit in Amoore's (1964) stereochemical model. Also the importance of contact area, used here to account for aniline's lack of odor, is in line with Davies' (1959) correlation of the olfactory threshold with molecular cross-sectional area.

This is a progress report on a theory whose postulates have now, for the first time, been spelled out in detail in relation to a particular odor. When these postulates have been tested by applying them to a different odor, we will know whether this has been the end of the beginning or the beginning of the end of the vibrational theory.

PART 3

(A. DEMERDACHE and R. H. WRIGHT)

DISCUSSION OF PRINCIPLES

A. D. I feel that your invoking of the difference frequencies as recently as six weeks ago has left too little time for their significance to be properly investigated. It would have been far better to wait until more experimental evidence was gathered before putting so much weight on new postulates based on the difference frequencies.

R. H. W. It has been known for at least thirty years that some of the vibra-

tional movements of complex molecules can be activated at the ordinary temperature. The evidence comes from specific heat data and from the study of reaction rates of gases. What I have done is to import a well-established concept from one department of science into another. I am sorry I did not do it sooner.

A. D. No one can deny their existence, but was not your introduction of difference frequencies done to rescue the vibration theory when the normal mode data failed to confirm it?

R. H. W. Before we had tolerably complete and precise information about the normal modes, it was neither possible nor necessary to take difference frequencies into account. When the complete data did become available, it became not only possible but also legitimate to do so.

A. D. Nevertheless, such a step should not have been based simply on the spectra of three bitter-almond compounds.

R. H. W. The hint came from three, but it was immediately extended to something over a dozen.

A. D. In all your previous papers your conclusions were based on meagre and incomplete data. Eleven years have passed since you put forward the possible relevance of low frequencies. At this meeting, would it not have been better to report the work of the past two years only? The difference frequencies should have been mentioned only briefly and at the end of the paper as one of the avenues under investigation. This is a question of methodology: the orderly arrangement of scientific thought.

R. H. W. The role of difference frequencies is neither more nor less clear than the role of any other kind of frequency. I feel that there *is* enough factual evidence for the real existence of these difference frequencies. We know the energies available in the molecular collisions, and we know the energies of the various normal modes of oscillation. Provided their combined energies are low enough, there is no reason why two normal modes should not be excited in the same molecule at the same time. If they are, they will come in and out of phase with a certain periodicity—which means there is a difference frequency.

A. D. I concede the existence of spectroscopic bands due to the sums as well as the differences of normal modes, but are they not secondary? Are they not much weaker than the normal mode frequencies? Difference bands are particularly weak because of the Boltzmann Factor in their intensity derivation.

R. H. W. The relation between spectroscopic intensities and the populations of the various levels is the vital point that needs to be cleared up. What governs the spectroscopic intensities of combination bands?

A. D. The theory of infrared intensity is still in its infancy. Combination bands seem to be governed among other things by selection rules, anharmonicities, and the populations of levels. As regards selection rules, symmetry is mainly the dominant factor. All the difference bands are allowed in the Raman spectra, and almost all in the infrared in the C_{2v} symmetry group. Do you believe that your difference band has a symmetry like the fundamental?

R. H. W. A molecule can have symmetry, and a vibrational movement can have symmetry. A "difference band" is a line observed in the spectrum and attributed to a molecular transition between two levels of vibrational excitation. Please refine your question.

A. D. The symmetry species of the overtones and combination tones are obtained readily from the species of the fundamentals. The rules are given in any standard treatise.

R. H. W. Spectroscopic bands are caused by transitions between energy levels. Does the band intensity depend on the symmetry at both ends of the transition or just at one of them?

A. D. It depends on the symmetry at both ends. In the case of a difference band in the spectrum two more factors are involved, namely, anharmonicity in the fundamentals from which it is derived, and the population of the levels. Without the anharmonicity the difference band would not exist.

R. H.W . This is the vital point. Let us look at it carefully. If the molecule is excited in two normal modes simultaneously, the two oscillations will not interact—there will be no exchange of energy back and forth between them—unless there is some anharmonicity in both.

A. D. Yes.

R. H. W. Then a radiative transition is not likely to take place into two normal modes *simultaneously* unless they are coupled through their anharmonicities in this way. If they are not so coupled, radiative transitions will be into one or other of the two normal modes, and not into both together. The combination band due to a transition into both will therefore be absent from the spectrum when there is no anharmonicity.

A. D. In the absence of anharmonicity, the motion is that produced by the two normal mode motions. It is not spectroscopically observable and should therefore exist feebly, if at all, both classically and quantum mechanically.

R. H. W. But is this kind of two-mode excitation physically possible, even if it is not spectroscopically observable?

A. D. If any vibration produces a change in the dipole moment, or in the polarizability of the molecule, it must manifest itself somehow in the spectra

where, as here, the symmetry allows it. Therefore I cannot see how such changes could fail to appear. My objection is that these summation, difference and overtone bands are nearly always missing from the spectra I have recorded, and they are likewise seldom reported in the literature. If they are there they must be very weak. I feel it is illogical to put the effect of these secondary bands on the same level as the main ones.

R. H. W. The weakness of the bands is due to the absence of anharmonicity and not to the absence of doubly-excited molecules.

A. D. This is a matter of conjecture. Coming back to the symmetry of the combination, certain bands which you have disregarded as "osmically inactive" on the grounds that they are in-plane motions, do not follow the derived symmetry rules for combination bands. For example, some combinations which you have assumed to be in-plane appear to be out-of-plane if the symmetry rules are obeyed, and *vice versa.*

R. H. W. I cannot see how the simultaneous excitation of two in-plane oscillations can produce an out-of-plane movement, and further, I think that combining an in-plane mode with an out-of-plane mode would not affect the frequency of the out-of plane *component* of the combined motion. It seems to me that this is a matter of elementary mechanics.

A. D. I realize your difficulty in visualizing these very complex motions, and indeed, it is simply not possible in many cases.

R. H. W. The absence of difference bands means only that two modes are not being excited by a single radiative event, but they can be excited by two independent events that are spectroscopically observable. However, radiative excitation is not the only source of vibrational energy, and in collisional excitation the molecules must be so pushed out of shape at the moment of impact that there will be all the anharmonicity and all the lack of symmetry needed for two modes to become excited simultaneously, always provided there is enough energy in the impact. The anharmonicity only exists at the instant of collision, which is a small fraction of the time, and so does not appear in your spectra.

A. D. I assume that when one records the spectrum of a gas or liquid, it includes the molecules that are in collision. However, let us not get enmeshed in too many theoretical arguments that may turn out to be superfluous. I have done already the far infrared spectra of nine disubstituted compounds with "bitter almond" smell. Furthermore, I have found complete assignment for most of the disubstituted halogens and xylenes. A preliminary correlation using the difference bands has not been to successful in improving the picture obtained by using only the observed band and the normal modes whenever known. It will not take long to find out whether the disub-

stituted benzene derivatives—some of which have the almond odor—confirm or confound your theory. At this stage, let us stick to the facts.

R. H. W. I agree that tomorrow we will know more than we do today. This has been a progress report, not a final one. If it has done nothing else, it has spelled out the postulates of the vibrational theory more precisely than ever before, and this can only help us to devise the definitive tests that are needed so urgently.

ACKNOWLEDGMENTS

This work was supported in part by the Canada Department of Agriculture, and the far infrared spectrophotometer was purchased under a contract with the U.S. Army Medical Research and Development Command. A. Boake, Roberts and Co., Firmenich Inc., the Givaudan Corporation, and International Fragrances and Flavors Inc., have generously provided samples and expert olfactory evaluations. Dr. P. N. Daykin, Dr. C. Reid, Prof. R. E. Burgess, Prof. K. B. Harvey, and Prof. Neil Bartlett have regularly helped with comments and criticisms. Particular thanks are due also to Dr. R. S. Stuart of Charles E. Frosst and Co. for evaluating the odors of normal and deuterated naphthalene.

REFERENCES

AMOORE, J. E. 1964. Current Status of the Steric Theory of Odor. *Ann. N.Y. Acad. Sci.* **116**, 2, 457–476.

BECK, L. H. and W. R. MILES, 1947. Some Theoretical and Experimental Relationships Between Infrared Absorption and Olfaction. *Science* **106**, 511, Nov. 28.

BEETS, M. G. J., 1957. *Structure and Odor*, S.C.I. Monograph No. 1.

DAVIES, J. T. and F. H. TAYLOR, 1959. The Role of Adsorption and Molecular Morphology in Olfaction; the Calculation of Olfactory Thresholds. *Biol. Bull.*, **117**(2), 222–238.

KLOUWEN, M. H. and A. H. RUYS, 1963. Chemical Constitution and Odor. *Parfum. Cosmet. Savons.*, **6**, 6–12.

SCHUTZ, H. G., V. G. VELY and R. B. IDEN, 1961. Relations of Odor to Physico-Chemical Properties of Compounds. Batelle Memorial Institute, Report No. 4, Feb. 1, 1961. 29 pp, 9 Tables, 3 Figures. Contract No. DA-19-129-QM-1500.

WRIGHT, R. H. 1954. Odor and Molecular Vibration. I. Quantum and Thermodynamic Considerations. *J. Appl. Chem.* **4**, 611–615.

WRIGHT, R. H. and R. S. E. SERENIUS, 1954, Odor and Molecular Vibration. II. Raman Spectra of Substances with the Nitro-Benzene Odor. *J. Appl. Chem.* **4**, 615–621.

WRIGHT, R. H. 1964. *The Science of Smell.* London, George Allen and Unwin, New York, Basic Books, Inc.

WRIGHT, R. H., A. DEMERDACHE and H. B. RAYNER, 1964. Annual Report to the Canada Department of Agriculture on E.M.R. 141.

WRIGHT, R. HUEY and K. M. MICHELS, 1964. Evaluation of Far Infrared Relations to Odor by a Standards Similarity Method. *Ann. N.Y. Acad. Sci.* **116**, Art. 2; 535–551.

APPENDIX 1

In these detailed tables, we have tried to reproduce *exactly* the description given by each individual author to the bands they have observed in the Raman infrared for the vapour, liquid and solid state, whichever has been observed. The values we have chosen and their assignments are those which enjoy the unanimous agreement of the different authors. The latest papers, especially if the far infrared spectrum was obtained by an instrument specifically designed for that region of the spectrum, played a decisive role whenever a difference of opinion arose between the authors.

GENERAL REMARKS:

Intensities

In both the infrared and Raman spectra, intensities are described as follows:

s	strong
m	medium
w	weak
vw	very weak

In certain cases reported intensities for Raman data use a numerical scale from 0 to 10

Qualitative Band Description

To help in the visual conception, whenever possible, a few authors have added de scriptions of the main movement taking place during the vibration. Those of Randle and Whiffen (1955) are the most widely used.

β(C−H):	C−H deformation in the plane
γ(C−H):	C−H deformation out of the plane
$\alpha-\widehat{ccc}$:	ring deformation in the plane
$\phi c-c$:	ring deformation out of the plane

Symmetry Species

Monosubstituted benzenes belong mainly to two symmetry classes:

1. C_s symmetry: One plane of symmetry perpendicular to the plane of the ring (σ_{zx}). The symmetry species are:

a' :	Totally symmetrical vibration
a'':	has no plane of symmetry

2. C_{2v} symmetry: In this group the elements of symmetry are the two-fold (C_2) axis of symmetry (z-axis), the plane of the ring (σ_{zy}) and the plane perpendicular to the ring (σ_{zx}). The symmetry species are:

a_1: totally symmetrical

a_2: C-axis and no planes of symmetry

b_1: no axis of symmetry, $\sigma_{(xz)}$ as a plane of symmetry

b_2: no axis of symmetry, $\sigma_{(zy)}$ as a plane of symmetry

Raman Data

The Raman data of Lebas (1964), Delorme (1964), and Whiffen (1956) is taken from Kohlrausch *et al.* The depolarization ratio ϱ gives a measure of the degree of symmetry of the molecule. This means that ϱ for all vibrations which are not in the totally symmetric species will be 6/7, while only those in the totally symmetric species can have a value of ϱ less than 6/7.

Instruments Used

The spectrum obtained depends on the type of instrument used. Therefore, the instruments used by each author are indicated below.

Green (1962)IR Grubb and Parson GS3..
Raman Hilger FL1 Raman source and E-612 spectrograph.

Stephenson (1961)—700–285 cm^{-1}, P.E. 21, Cs Br prism
Hilger double prism Raman spectrograph, model E-612.

Lebas (1962)—700–300 cm^{-1}, P.E. double beam used as such, Br Cs prism
400–200 cm^{-1}, P.E. double beam used as single beam, I Cs prism.

Delorme (1964)—660–470 cm^{-1}, P.E. 112, K Br prism for 660–470 cm^{-1}
470–220 cm^{-1}, P.E. 12 C, I Cs prism for 470–220 cm^{-1}.

Grating instrument (Delorme, Ph.D. thesis, Paris, 1963).

BENZONITRILE

The paper of Jakobsen (1965) formed the basis of this assignment because of its thoroughness. In the bibliography, a number of earlier papers are mentioned.

IR		Raman liquid	Assignment	Description of mode
Vapour	Liquid			
—	462 w	461 mw, p	a_1 fund., 6*a*	X sens $\alpha\hat{ccc}$
—	401 vw	—	a_2 fund., 16*a*	ϕ $c-c$
—	381 mw	380 w, dp	b_2 fund., 18*b*	X sens ($\beta c-c\equiv n$)
	172 m	172 mw, dp	b_1 fund., 11	X sens ($\gamma c-c\equiv n$)
	162 sh	162 sh	b_1 fund., —	side-chain $\gamma c\equiv n$

Values chosen for this paper	
Frequency	Assignment
462	a_1
401	a_2
381	b_2
172	b_1
162	b_1

NITROBENZENE

Raman liquid	Infrared liquid	Assignment	Mode Wilson	Description Whiffen
GREEN (1961)				
435 (w)	420 (w)	b_1	16*b*	X sens
	420 (w)	b_2	—	NO_2 rock in-plate
397 (m, p)	—	a_1	1	X sens
		a_2	16*a*	ϕ (*cc*)
252 (w)	—	b_2	18*b*	X sens
176 (m)	—	b_1	11	X sens
STEPHENSON *et al.* (1961)				
139 vw	—	a_2	—	NO_2 torsion
182 w, dp	—	b_1	16*b*	(*cc*)
251 vw	—	b_2	18*b*	β(*c*—NO_2)
397 vw, p ?	396 m	a_1	6*a*	$\alpha(\hat{ccc})$
442 vw	—	b_1	—	NO_2 rock out-of-plane
LEBAS (1962)				
176 (4, dp)	—	b_1	10*b*	γ(*c*—X)
—	238??	—		
255 (0)	255 (s)	b_2	15	β(*c*—X)
—	360?			
397 (2, p)	399	a_2 and a_1	16*a*, 6*a*	
—	420 (m to s)	b_1	16*b*	
DELORME (1964)				
139		a_2		torsion
182 (4, dp)	182 (s)	b_1		
247 (0)	255 (s)	b_2	18*b*	β(*c*—X)
			15	
—	420 F	b_1	17*b*	γ(*c*—X)
			10*b*	

Frequencies chosen for this paper	
Frequencies	Assignment
420	b_1 and b_2
397	a_2
255	b_2
176	b_1
139	a_2

BENZALDEHYDE

Raman	IR	Assignment	Mode
LEBAS (1962)			
225 (2, *dp*)	226 (s)	b_1	10*b*
237 (2, *dp*)	237 (s)	b_2	15
400 (0, *dp*)	402 (w)	a_2	16*a*
437 (5·0, 4·0)	437	a_1	6*a*
—	450	b_1	16*b*
DELORME (1964)			
	130 (s)	a_2	torsion
225 (2, *dp*)	223 (s)	b_1	16*b*
234 (2, *dp*)	237 (s)	b_2	$\beta(c—X)$
	450 (m)	b_1	$\gamma(c—X)$
GREEN (1962)			
130	130 (s)	—CHO torsional	
225	224 (s)		
237	235 (s)		

FATELEY *et al.* (1965)

The torsional frequency of benzaldehyde in the far infrared provides another extreme example of frequency shift on condensation. In the gas the torsion is 111 cm^{-1}, for the liquid the frequency is 133 ± 2 cm^{-1}, i.e. a difference of 21.

HALOGENATED BENZENES

Wilson notation	Sym. species	F				Cl	
		I–R	IR	R^2	R^1	Ir	R
WHIFFEN (1956)							
16*b*	b_1			241	241		196
17*b*	b_1					467	467
18*b*	b_2	405	406	407	407	297	297
6*a*	a_1					515	418
16*a*	a_2		(400)				(400)
LEBAS (1961)							
10*b*	b_1		240 (s)	241	(12,0·83)	196 (s)	196 (8,0·66)
18*b*	b_2		402 (s)	407	(00)	296 (s)	297 (3,0·72)
16*a*	a_2			407	(00)	398 (w)	—
16*b*	b_1		(495 (s))	(496	(2·0))	464 (s)	467 (0)
6*a*	a_1				—	414 (s)	418 (8,0,0·28)
DELORME (1964)							
			241		241	197 (s)	196 (8,0·66)
			402 (s)			296 (f)	
			495			464 (f)	
WYSS (1964)							
	b_1					195	
	b_2					297	
	a_1					418	
	a_2					392	
	b_1					467	
WHIFFEN (1956)							
16*b*	b_1			181			166
17*b*	b_1		458	460		488	450
18*b*	b_2		—	254		—	220
6*a*	a_1		314	315		—	260
16*a*	a_2		—	409		—	398
LEBAS (1961)							
10*b*	b_1			181	(10,0·7)		166 (5,0·76)
18*b*	b_2	254	(4,0·65)		(250)	218 (w)	220 (2,0·53)
16*a*	a_2		409 (00)			390	398 (00)
16*b*	b_1	454 (s)		454	(00)	447 (s)	450 (00)
6*a*	a_1	313 (s)		315	(12,0·24)	268 (s)	266 (10,0·26)
DELORME (1964)							
		183 (s)				170 (s)	
		250 (w)				218 m	
		454 (s)				447 (s)	
WYSS (1964)							
	b_1	180 (s)					
	b_2	251 (w)					
	a_1	314 (s)					
	a_2	404 (sl)					
	b_1	457 (s)					

TOLUENE

Sym. species	Wilson notation	Pitzer 1943		Bogomolov 1960		Lebas 1961		Wyss 1964		Delorme 1964	
		R	IR	R	IR	R	IR	R	IR	R	IR
a_1	6*a*	521 (p)	?	521	499	521 (6·0,0·6)	—		521	—	—
b_2	15			347	346	344 (0·5,1·22)	344 (s)	345			
	18*b*	340 (d)	—								
	18*b* or 15									344	344
b_1	16*b*			217	207				216 (s)	216 (5·0,0·68)	217 (s)
	11	216	—			2·6 (5·0,0·68)	2·8 (s)				
	10*b*										
b_1	11			465	445				464		
	16*b*	467					465 (mw)				
	10*b* or 17*b*									446	465
a_2	16*a*	405		405	465	400	405		408		

a_1	521	Values chosen
b_1	465	
a_2	405	
b_2	345	
b_1	217	

ETHYL BENZENE

The assignment of this compound is based mainly on the work of Green (1962). It seems to be of doubtful assignment.

Raman liquid	IR liquid	Assignment Cs symmetry	Assignment C_{2v} symmetry
158 (6) 0·63	—	a' fund.	X sens 11 (b_1)
314 (3) 0·53	295 vw	a'' fund.	$18b$, b_2
349 (00) ?	365 vw	δ ($c\hat{c}c$) a' fund.	δ $c\hat{c}c$, in plane
399 (w)	404 (vw)	a'' fund.	$16a$, a_2
	455 (w)	295+158 = 453 (A'')	—
487	486 (s)	a' fund.	$16a$, a_1
557	556	a' fund.	$16b$, b_1

Note that the combination band is stronger than several fundamentals.

Chosen values of the frequencies and their values	
cm^{-1}	Assignment
158	b_1
314	b_2
347	b_2
404	a_2
455	combination band
486	a_1

ANISOLE
(Detailed Table)

Raman $\Delta\nu$	Raman ϱ*	Infrared liquid	Assignment	Value chosen
GREEN (1962)				
209 (m)	(0·70)	221†	a' fund. (11, b_1)	209
258 (m)	(0·70)	259†	a'' fund. ($18b$, b_2)	258
—	—	352†	β ($\hat{c}oc$), a' fund. (in-plane def.)	352
444 (s)	0·38	439 w	a' fund. ($6a$, a_1)	444
512 (m)	0·86	510 m	a' fund. ($16b$, b_1)	511
554	—	553 m	209+352 = 561 (A')	

* Depolarization O. Paulsen (1939).
† Barchewitz and Parodi (1939).

Raman $\Delta\nu$	Raman ϱ*	Infrared liquid	Assignment	Value chosen
STEPHENSON (1961)				
210 (w)	dp	—	b_1 fund., 16*b*	210
258 (w)	dp?	—	b_2 fund., 18*b*	258
390 (vw)		—	fund., *côc* bending?	
415 (vw)		415 vw	a_2 fund., 16*a*	415
443 (s)	dp	441 w	a_1 fund., 6*a*	441
515 (w)		511 m	b_1 fund., 17*b*	511
556		554	fund., *côc* bending?	
LEBAS (1962)				
209 (2)	0·70	209 w	b_1 fund., γ(*c*—X)	
555 (1)	0·76	551 s	b_2 fund., β(*c*—X)	
264 (2)	0·68	263 s	—	
LEBAS (1962)				
415 vw		415 w	a_2 fund.	
512½	0·86	507 s	b_1 fund.	
443 (2)	0·38	440 m	a_1 fund.	
555 (1)	0·76	551 s	—	

*Raman values from Kohlrausch *et al.*

DELORME (1964)				
		113 m	a_2, torsional	
209, 2	0·70	—	b_1 fund., 16*b*	
264, 2	0·68	263	b_2 fund., β(*c*—X) 18*b* or 15	
512, ½	0·86	507 s	b_1 fund., γ(*c*—X) 17*b* or 10*b*	

Chosen values for present paper	
Frequency	Assignment
113	a_2
209	b_1
263	b_2
290–350	b_2
414	a_2
443	a_1
512	b_1

ACETOPHENONE

IR	Raman	Assignment
LEBAS (1961)		
—	163, (6), (0·56)	b_1, 10b, γ(c—X)
—		β(c—X)
222 s	255 (0)	—
256 w	—	—
277 w	—	—
353 w	—	—
406 s	406 $\frac{1}{2}$	a_2, 16a
464 s	464 $\frac{1}{2}$	b_1, 16b
366 w	368, (3), (p)	a_1, 6a

IR	Raman*	Assignment
DELORME (1964)		
—	163, 6–0, 56	b_1, 16b
222 (s)	225 (0)	b_1, 18b or 15, β(c—X)
464	464, $\frac{1}{2}$	b_2, 17b or 10b, β(c—X)

* Raman data of both cases from Kohlrausch *et al.*

Values chosen for present work	
Frequency (cm^{-1})	Assignment
464	b_1
406	a_2
366	a_1
225	b_2
163	b_1

PHENYL ISOTHIOCYANATE

The values chosen for the wavenumbers of phenyl isothiocyanate and their assignments are based on the work of Stephenson *et al.* (1961).

Infrared (cm^{-1})	Raman (cm^{-1})	Assignment
—	180 vw	b_1 Fundamental
—	245 m, dp	b_2 Fundamental
355 m	357 m, p	a_1 Fundamental
396 vw	396 vw, dp	a_2 Fundamental
443 w, br	437 vw, dp	b_1 Fundamental, —NCS out-of-plane bending
491 m	491 w, dp	b_1 Fundamental

The above values of the frequencies and their assignments have been chosen for the present studies. A spectrum of the once purified commercial sample (Eastman Kodak Co.) was run on the Perkin–Elmer Model 301 Far Infrared Spectraphotometer. The 180 cm^{-1} peak did not appear.

APPENDIX 2

BIBLIOGRAPHY ON LOW-FREQUENCY VIBRATIONS OF MONOSUBSTITUTED BENZENES

The number of publications dealing with the vibrational spectra of benzene and its substitution products is very large. This bibliography lists only those papers which have been found most useful.

The most complete list of references on the subject of the low-frequency vibrations of monosubstituted benzenes is in Delorme's 1964 paper, which lists 105 references spanning 30 years.

One of the difficulties in approaching a new subject is to find the "key papers", i.e. those which are essential in understanding it. Most often they are the early ones. Ingold's series of 20 papers is an example. Part XI of this series, entitled "Introductory consideration of the spectral properties of the vibrations of some deuterated benzenes" touches upon every aspect of the subject. Furthermore, it is written in a clear and lucid style.

A major problem is the use of different symbols by various authors to describe the modes of vibration of the benzene ring. Delorme (1964) and Stephenson (1961) each give a table connecting all the different systems used.

The references are arranged under major headings:

Under the heading "General Articles" are listed a number of papers in French and English which deal with all the aspects of far infrared spectroscopy. The most useful are those of Hadni (1964) and Wood (1963).

Palik's bibliography (1963) is extremely thorough. It gives both subject and author indexes and lists the papers year by year from 1889 to 1962. There are 650 listed references.

Under the heading "Vibration of aromatic compounds" are listed the papers which touch on low-frequency vibration, although sometimes only incidentally. The most useful

introduction to the characteristic vibrations of the benzene ring is the work of Randle and Whiffen (1955). Bogomolov (1960–61) develops the subject further in a series of papers. The most up-to-date reference on the spectra below 180 cm^{-1} is that of Delorme (1964). He examines 20 compounds and gives a detailed discussion. Stanevich's paper, while treating mainly with the OH bond in the far infrared, gives a most useful discussion of the effect of changing the physical state and temperature of the sample. In the planar vibrations of chlorinated benzenes, Scherer gives the amplitude of the vibrations of benzene, benzene-d_6, and all the chlorinated benzenes as well as their deuterated derivatives. The normal coordinates are calculated by two standard methods (VFF and UBFF). This is a most useful publication, but only a limited number of copies are available.

Under "Monumental Works" are listed three authors who have worked on the problem of vibrational spectra of aromatic compounds over a long period. Kohlrausch studied the Raman spectra, and Lecomte the infrared, and both have published books. Ingold's main interest was to establish the structure and symmetry of the benzene ring. At the end of his investigation he arrived at the complete attribution of the 20 benzene vibrations.

A. Infrared and Raman Reference Works

American Petroleum Institute, "Research Project 44", Catalogue of Infrared and Raman Spectra.

LANDOLT–BÖRNSTEIN, Tabellen: I Band, *Atom und Molekular Physik*, **2**, Teil, Molekln I.

MECKE, R., *Documentation of Molecular Spectroscopy No. 3368*, Butterworths, London, (1958).

B. General Articles Dealing with Different Aspects of Far-Infrared Spectroscopy

BENTLEY, F. F., E. F. WOLFARTH, NORA E. SRP and W. R. POWELL: "Analytical Applications of Far Infrared Spectra: I. Historical Review, Apparatus and Technique", *Spectrochim. Acta*, **13**, 1–30, 1958.

BENTLEY, F. F. and E. F. WOLFARTH "Analytical Applications of Far Infrared Spectra: II Spectra-Structure Correlations for Aliphatic and Aromatic Hydrocarbons in the Cesium Bromide Region", *Spectrochim. Acta*, pp. 165–205, 1959.

HADNI, A.: "L'Elargissement Actuel du Domaine des Applications de l'Infrarouge Lointain", *Ann. Phys.* **9**, 9–27, 1964.

LECOMTE, J.: "Quelques Resultats Obtenus dans l'Infrarouge Lointain", *Boll. Sci. Fac. Chim. Ind. Bologna*, 21(1), 51–70 (1963).

WOOD, J. L.: "Far-Infrared Spectroscopy", *Quart. Rev.*, **27**, 362–382, 1963.

WILKINSON, G. R., S. A. INGLIS and C. SMART: "Far Infrared Spectroscopy", *Spectroscopy*, the Institute of Petroleum, London, 1962.

C. Far Infrared Bibliography

PALIK, E. D.: "*A Far Infrared Bibliography*", NRL Bibliography No. 21, 1963.

ULRICH, W. F.: "*Bibliography of Infrared Applications*", Beckman Bulletin No. 7037.

D. Vibrational Spectra of Aromatic Compounds

1. *Monumental Works over an Extensive Period of Time*

INGOLD, C. K. *et al.*, *J. Chem. Soc.*, between 1936 and 1946.

KOHLRAUSCH, K. W. F. *et al.* in *Monatsh. Chem.*, between 1926 and 1946.

LECOMTE, J. *et al.*, *J. de Phys. et le Radium*, 1936–1946.

2. *Characteristic Vibrations of the Benzene Ring*

RANDLE, R. R. and D. H. WHIFFEN: "The Characteristic Vibration Frequencies of Substituted Benzenes", *Molecular Spectroscopy*, The Institute of Petroleum, 1955.

BOGOMOLOV, A. M.; "Vibrational Spectra of Aromatic Compounds, VII Characteristic Vibrations of Monosubstituted Benzenes", *Optics and Spectroscopy*, **9**, 162–166, 1960.

3. *Far-Infrared Spectra of Group of Monosubstituted Benzenes*

DELORME, P.: "Etude des Vibrations Fondamentales de Basse Frequence d'une Serie de Derives Benzeniques Monosubstitués", *J. Chim. Phys.*, **61**, 1439–1456, 1964 .

LEBAS, J. M.: "Spectres de Vibrations d'une Série de Dérivés Monosubstitués du Benzene Region 200–700 cm^{-1}", *J. Chim. Phys.*, **59**, 1072–1083, 1962.

GREEN, J. H. S., W. KYNASTON and H. A. GEBBIE: "Far Infrared Spectroscopy of Benzene Derivatives", *Spectrochim. Acta*, **19**, 807–810, 1963.

WILCOX, W. S., C. V. STEPHENSON and W. C. COBURN, JR.: "Far Infrared Spectra of Substituted Aromatic Hydrocarbons", Wadd Technical Report, 60–333, 1960.

WYSS, H. R., R. D. WERDER and Hs. H. GUNTHARD: "Far Infrared Spectra of Twelve Organic Liquids", *Spectrochim. Acta*, **20**, 573–579, 1964.

4. *Monographs*

(a) *Nitrobenzene*

GREEN, J. H. S., W. KYNASTON and A. S. LINDSAY, *Spectrochim. Acta*, **17**, 486, 1961.

STEPHENSON, C. V., W. C. COBURN JR. and W. S. WILCOX, *Spectrochim. Acta*, **17**, 933, 1961.

WITTEK, H., *Z. Phys. Chem.*, B 52, 315, 1942.

(b) *Benzonitrile*

BAK and NIELSEN, *Z. für Electrochem.*, **64**, 650, 1960.

BARCHEWITZ, P. and PARODIE, *J. Phys. et le Radium*, **10**, 143, 1939.

GREEN, J. H. S., *Spectrochim. Acta*, **17**, 607, 1961.

JAKOBSEN, R. J., Technical Documentary Report No. ASD-TDR-63-869, Wright–Patterson Air Force Base, Ohio (1963).

JAKOBSEN, R. J., *Spectrochim. Acta*, **21**, 122–131, 1965

(c) *Benzaldehyde, Acetophenone and Benzoylchloride*

GARRIGOU-LAGRANGE, C., N. CLAVERIE, J. M. LEBAS et M. L. JOSIEN, *J. Chim. Phys.* **58**, 559, 1961.

(d) *Thiophenol*

SCOTT, D. W. *et al.*: "Benzenethiol: Thermodynamic Properties in the Solid, Liquid and Vapour States, Internal Rotation of the Thiol Group", *J. Amer. Chem Soc.*, **78**, 5463, 1956.

(e) *Other Compounds*

GREEN, J. H. S.: "Anisole, Ethylbenzene, Phenetole, Methyl Phenyl Sulphide, and Ethyl Phenyl Sulphide", *Spectrochim. Acta*, **18**, 39–50, 1962.

STEPHENSON, C. V.: "Infrared and Raman Spectra of *a*, *a*, *a*-Trichlorotoluene, $C_7H_5Cl_3$" *J. Chem. Phys.*, **42**, 35, 1965.

EVANS, J. C. and R. A. NYQUIST: "The vibrational spectra of ethynyl benzene and ethynyl benzene-d", *Spectrochim. Acta*, **16**, 918–928, 1960.

STEELE, D., E. R. LIPPINCOTT and J. XAVIER: "Vibrational spectra of fluorobenzene-d_s" *J. Chem. Phys.* **33**, 1242, 1960.

(f) *Hydrogen Bonding*

STANEVICH, A. E.: "Hydrogen Bonding and Long-Wavelength Infrared Absorption Spectra of Some Benzene Derivatives I", *Optics and Spectroscopy*, 425–429, 1964 (English edit.).

(g) *Miscellaneous*

FATELEY, W. G., R. K. HARRIS, F. A. MILLER and R. E. WITKOWSKI: "Torsional frequencies in the far infrared-IV. Torsions around the *c-c* single band in conjugated molecules", *Spectrochim. Acta*, **21**, 231–244, 1965.

CHANTRY, G. W., A. ANDERSON and H. A. GEBBIE: "Far infrared spectra of naphthalene h_8 and d_8", *Spectrochim. Acta*, **20**, 1465–1466, 1964.

ROW, C. N. R.: "Chemical Applications of Infrared Spectroscopy, Academic Press, New York and London, 1963.

SCHERER, J. R.: *Planar Vibrations of Chlorinated Benzenes*, The Dow Chemical Co., 1963.

SIRKAR, S. C., D. K. MUKHERJEE and P. K. BISHUI: "On the Infrared Spectra of the Fluoro-, Chloro-, Bromo- and Iodobenzene in the Vapour State", *Indian J. Phy.*, **38**, 610–622, 1964.

ARE EOG's GENERATOR POTENTIALS?

SADAYUKI F. TAKAGI

Department of Physiology, School of Medicine,
Gunma University, Maebashi, Japan

INTRODUCTION

A slow electronegative potential appears in the olfactory epithelium when an odour is applied. This potential was discovered by Hosoya and Yoshida in the dog in 1937. Seventeen years later Ottoson studied this potential in the rabbit (1954) and very extensively in the frog (1956, 1958). He named it an "electro-olfactogram" (EOG) (1956). A similar potential was found in the olfactory epithelium of amphibians at the cessation of odour application (Takagi and Shibuya, 1959, 1960a, b, c; Shibuya and Takagi, 1963a, b; Higashino and Takagi, 1964). This was called "off"-potential (Fig. 1A, *b*, *c*), while the one found by Ottoson was called "on"-potential (Fig. 1A, *a*). Then, an electropositive potential was found when some kinds of odours were applied (Takagi, Shibuya, Higashino and Arai, 1960; Higashino and Takagi, 1964; Shibuya and Takagi, 1963a, b; Gesteland, 1964; Takagi, Wyse and Yajima, 1966). Thus, three kinds of EOG's were found in the olfactory epithelium of amphibians.

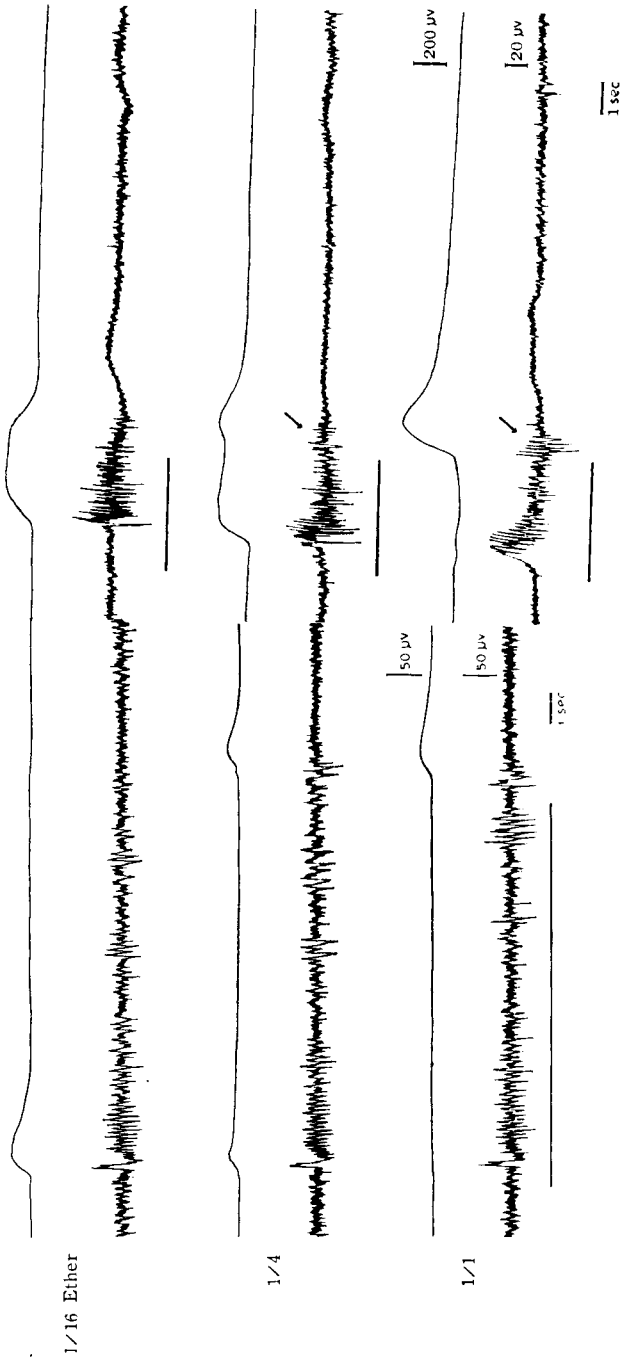
Ottoson (1956, 1963) assumed that the electronegative on-potential is a generator potential which elicits afferent discharges in the olfactory nerve. However, when the EOG's were recorded together with the induced wave in the olfactory bulb, some questions were raised regarding this hypothesis (Takagi *et al.*, 1960).

(1) Change in amplitude of the negative on-potential is not parallel with that of the induced wave in the olfactory bulb (Fig. 1B).

(2) Even when an electropositive potential appears in the olfactory epithelium, an induced wave appears in the olfactory bulb (Fig. 1A, *d*).

(3) The off-induced wave in the olfactory bulb precedes the onset of the "off"-potential in the epithelium (Fig. 1A, *b*, *c*, *d*).

Later, similar findings were obtained by Mozell (1962). Facts which are favourable and unfavourable for the hypothesis are considered below.


1/16 Ether
1/4
1/1
200 μv
20 μv
50 μv
50 μv
1 sec
1 sec

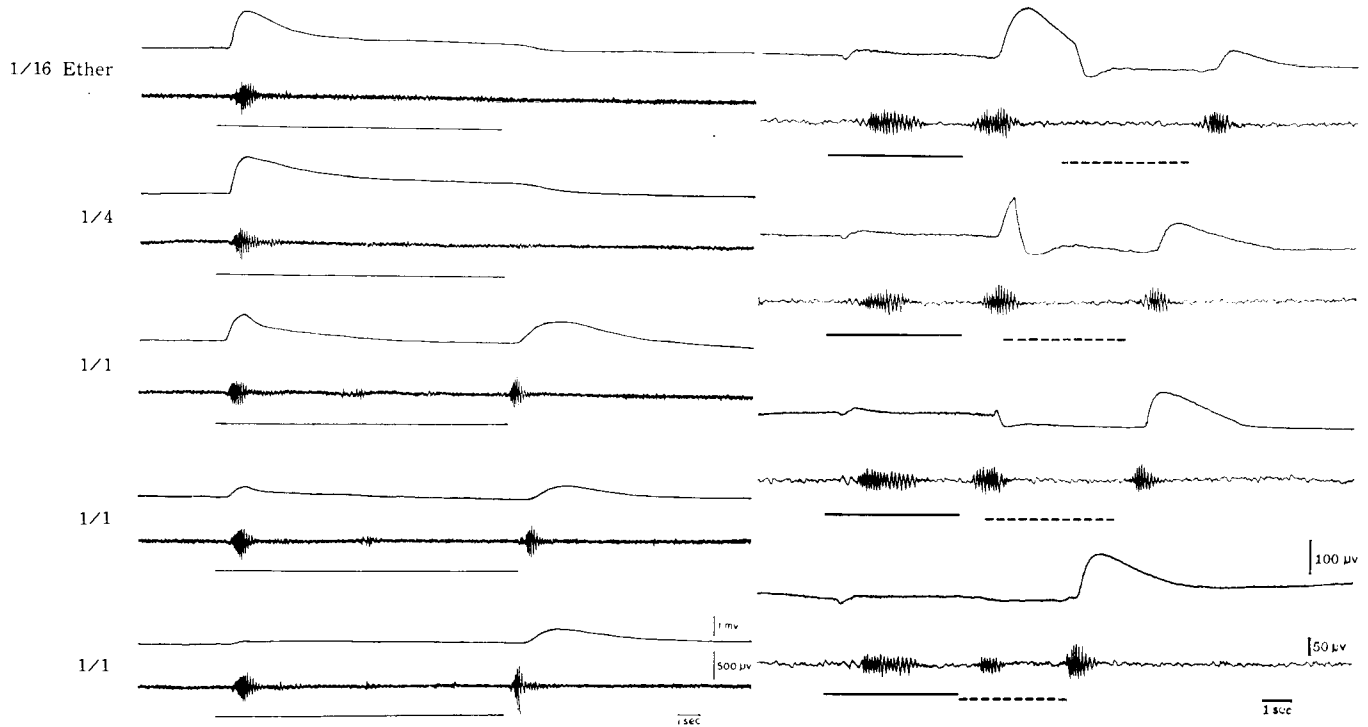


FIG. 1B. Findings which contradict the generator potential hypothesis. Top left, the EOG decreased in amplitude, when the concentration of ethyl ether was increased.

Top right, the EOG similarly decreased and disappeared, when the velocity of odorous air was increased.

Bottom left, the EOG decreased in amplitude, when saturated ethyl ether vapour ($^1/_1$) was applied three times, though sufficient intervals were interposed. In all these cases, the induced waves in the olfactory bulb appeared with nearly the same magnitude though the EOG's decreased and disappeared.

Bottom right, the off-EOG elicited by ethyl ether vapour was abolished by chloroform vapour when the interval between these two stimulations was shortened. Even when the off-EOG disappeared, the off-induced wave survived. Reprinted from *Japanese Journal of Physiology*, **10,** 571–584, 1960.

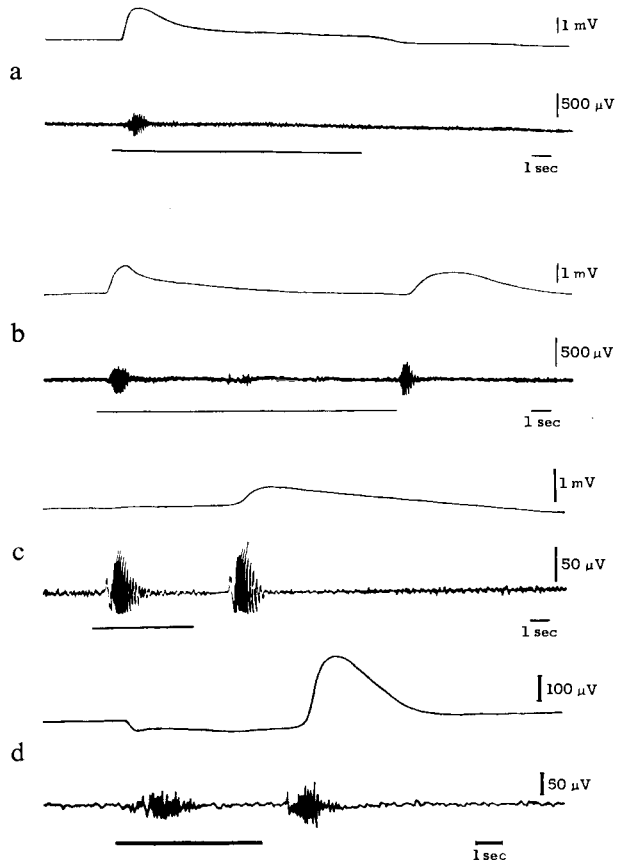


FIG. 1A. Three kinds of EOG's (top) and the induced waves simultaneously recorded in the olfactory bulb (bottom).
a. On-potential found by Ottoson (1956).
b, c. On–off-potentials.
d. Electropositive potential accompanied by a negative off-potential.
It is noted that the on-induced wave appears even when a very small on-potential or a positive on-potential is elicited in the olfactory epithelium in *c* and *d*, and that the off-induced discharges precede the onset of the off-potential in *b*, *c* and *d*.

RETROGRADE DEGENERATION OF THE OLFACTORY CELL

If the EOG's are generator potentials, they should be elicited in the olfactory cells. There is another kind of cell, named "sustentacular cell", in the olfactory epithelium. This is an ependymal cell and belongs to a glial cell. It has been a center of discussion in neurophysiology whether a glial cell produces a potential change. It is probable that the sustentacular cell

produces a potential change in response to olfactory stimulation. If this is the case, the generator potential hypothesis becomes improbable.

When the olfactory nerve was sectioned, retrograde degeneration occurred in the nerve and in the olfactory cell. Under the light microscope, decrease in number of the olfactory cells became more and more manifest day by day (Fig. 2), while no considerable change was found in the sustentacular cells. The EOG's began to decrease in amplitude and disap-

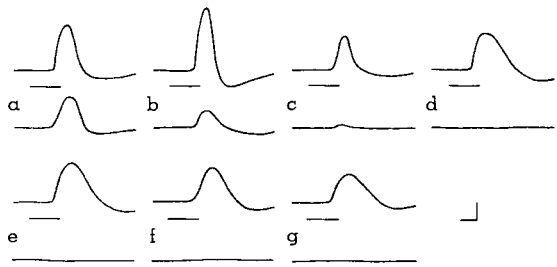


FIG. 2. Changes in amplitude of the EOG's. In each pair of records, the slow potential at the top was obtained as a control in the olfactory epithelium of the normal (left) side and the potential at the bottom was recorded in the sectioned (right) side of the same bullfrog. The responses were obtained 5 days after the nerve section in *a*, 6 days after in *b*, 7 days after in *c*, and 8 days after in *d*. Here the EOG completely disappeared. In *e*, *f*, and *g*, which were obtained 9, 10, and 12 days after respectively, no response could be found in the sectioned side (at the bottom). Reprinted by permission of the Rockefeller Institute Press from the *Journal of General Physiology*, **48**, (4), 562, March 1965.

peared in 8 days after the section in summer (Fig. 3) and in 16 days in winter (Takagi and Yajima, 1964, 1965). This finding was very favourable to the "generator potential hypothesis". At that time it was even concluded that the EOG's are elicited in the olfactory cell, although the discrepancies stated above yet remained to be explained.

REMOVAL OF OLFACTORY MUCUS

Shibuya (1964) separated a very thin bundle (20 μ in diameter) of olfactory nerve fibres from the main bundle in the gopher tortoise. By laying this bundle on a stimulating electrode, it was possible to evoke nerve action potentials which reach the olfactory epithelium antidromically. By means of a microelectrode, he could record the action potentials and located a small area of the olfactory epithelium which was innervated by the separated olfactory nerve fibres (Fig. 4).

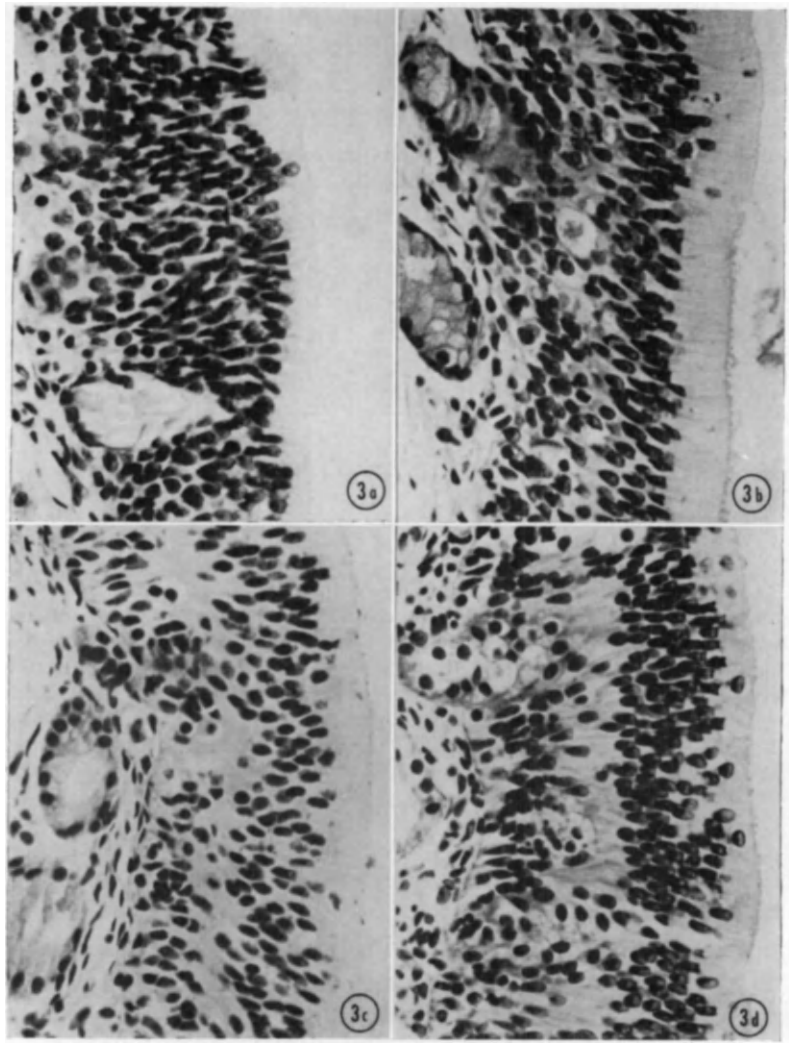


FIG. 3. Sections of the normal and degenerating olfactory epithelia. *a*. A section of normal olfactory epithelium as a control. Slender nuclei of the supporting cells are found in the upper part and round nuclei of the olfactory cells are found below them. *b–d*. Sections of degenerating epithelia. 6 days in *b*, 9 days in *c*, and 16 days in *d* after the nerve section, respectively. Slender nuclei are seen in the upper part and the round ones in the lower part. Between them a spacial gap is noted. It becomes more and more striking as days elapse. In *d*, the number of the supporting cells is far larger than that of the nuclei in the lower part. The olfactory cell has degenerated considerably and the number is very small in this stage. Most of the nuclei found in the lower part and the round nuclei in the uppermost part are supposed to be of different origin. Further explanation in the text. Reprinted by permission of the Rockefeller Institute Press from the *Journal of General Physiology*, **48,** (4), 564, March 1965.

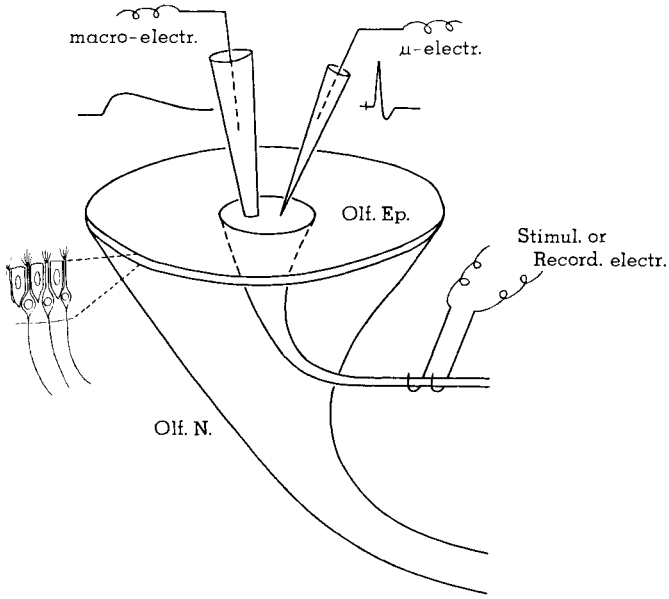


FIG. 4. Olfactory epithelium-nerve twig preparation.
A very thin bundle of the olfactory nerve fibres was separated from the main bundle. It was electrically excited with a stimulating electrode and the antidromically conducted spike potentials were recorded on the surface of the olfactory epithelium by means of microelectrode. Thus, a small area can be located which is innervated by the small olfactory nerve twig. By means of a macroelectrode put in the center of this small area, normal EOG is recorded and simultaneously the afferent olfactory nerve discharges are recorded in the small olfactory nerve twig.

FIG. 5. Dissociation of the EOG from the olfactory nerve discharges.
A, Negative EOG (top) and olfactory nerve twig discharges in the tortoise elicited by small puff of amyl acetate near vapour saturation. *B*, Negative EOG and nerve twig discharges recorded after absorbent paper was put on the epithelium for 4 minutes and then taken off. The EOG nearly disappeared, but the olfactory nerve twig discharges did not change much. Voltage calibration: about 1 mV; below, 40 μV. Time calibration, 0·5 sec. *C* and *D*, Integrated curve of nerve twig discharges in *A* and in *B* respectively. Time calibration, 2 sec. Reprinted from *Science*, **143**, 1338, 1964.

EOG's were recorded from the small area with macroelectrode. Simultaneously, using the above stimulating electrode as a recording electrode, olfactory nerve discharges were recorded in the very thin bundle. After a small piece of soft paper (Kimwipe) was put on the olfactory area and the mucus on it was absorbed, the same experiment was repeated. It is surprising that the EOG disappeared, while the olfactory nerve discharge appeared without considerable change (Fig. 5). Such dissociation of the EOG from the olfactory nerve discharges seems to be a most definite finding against the hypothesis that the EOG's are generator potentials.

IONIC MECHANISMS OF EOG'S

When the ionic mechanisms of the EOG's were studied, it was made clear that the negative EOG's depend upon the entry of Na^+, and the positive EOG mainly upon the exit of Cl^- through the olfactory receptive membrane (Takagi and Wyse, 1965a, b; Takagi, Wyse and Yajima, 1965). The resemblance in shape and ionic mechanism of the negative EOG's with the generator potentials of the Pacinian corpuscle (Diamond, Gray and Inman, 1957, 1958), of the stretch receptor neuron (Edwards, Terzuolo and Washizu, 1963) and of the muscle spindle (Ottoson, 1963, 1964) strongly suggests that the negative EOG's are generator potentials. Similarly, the resemblance in shape and ionic mechanism of the positive EOG with inhibitory potentials of the spinal motoneurones (Coombs, Eccles and Fatt, 1955), of the crustacean neuromuscular junction (Fatt and Katz, 1953; Edwards and Hagiwara, 1959), of the snail (Kerkut and Thomas, 1963, 1964) and of the Mauthner cell of fish (Asada, 1963) suggests that the positive EOG is an inhibitory potential. These findings on the negative EOG's are favourable to the generator potential hypothesis, while the finding on the positive EOG indicates the presence of an inhibitory mechanism in the olfactory receptor. The negative and positive EOG's are supposed to induce "on"-, "off"- discharges and inhibition of the spontaneous discharges in the olfactory cell respectively (Fig. 6) (Takagi and Omura, 1963).

RECORDING WITH MICROELECTRODE

Ottoson (1956) inserted a microelectrode into the olfactory epithelium and found that the negative EOG decreases in amplitude and disappears as the electrode is advanced. Shibuya (unpublished data) confirmed this finding and found that an electropositive slow potential together with

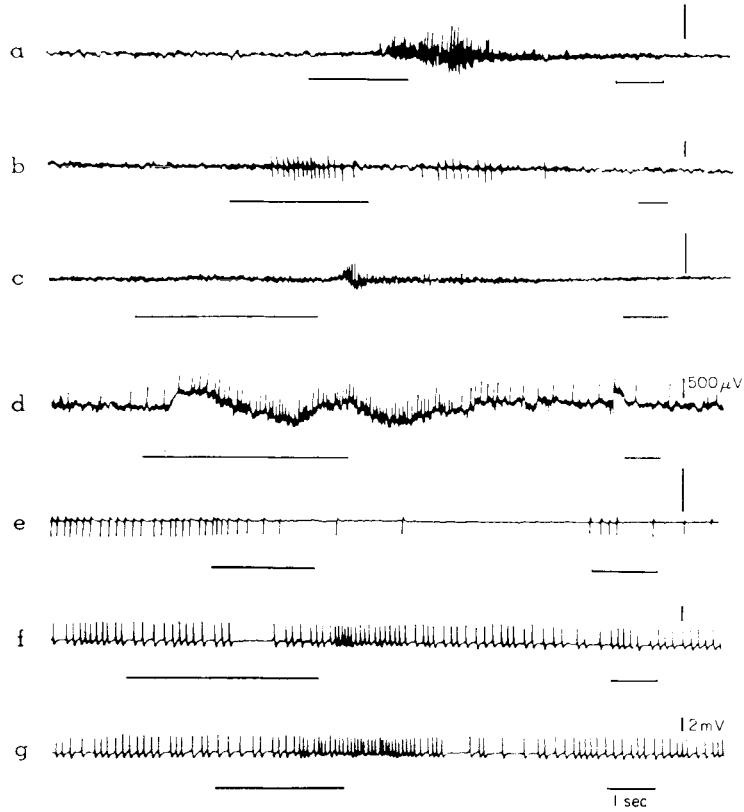


FIG. 6. Seven types of the responses of the olfactory receptor cell. Induced discharges in resting cells in *a*, *b*, and *c*. *a*, induced "on"-type. *b*, induced "on–off"- type. *c*, induced "off"-type. Spontaneous discharges in *d*, *e*, *f* and *g*. *d*, "on"-type. Increase of discharges of probably the same origin is observed during olfactory stimulation. Recording was made by means of an amplifier with a longer time constant (0·3 sec). *e*, "simple inhibition"-type. *f*, "post-inhibitory excitation" type. *g*, "post-excitatory inhibition"-type. Vertical lines on the right indicate 500 μV in *a* to *d*, and 2 mV in *e* to *g*. Horizontal lines indicate 1 sec.
Reprinted from *Proceedings of the Japan Academy*, **39**, 253–255, 1963.

single unit spikes appears as the microelectrode is advanced further. These findings are also favourable to the generator potential hypothesis. However, he observed with microelectrode that the electronegative slow potential disappears after absorbing the olfactory mucus, while the electropositive slow potential remains (unpublished data). This finding is again inconsistent with the hypothesis. Besides, he noticed that the electropositive potential appears superimposed upon the EOG but that the onset of the positive potential is always delayed (unpublished data). The finding of

the superposition and the delay between the onsets of both potentials is important, because such phenomena may show that the two potentials are of different origin.

DISCUSSION

The presence of a secretory mechanism in the sustentacular cell has been indicated by data from electron microscopy (Hama, personal communication).

Although the Bowmann's gland is better known as a secretory gland of the olfactory mucus, there is a possibility that the EOG is a secretory potential of the sustentacular cell. If odours depolarize the receptive membrane of the olfactory cell first and this depolarization in turn induces the sustentacular cell to secrete olfactory mucus, then degenerated olfactory cell due to nerve section (Takagi and Yajima, 1964, 1965) could no longer induce the secretion by the sustentacular cell. This may explain why the EOG disappears in the olfactory epithelium after section of the olfactory nerve. Thus, the experiment on the retrograde degeneration of the olfactory cell is not enough to negate the origin of the EOG in the sustentacular cell.

The next experiment which looks favourable to the generator potential hypothesis was the discovery of the ionic mechanisms in the three kinds of EOG's which are the same as those found in the other receptive membranes. In this experiment, it was shown that the Na^+ and Cl^- in the mucus are essential to the negative and positive EOG's. From this finding, it is natural that the negative EOG decreased in amplitude and disappeared in Shibuya's experiment (1964) when the mucus was removed.

The sustentacular cell is supposed to be a glial cell. The electrical activity of the glial cell has been a centre of discussion for many years, and is still a problem yet to be solved. There is a possibility that the glial cell shows an electrical activity similar to that of the neuron. If so, the glial cell action potential may have a similar underlying ionic mechanism, and the experiments on the ionic mechanisms of the EOG's are not enough to negate the origin of the EOG in the sustentacular cell.

Shibuya (1964) showed that the EOG can be dissociated from the olfactory nerve discharges when the olfactory mucus is absorbed with a soft absorbent paper.

Concerning this experiment, however, one can ask if the removal of the olfactory mucus may make it difficult to record the EOG's which are being generated with the same magnitude as before. It is conceivable that the decrease of the mucus results in a change of the recording condition of the EOG's. One of the possibilities for this is the change of the resistance of

the mucus. The olfactory mucus is not a simple solution but looks to be a kind of sol (especially in the case of the frog). If the water in it is absorbed with the paper, the electric resistance of the mucus may be increased. If this is the case, the amplitudes of the EOG's may be reduced gradually as the water in the mucus is absorbed.

It should be emphasized here that Shibuya from his data does not negate the possibility of a generator potential itself in the olfactory cell, but he suggests that the EOG's are the composite potentials of the generator potential and other unknown potentials. He believes that only the latter potential was "absorbed" in his experiment. His idea means that only the secretory mechanism of the sustentacular cell was damaged with the soft absorbent paper, leaving the generator mechanism of the olfactory receptive membrane intact. If this idea is admitted, the dissociation of the EOG from the olfactory nerve discharge found in his experiment is easily explained. Furthermore, the discrepancies between the EOG's and the induced waves in the olfactory bulb stated at the beginning can also be explained. However, whether the secretory mechanism in the sustentacular cell is really damaged or not in Shibuya's experiment (1964) is a problem to be solved in future.

SUMMARY

When the negative olfactory receptor potential (EOG by Ottoson, 1956) was studied, it was assumed to be a generator potential for the olfactory nerve discharges. However, subsequent studies disclosed several questions to this hypothesis. In the light of the recent investigation, origin of the EOG's was discussed.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Dr. T. Shibuya in my laboratory for his cooperation in writing this paper, and to Dr. S. Fox in the University of Iowa for his correction of the English.

REFERENCES

ASADA, Y. (1963). Effects of intracellularly injected anions on the Mauthner cells of goldfish. *Jap. J. Physiol.* **13**, 583–598.

COOMBS, J. S., ECCLES, J. C. and FATT, P. (1955). The specific ionic conductances and the ionic movements across the motoneuronal membrane that produce the inhibitory post-synaptic potential. *J. Physiol.* **130**, 326–373.

DIAMOND, J., GRAY, J. A. B. and INMAN, D. R. (1957). Receptor potentials and Na concentration. *J. Physiol.* **138**, 31–32P.

DIAMOND, J., GRAY, J. A. B. and INMAN, D. R. (1958). The relation between receptor potential and the concentration of sodium ions. *J. Physiol.* **142,** 382–394.

EDWARDS, C. and HAGIWARA, S. (1959). Potassium ions and the inhibitory process in the crayfish stretch receptor. *J. gen. Physiol.* **43,** 315–321.

EDWARDS, C., TERZUOLO, C. A. and WASHIZU, Y. (1963). The effect of changes of the ionic environment upon an isolated crustacean sensory neuron. *J. Neurophysiol.* **26,** 948–957.

FATT, P. and KATZ, B. (1953). The effect of inhibitory nerve impulses on crustacean muscle fibre. *J. Physiol.* (London) **121,** 374–389.

GESTELAND, R. C. (1964). Initial events of the electro-olfactogram. *Ann. N. Y. Acad. Sci.* **116,** 440–447.

HIGASHINO, S. and TAKAGI, S. F. (1964). The effect of electrotonus on the olfactory epithelium. *J. gen. Physiol.* **48,** 323–335.

HOSOYA, Y. and YOSHIDA, H. (1937). Über die bioelektrische Erscheinungen an der Riechschleimhaut. *Jap. J. med. Sci. III. Biophysics,* **5,** 22–23.

KERKUT, G. A. and THOMAS, R. C. (1963). Anion permeability of the inhibitory post-synaptic membrane of *Helix* neurones. *J. Physiol.* **168,** 23–24P.

KERKUT, G. A. and THOMAS, R. C. (1964). The effect of anion injection and changes in the external potassium and chloride concentration on the reversal potentials of the IPSP and acetylcholine. *Comp. Biochem. Physiol.* **11,** 199–213.

OTTOSON, D. (1954). Sustained potentials evoked by olfactory stimulation. *Acta Physiol. Scand.* **32,** 384–386.

OTTOSON, D. (1956). Analysis of the electrical activity of the olfactory epithelium. *Acta Physiol. Scand.* **35,** Supp. 122, 1–83.

OTTOSON, D. (1958). Studies on the relationship between olfactory stimulating effectiveness and physico-chemical properties of odorous compounds. *Acta Physiol. Scand.* **43,** 167–181.

OTTOSON, D. (1963a). Generation and transmission of signals in the olfactory system. *Proc. First Intern. Symp. on Olfaction and Taste,* ed. ZOTTERMAN, Y., Pergamon Press.

OTTOSON, D. (1963b). Activity of muscle spindle and sodium concentration. Abstr. XI. Scand. Congr. Physiol. *Acta Physiol. Scand.* **59,** 118.

OTTOSON, D. (1964). The effect of sodium deficiency on the response of the isolated muscle spindle. *J. Physiol.* **171,** 109–118.

SHIBUYA, T. (1964). Dissociation of olfactory neural response and mucosal potential. *Science,* **143,** 1338–1340.

SHIBUYA, T. and TAKAGI, S. F. (1963). Electrical response and growth of olfactory cilia of the olfactory epithelium of the newt in water and on land. *J. gen. Physiol.* **47,** 71–82.

TAKAGI, S. F. and OMURA, K. (1963). Responses of the olfactory receptor cells to odours. *Proc. Jap. Acad.* **39,** 253–255.

TAKAGI, S. F. and SHIBUYA, T. (1959). "On"- and "off"-responses of the olfactory epithelium. *Nature, Lond,* **184,** 60.

TAKAGI, S. F. and SHIBUYA, T. (1960a). The "on" and "off" responses observed in the lower olfactory pathway. *Jap. J. Physiol.* **10,** 99–105.

TAKAGI, S. F. and SHIBUYA, T. (1960b). The electrical activity of the olfactory epithelium studied with micro- and macro-electrodes. *Jap. J. Physiol.* **10,** 385–395.

TAKAGI, S. F. and SHIBUYA, T. (1960c). Electrical activity of lower olfactory nervous system of toad. In *Electrical Activity of Single Cells,* ed. KATSUKI, Y. Bunkyo-ku, Tokyo, Igaku-Shoin.

TAKAGI, S. F., SHIBUYA, T., HIGASHINO, S. and ARAI, T. (1960). The stimulative and anaesthetic actions of ether on the olfactory epithelium of the frog and the toad. *Jap. J. Physiol.* **10,** 571–584.

TAKAGI, S. F. and YAJIMA, T. (1964). Electrical responses to odours of degenerating olfactory epithelium. *Nature, Lond.* **202,** 1220.

TAKAGI, S. F. and YAJIMA, T. (1965). Electrical activity and histological change of the degenerating olfactory epithelium. *J. gen. Physiol.* **48,** 559–569.

TAKAGI, S. F. and WYSE, G. A. (1965a). Ionic mechanisms of olfactory receptor potential. Abstracts of Papers presented at XXIII Intern. Congr. of Physiol. Sci., Tokyo, Japan.

TAKAGI, S. F., and WYSE, G. A. (1965b). The effect of sodium ion on the olfactory receptor potentials (in preparation).

TAKAGI, S. F., WYSE, G. A. and YAJIMA, T. (1966). Anion permeability of the olfactory receptive membrane *J. gen. Physiol.* (in the press).

SLOW POTENTIAL CHANGES INDUCED IN THE OLFACTORY BULB BY CENTRAL AND PERIPHERAL STIMULI*

M. CARRERAS, D. MANCIA, and M. MANCIA

Clinica delle Malattie Nervose e Mentali,
Università di Parma ed Istituto di Fisiologia Umana
—Università di Milano—
Imprese di Elettrofisiologia del CNR, sezioni di Milano e Parma, Italia

INTRODUCTION

D. C. potential recording has been largely employed in the study of CNS physiology. Particularly slow potential changes have been observed in the cerebral cortex following sensory (Arduini *et al.* 1957, Vanasupa *et al.* 1959), reticular (Arduini *et al.* 1957, O'Leary *et al.* 1958, Vanasupa *et al.* 1959), thalamic (Goldring and O'Leary 1957, Brookhart *et al.* 1958), cortical (Goldring *et al.* 1961) and cerebellar (Dondey and Snider 1955) stimulations as well as during the physiological sleep-wakefulness rhythms (Caspers 1963, Tabushi 1965, Kawamura and Sawyer 1964, Wurtz 1965) and in other experimental conditions (O'Leary and Goldring 1964).

D. C. shifts have been generally considered as due to neuronal activity although an extra neuronal origin of the phenomenon cannot be with certainty ruled out (O'Leary and Goldring 1964).

Various lines of evidence suggest that surface negative shift envelops a sustained depolarization of underlying neurons (O'Leary and Goldring 1964). Other authors (Roitbak 1955, Li and Chow 1962, see Aladjalova 1964, O'Leary and Goldring 1964) consider the surface negative potential changes an expression of deep cell layer hyperpolarization.

The aim of the present investigation has been to study the slow potential changes induced in a relatively simple and rather well known structure such as the olfactory bulb by central and peripheral stimulations. Particularly our interest has been concentrated on the study of SP (steady potential) shift induced in the olfactory bulb by stimulation of the contralateral bulb and of other central structures which are known to be functionally connected in a centrifugal way with the olfactory bulb.

* Research supported by funds of CNR—Italy.

The results which will be briefly presented here have been already reported in a preliminary note (Carreras *et al.* 1965) and will be published *in extenso* (Carreras *et al.* in preparation).

METHODS

The experiments were carried out in *encephale isolé* cats. Under ether anaesthesia the animals were cannulated and the spinal cord transected at C2. The cats were then artificially ventilated and curarized. The periorbital region of one side or bilaterally was infiltrated with procaine and the eye emptied so that the medial wall of the orbital cavity was widely exposed. The bone of this side was then drilled and the olfactory bulb exposed without any damage.

Potential changes at the surface of the bulb were recorded monopolarly. The recording electrode consisted of a helix of chloride silver wire contained in a glass vessel and in contact with the tissue through a bridge of Ringer-soaked solution. The reference electrode was buried under the skin over the temporal muscle. When not in use, the electrodes were stored as short-circuited couples in Ringer's solution. They were essentially isopotential and free from drift. The d.c. recording system had a spontaneous drift of about 100μV in 5 minutes so that it was not troublesome for the short periods during which records were taken.

In many experiments the same electrodes and d.c. recording system were used to record the EEG and slow potential changes from the frontal cortex.

The electrical stimulation of the midbrain reticular formation, medial thalamus and controlateral olfactory bulb was performed through concentric bipolar electrodes. Bipolar electrodes were also used to stimulate the mucosa of the gum. The olfactory mucosa was stimulated through two polythene cannulae inserted into the nostrils by blowing odorified (Xilene) air. At the end of the experiment the brain was removed, fixed in formaline and the points of stimulation checked histologically by Nissl stained sections.

RESULTS

1. General Remarks

The baseline which recorded the d.c. potential from the olfactory bulb was fairly stable during the experiment. Care was taken to keep the conditions of the cat as steady as possible. The condition of the bulb was regu-

larly tested by applying a natural stimulation to the olfactory mucosa which constantly yielded the Adrian's induced waves. A positive d.c. shift electrotonically transmitted from the olfactory mucosa was also generally recorded following natural olfactory stimulation (Ottoson 1954).

2. *D.C. Shift Induced by Stimulation of the Cantrolateral Bulb*

Low-frequency stimulation (5–15/sec, 0·1–0·5 msec, 2–5 V) of the bulb of one side induced a clear-cut negative shift on the surface of the contralateral bulb when the frequency of stimulation reached about 10–15/sec.

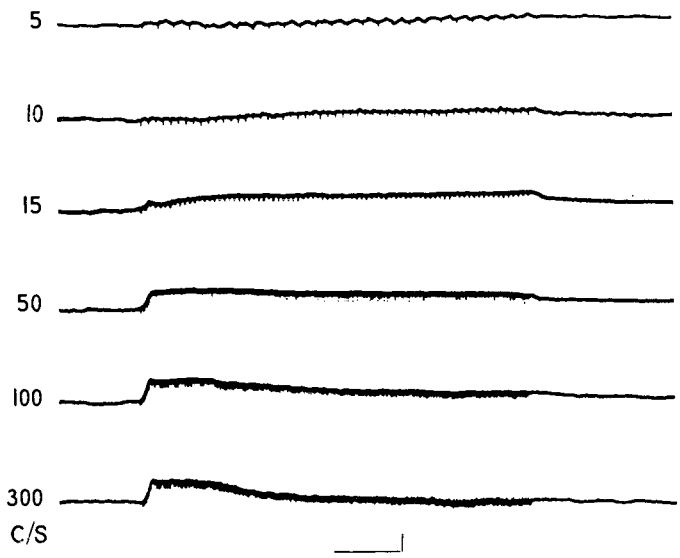


FIG. 1. D.C. potential changes induced in the olfactory bulb by contralateral bulb stimulation at different frequencies.

Encéphale isolé cat. In this and following figures negativity is shown upward except for Fig. 3. Numbers indicate the frequency of stimulation. Other parameters: 0·5 msec, 4 V. Stimulus duration 5 sec. Calibration: 1 sec, 100 μV.

This shift was generally of the order of 70 mV and sustained throughout the entire period of stimulation (Fig. 1). As the frequency increased (50–300/sec) a higher d.c. potential change of about 100–150 μV occurred on the contralateral bulb (Fig. 1). This shift increased as the intensity of stimulation was raised (Fig. 2) and often reached the maximal value of 0·5 μV (Fig. 3). The potential changes occurred as the stimulation was applied and quickly reached a plateau (Fig. 3). Subsequently it declined even if the stimulation was maintained (see Figs. 1, 2 and 3C). As the stimuli ceased the

baseline went to the original level in about 0·5–1 sec. Generally following strong stimulation, after-effects could be seen (Fig. 3C).

All these effects disappeared after acute section of the anterior commissure.

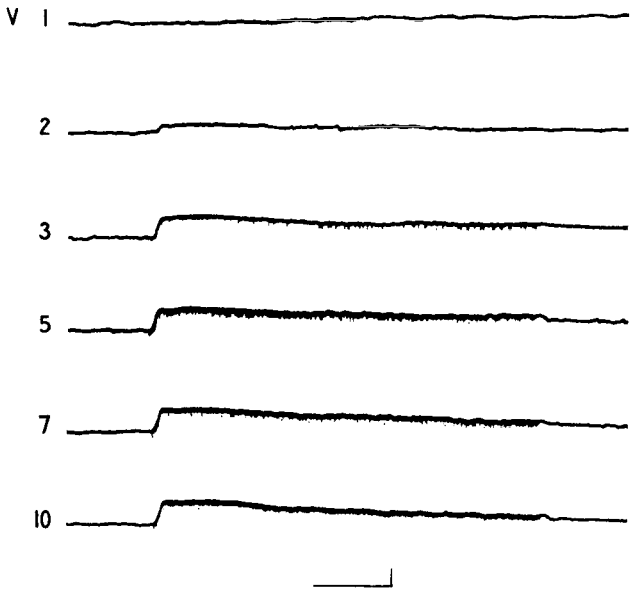


FIG. 2. D.C. potential changes induced in the olfactory bulb by contralateral bulb stimulation.

Encéphale isolé cat. Numbers indicate the voltage of stimulation. Other parameters: 0·5 msec, 300/sec. Stimulus duration 5 sec. Calibration: 1 sec, 100 μV.

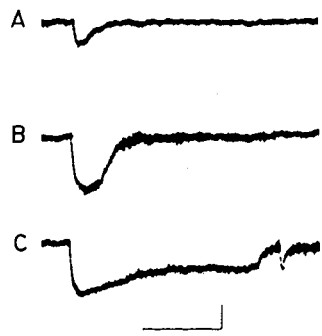


FIG. 3. D.C. potential changes induced in the olfactory bulb by contralateral bulb stimulation.

Encéphale isolé cat. Negative is down. A: stimulation at 300/sec, 0·5 msec, 2 V. B and C: stimulation at 300/sec, 0·5 msec, 8 V. Stimulus duration in A and B 2·5 sec. In C: 5 sec. Note the after-effects in C after the stimulus is ceased. Calibration: 2 sec, 200 μV.

3. D.C. Shifts Induced by Midline Thalamic Stimulation

Low frequency stimulation (8–10/sec, 0·1–1·5 msec, 2–8 V) of the midline thalamic nuclei which evoked on the sensorimotor cortex the classical recruiting potentials (Dempsey and Morison) (Fig. 4A) were also able to

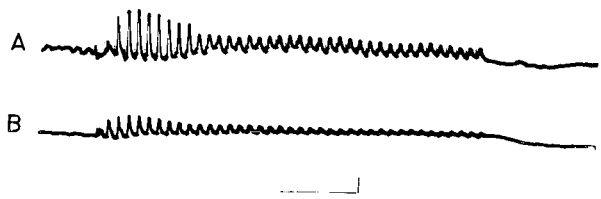


FIG. 4. Recruiting potentials induced in the frontal cortex (A) and olfactory bulb (B) by 8/sec, 0·5 msec, 5 V stimulation of midline thalamic nuclei. *Encéphale isolé* cat. Calibration: 1 sec, 100 μV.

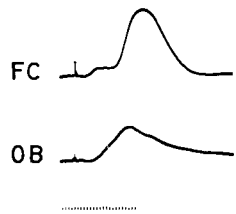


FIG. 5. Latency of the third wave recruited in the frontal cortex (FC) and olfactory bulb (OB) by 8/sec, 0·5 msec, 5 V stimulation of midline thalamic nuclei. *Encéphalé isolé* cat. Signal calibration: 500/sec.

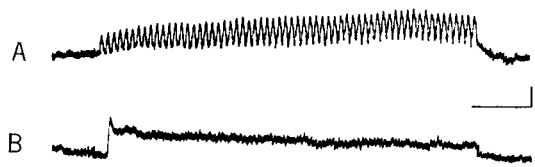


FIG. 6. Recruiting responses and d.c. negative shift induced in the olfactory bulb by 8/sec (A) and 300/sec (B) 0·5 msec, 5 V stimulation of midline thalamic nuclei. *Encéphale isolé* cat. Calibration: 1 sec, 100 μV.

produce on both olfactory bulbs similar recruiting responses (Fig. 4B) as reported by Arduini and Moruzzi (1953). The recruiting waves appeared on the bulb with the peak at 36 msec from the stimulus, slightly earlier than the recruiting responses recorded from the frontal cortex (Fig. 5). These potentials were mainly negative in polarity, grew slowly and reached a full size after 4–6 stimuli. Usually they showed a waxing and waning variation

of amplitude. The recruiting potentials were regularly superimposed on a negative shift of about 100 μV amplitude which increased gradually and returned in 0·5–1 sec to the original level, as the stimulus was disconnected (Fig. 6A).

High frequency stimulation (30–300/sec) of the same thalamic loci elicited a sustained slow potential change of 150–200 μV amplitude (Fig. 6B). A relationship was noted between amplitude of d.c. shift and intensity of stimulation. The shift usually appeared as the stimulation was started, slowly declining when the stimulation was maintained for a long period.

The effects reported by thalamic stimulation remained after bilateral large suction of the frontal cortex while disappeared after cutting the olfactory peduncle. This procedure did not impair the olfactory bulb activity as revealed by the Adrian's induced waves regularly evoked by olfactory stimulation.

4. D.C. Shift Induced by Reticular and Peripheral Stimulations

High frequency activation (300/sec, 0·5 msec, 2–8 V) of midbrain reticular formation induced a d.c. shift of about 100 μV on the olfactory bulb (Fig. 7B and 8 A & B) which was paralleled by a generalized arousal, viz.: EEG desynchronization, d.c. potential shift on sensory motor cortex (Fig.

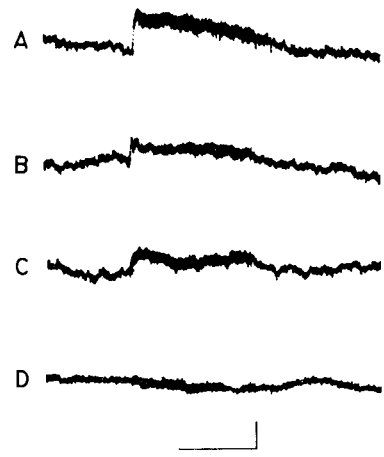


FIG. 7. D.C. shift evoked in frontal cortex (A) and olfactory bulb (B) by stimulation of midbrain reticular formation (300/sec, 0·5 msec, 4 V). C: D.C. shift induced in the olfactory bulb by electrical stimulation (300/sec, 0·5 msec) of the trigeminal territory. D: Disappearance of reticular induced d.c. shift in the olfactory bulb after cutting the olfactory peduncle. *Encéphale isolé* cat. Stimulus duration 3 sec. Calibration: 1 sec, 100 μV.

7A) and pupillary dilatation. The slow potential changes interested the two bulbs, were already present at the very beginning of the stimulation and remained sustained for about one second after the stimulus ceased. The effect was still present after removal of the frontal cortex while disappeared following section of the olfactory peduncle (Fig. 7D). This procedure did not alter the condition of the bulb as revealed by Adrian's induced waves evoked by olfactory stimulation.

Similar shifts could be obtained by electrical stimulation (300/sec, 0·5 msec, 2–6 V) of the mucosa of the oral cavity (Fig. 7C). Slow potential changes were also induced by natural stimuli, although the amplitude was slightly smaller as compared to the shifts evoked by electrical stimuli.

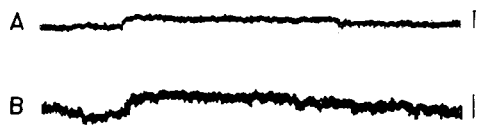


FIG. 8. D.C. shift induced in the olfactory bulb by midbrain reticular stimulation (300/sec, 0·5 msec, 5 V) in two different *encéphale isolé* cats. Stimulus duration 3 sec. Calibration: 1 sec, 100 μV.

DISCUSSION

The results which have been briefly reported here show that following central and peripheral stimuli a SP (steady potential) negative shift can be induced in the olfactory bulb. It is likely that this effect is due to electrophysiological processes within the olfactory bulb since it remains after suction of the frontal cortex while it disappears after cutting the olfactory peduncle or the anterior commissure in the case in which the contralateral bulb was stimulated. The SP shift cannot be due to variations of systemic blood pressure or to other unspecific causes since the experiments were performed on *encéphale isolé* and curarized preparations.

The slow potential shift obtained from one bulb by stimulation of the controlateral one is usually higher than those produced by other stimulations. It can be attributed either to orthodromic or to antidromic activation of commissural fibers although the possibility that other central circuits are involved cannot be excluded. The shift is already present for stimulation at 15/sec and is enhanced by increasing the stimulus frequency. It appears to be due to a progressive summation of slow negativity evoked by each stimulus until a plateau is reached when the stimulus frequency gets up to 100/sec.

The observation that recruiting responses can be evoked from the bulb by low frequency stimulation of the intralaminar thalamus confirms the observations reported by Arduini and Moruzzi (1953). At 8–10/sec stimulation the recruiting potentials are superimposed on a clearcut negative shift which becomes higher as the frequency of stimulation is increased. This slow potential change like the shift produced by activation of the contralateral bulb seems to be due to a progressive summation of negative after-effects. This mechanism would also account for the brief persistence of the negative change as the stimulus ceases.

It is likely that the intralaminar thalamus influences the olfactory bulb either directly or through other control pathways. However, an antidromic activation of reticular neurons by the thalamic stimuli cannot be ruled out although the comparison between reticular and thalamic shift amplitude makes this hypothesis rather unlikely.

The slow potential change induced in the bulb by reticular stimulation shows that the olfactory bulb is under a centrifugal control from the reticular neurons. This shift is usually of low amplitude compared to other shifts and may be due either to direct pathways to the bulb or to activation of other central structures. The effects we reported confirm the observations made on the bulb with macro (Yamamoto and Iwama 1961) and microelectrodes (Mancia *et al.* 1962) recording following reticular activation.

The similarity between the reticular shift and that obtained by natural or electrical stimulation of the peripheral receptors makes it likely that the latter is mediated by the reticular system. Other extrareticular pathways may, however, be involved in the production of this potential change.

As far as the origin and the nature of the olfactory bulb shift is concerned it is not possible for the moment to advance more than hypothesis. By analogy with the cerebral cortex where changes in membrane potential of single neurons were found to be closely related to slow potential changes recorded extracellularly (Sugaya *et al.* 1964) we may assume that also in the olfactory bulb the SP shift represents a slow neural event although an extraneural origin of the phenomenon cannot be with certainty excluded (see O'Leary and Goldring 1964).

Considering the slow potential change as an expression of neural activity, it has been generally related to postsynaptic events depolarizing in nature (Von Euler *et al.* 1958, Marshall 1959, Gloor 1962, Gloor *et al.* 1962, Sugaya *et al.* 1964, see O'Leary and Goldring 1964 for complete ref.). A presynaptic depolarization has also been indicated as responsible for negative d.c. potential changes (see O'Leary and Goldring 1964). Other evidences indicate that these slow negative changes can be related to an arrest

of spontaneous firing of underlying neurons (Li and Chow 1962, Stohr *et al.* 1963, Li and Salmoiraghi 1963) or to postsynaptic hyperpolarization (Roitbak 1955, see Aladjalova 1964 for ref.).

It is likely that a surface negative d.c. shift is an expression of an algebraic summation of depolarizing (pre- and postsynaptic) and hyperpolarizing events.

As far as the d.c. shift induced in one bulb by activation of the contralateral one is concerned, it is suggested that this is related in some way to inhibitory phenomena which have been shown to occur in the bulb following stimulation of the contralateral one or the anterior commissure (Kerr and Hagbarth 1955, von Baumgarten *et al.* 1962, Yamamoto *et al.* 1963).

At the present moment, the significance of the d.c. shifts induced in the bulb by thalamic and reticular stimulations is uncertain. They indicate that these central structures are able to influence the olfactory bulb activity, as also suggested by older observations (Arduini and Moruzzi 1953, Yamamoto and Iwama 1961, Mancia *et al.* 1962, see Mancia 1963). However, the understanding of the relationship between this centrifugal control and bulbar slow potential changes needs further investigation.

SUMMARY

1. Low frequency stimulation of one bulb evokes in the contralateral one a sustained negative slow potential change of about 70 μV amplitude. The shift increases (up to 200 μV) as the frequency is raised and is proportional to a certain extent to the intensity of stimulation.

The effect disappears following section of the anterior commissure.

2. Low frequency stimulation of the intralaminar thalamus evokes in the bulb recruiting potentials similar to those recorded from the frontal cortex. The bulbar recruiting potentials are superimposed on a sustained negative d.c. change which increases as the frequency of stimulation is raised.

The effect is present after removal of frontal cortex while disappears after section of the olfactory peduncle.

3. High frequency activation of the midbrain reticular formation induces in the bulb a negative d.c. shift of 100 μV amplitude paralleled by a generalized arousal. Similar slow potential changes follow electrical stimulation of the oral cavity and natural stimuli which induce an EEG arousal.

These effects remain after removal of frontal cortex while disappear after section of the olfactory peduncle.

REFERENCES

ALADJALOVA, N. A., Slow electrical processes in the brain. In: *Progress in brain research*, **7**, Elsevier, Amsterdam 243, 1964.

ARDUINI, A., MANCIA, M. and MECHELSE, K., Slow potential changes elicited in the cerebral cortex by sensory and reticular stimulation, *Arch. ital. Biol.*, **95**, 127–138, 1957.

ARDUINI, A. and MORUZZI, G., Sensory and thalamic synchronization in the olfactory bulb, *Electroenceph. clin. Neurophysiol.*, **5**, 235–242, 1953.

BROOKHART, J. M., ARDUINI, A., MANCIA, M. and MORUZZI, G., Thalamocortical relations as revealed by induced slow potential changes, *J. Neurophysiol.*, **21**, 499–525, 1958.

CARRERAS, M., MANCIA, D. and MANCIA, M., D.C. potential changes induced in the olfactory bulb by central and peripheral stimuli (in preparation).

CARRERAS, M., MANCIA, D., MANCIA, M. AVANZINI, G., Variazioni lente di potenziale indotte nel bulbo olfattivo da stimoli centrali e periferici, *Atti Acc.naz. Lincei, Cl. Sci. fis. mat. nat.*, Serie VIII, 1965 (in press).

CASPERS, H., Shifts of the cortical steady potential during various stages of sleep. In: *Aspects anatomo-fonctionnels de la physiologie des états de sommeil.*, Lyon 1963, Ed. du Centre national de la recherche scientifique, Paris, 1965, 213–229.

DEMPSEY, E. W. and MORISON, R. S., The production of rhythmically recurrent cortical potentials after localized thalamic stimulation, *Amer, J. Physiol.*, **135**, 293–300, 1942.

DONDEY, M. and SNIDER, R. S., Slow potential shifts following cerebellar stimulation, *Electroenceph. clin. Neurophysiol.*, **7**, 265–272, 1955.

GLOOR, P. C., Die Neurophysiologische Mechanismus des epileptischen Anfalles, *Bull. Schweiz. Akad. Med. Wiss.*, **18**, 167–188, 1962.

GLOOR, P. C., VERA, C. L., SPERTI, L. and RAY, S. N., Investigations of the mechanism of epileptic discharge in the hyppocampus, *Epilepsia*, **2**, 42–62, 1961.

GOLDRING, S. and O'LEARY, J. L., Cortical D.C. changes incident to midline thalamic stimulation, *Electroenceph. clin. Neurophysiol.*, **9**, 577–584, 1957.

GOLDRING, S., O'LEARY, J. L., HOLMES, T. and JERVA, M. J., Direct response of isolated cerebral cortex of cat, *J. Neurophysiol.*, **24**, 633–650, 1961.

KAWAMURA, H. and SAWYER, C. H., D.C. potential changes during paradoxical sleep in the rabbit, *Physiologist*, **7**, 176, 1964.

KERR, D. I. B. and HAGBARTH, K. E., An investigation of olfactory centrifugal system, *J. Neurophysiol.*, **18**, 362–374, 1955.

LI, C. L. and CHOW, S. N., Cortical intracellular synaptic potentials and direct cortical stimulation, *J. cell. comp. Physiol.*, **60**, 1–16, 1962.

LI, C. L. and SALMOIRAGHI, G. C., Cortical steady potential changes: extracellular microelectrode investigations, *Nature (London)*, **198**, 858–859, 1963.

MANCIA, M., Specific and unspecific influences on the olfactory bulb, *Electroenceph. clin. Neurophysiol.*, supp. **24**, 1–12, 1963.

MANCIA, M., GREEN, J. D. and VON BAUMGARTEN, R., Reticular control of single neurons in the olfactory bulb, *Arch. ital. Biol.*, **100**, 463–475, 1962.

MARSHALL, W. H., Spreading cortical depression of Leão, *Physiol. Rev.*, **39**, 239–279, 1959.

O'LEARY, J. L. and GOLDRING, S., D.C. potentials of the brain, *Physiol. Rev.*, **44**, 91–125, 1964.

O'LEARY, J. L., KERR, F. W. L. and GOLDRING, S., The relation between spino-reticular and ascending cephalic systems, In: *Reticular formation of the brain*, Boston, Little, Brown Co. 1958, pp. 187–202.

OTTOSON, D., Sustained potentials evoked by olfactory stimulation, *Acta Physiol. scand.*, **32**, 384–386, 1954.

ROITBAK, A. I., *Bioelectrical phenomena in the cerebral cortex*, Tblisi, URSS, 1955.

STOHR, P. E., GOLDRING, S. and O'LEARY, J. L., Patterns of unit discharge associated with direct cortical response in monkey and cat, *Electroenceph. clin. Neurophysiol.*, **15**, 882–888, 1963.

SUGAYA, E., GOLDRING, S. and O'LEARY, J. L., Intracellular potentials associated with direct cortical response and seizure discharge in cat, *Electroenceph. clin. Neurophysiol.*, **17**, 661–669, 1964.

TABUSHI, K., Cortical d.c. potential changes associated with spontaneous sleep in cat, *Abstr. XXIII inter. Congr. physiol. Sci.*, Tokyo, 1965, p. 450.

VANASUPA, P., GOLDRING, S., O'LEARY, J. L. and WINTER, D., Steady potential changes during cortical activation, *J. Neurophysiol.*, **22**, 273–284, 1959.

VON BAUMGARTEN, R., GREEN, J. D. and MANCIA, M., Recurrent inhibition in the olfactory bulb. II Effects of antidromic stimulation of commissural fibers, *J. Neurophysiol.*, **25**, 489–500, 1962.

VON EULER, C., GREEN, J. D. and RICCI, G., The role of hippocampal dendrites in evoked responses and after discharge, *Acta Physiol. Scand.*, **42**, 87–111, 1958.

WURTZ, R. H., Steady potential shifts during arousal and deep sleep in the cat, *Electroenceph. clin. Neurophysiol.*, **18**, 649–662, 1965.

YAMAMOTO, C. and IWAMA, K., Arousal reaction of the olfactory bulb, *Jap. J. Physiol.*, **11**, 335–345, 1961.

YAMAMOTO, C., YAMAMOTO, T. and IWAMA, K., The inhibitory systems in the olfactory bulb studied by intracellular recording, *J. Neurophysiol.*, **26**, 403–415, 1963.

OLFACTORY RECEPTOR REACTION TO THE LESION OF THE OLFACTORY BULB

P. SEN GUPTA

Department of Anatomy—Unit No. I,
All India Institute of Medical Sciences,
Ansari Nagar, New Delhi-16, India

INTRODUCTION

An individual olfactory receptor cell has been reported showing graded differences in its morphology on the basis of its mitochondrial content, in the number of olfactory hairs, size of the terminal swellings, length and cross-sectional diameter of the rods and argentophil reactions (Clark and Warwick, 1946; Clark, 1957; De Lorenzo, 1957; Sen Gupta, 1964). Apart from these graded differences, Le Gros Clark (1957) divided the olfactory receptors into two major categories in roughly equal proportions—those which undergo almost immediate dissolution after destruction of the olfactory bulb and those which persist apparently unchanged for a period of at least six months. While unable to explain this difference in behavior of the olfactory receptor cells to the interruption of their axons, he suggested that this dissimilarity may be due to differences in their biochemical and biophysical properties.

Nagahara in 1940 also found similar results in mouse, and postulated that these "residual receptors" were resting elements and claimed, moreover, that in course of time these resting elements could by mitotic proliferation lead to a reconstitution of olfactory epithelium of normal appearance. These findings of Le Gros Clark (1957) and Nagahara (1940) have been accepted without question by several recent workers (Adey, 1959; Beidler, 1961; Moulton and Tucker, 1964; Gesteland *et al.*, 1963).

The proximal process of each of the olfactory receptor cells essentially synapses in the glomeruli of the olfactory bulb. Therefore, it is quite logical to expect a degenerative reaction in these receptors when the structural continuity of their axons are broken.

In the present paper similar experiments have been done with young albino rats and evidences are presented indicating that all the olfactory receptors undergo a rapid disintegration following a complete destruction of the olfactory bulb.

MATERIAL AND METHODS

Altogether fifteen albino rats were operated on under nembutal anaesthesia. Unilateral and bilateral lesions of the olfactory bulb were made in some by means of diathermy; in others by electrolysis and suction. These rats were secluded right after birth to minimize nasal infections. The rats were killed by perfusion with formol saline followed by a mixture of picric acid, alcohol and trichloracetic acid (Fitzgerald, 1964) under transitory ether anaesthesia after a survival period of 24, 28, 36, 48 and 72 hours and 5, 6, 8, 10, 12 and 25 days. Subsequently serial paraffin sections of the nose and olfactory chambers with the bulb were made after decalcification electrolytically. The sections were stained by means of the double silver impregnation technique of Fitzgerald (1964).

OBSERVATIONS

The serial sections of both bulbs were examined first to determine the extent of lesion and it was found that diathermy and electrolysis did not produce complete destruction of the bulb; in three out of fifteen cases there was incomplete destruction of the contralateral bulb also. Suction produced strictly unilateral and complete lesion in four. In one, the contralateral olfactory bulb was also damaged. The presence of atrophic glomeruli caused by the interruption of the olfactory nerves were also taken as the criterion of destruction apart from actual lesion in the bulb. In six out of eight cases of partial destruction, areas showing degeneration of the olfactory receptors were distributed roughly in a topographical manner according to the location of the olfactory bulb lesions, except in the other two cases where uniform reduction in the number of olfactory receptors on the ipsilateral side was present.

Changes in the olfactory mucous membrane on the side of the complete olfactory bulb destruction are described as follows:

Changes after twenty-four hours. The olfactory mucous membrane showed no change except the slightly mottled appearance of the olfactory nerves (Fig. 2).

Changes after twenty-eight hours. The mucous membrane appeared to have undergone a violent disruption. Olfactory rods were totally absent. The fragmented and pyknotic nuclei were scattered throughout the depth of the mucous membrane. Sustentacular cells were almost indistinguishable and so also were the basal cells. The basement membrane was broken up into pieces. The Bowman's glands showed an intense reaction. They

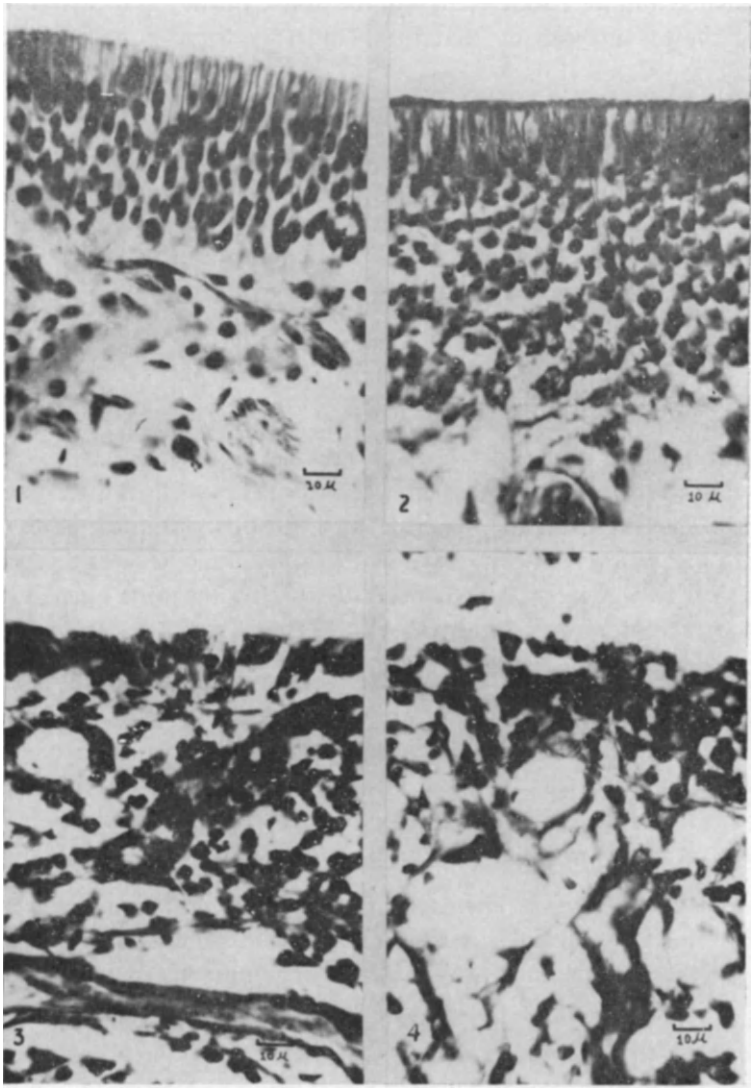


FIG. 1. Normal olfactory mucous membrane stained by Fitzgerald's double silver impregnation technique.

FIG. 2. Olfactory mucous membrane—after twenty-four hours of olfactory bulb destruction. Double silver impregnation of Fitzgerald.

FIG. 3. Olfactory mucous membrane—after twenty-eight hours of olfactory bulb destruction. Double silver impregnation of Fitzgerald.

FIG. 4. Olfactory mucous membrane after thirty-six hours of olfactory bulb destruction. Double silver impregnation of Fitzgerald.

were dilated and were wide open into the lumen of the nasal cavity. Blood vessels showed no sign of dilatation. Olfactory nerve bundles had completely disappeared (Fig. 3).

Changes after thirty-six hours. The disruption of the mucous membrane had gone a step forward. Pyknotic nuclei were strewn over the whole lamina propria and some of them were seen to be thrown outside into the lumen of the nose. The entire epithelial lining was broken into pieces. The acini were dilated and there was total absence of the basement membrane. Blood vessels did not show any change (Fig. 4).

Changes after forty-eight hours. Same chaotic picture as in the thirty-six hours specimen was seen.

Changes after seventy-two hours. The disrupted mucous membrane appeared to have attempted repair in few places and there the sustentacular cells were organized and distinguishable. These cells showed peculiar features. They were tall and hypertrophied, the cytoplasm taking up silver stain, quite a few of them having developed long cilia-like processes along their free borders. Glands were still dilated and wide open (Fig. 5). Olfactory receptors were totally absent.

Changes after five days. The olfactory receptor cells were absent. The total thickness of the mucous membrane was much less in comparison to the contralateral side and showed disrupted features in most of the places. Few more sustentacular cells had hypertrophied and had developed cilia-like processes. The basal cells were not recognizable. Basement membrane had not yet reformed. Glands were dilated (Fig. 6).

Changes after six days. There was relative increase in the number of the hypertrophied sustentacular cells. Basement membrane was reorganized to some extent. The lamina propria was comparatively free of pyknotic nuclei.

Changes after ten days. By the tenth day the mucous membrane more or less had reorganized itself. The epithelium was made up of tall sustentacular cells, with cilia-like growth at their free margins, arranged side by side resting on the basement membrane. There was no olfactory receptor cell. The lamina propria was almost clear except for the dilated glands, which appeared to have shrunken a little (Figs. 7, 8, and 9).

Changes after twelve days. The sustentacular cells were shorter and had started losing their cilia-like processes and even some sustentacular cells appeared to be atrophic. In places where the sustentacular cells were

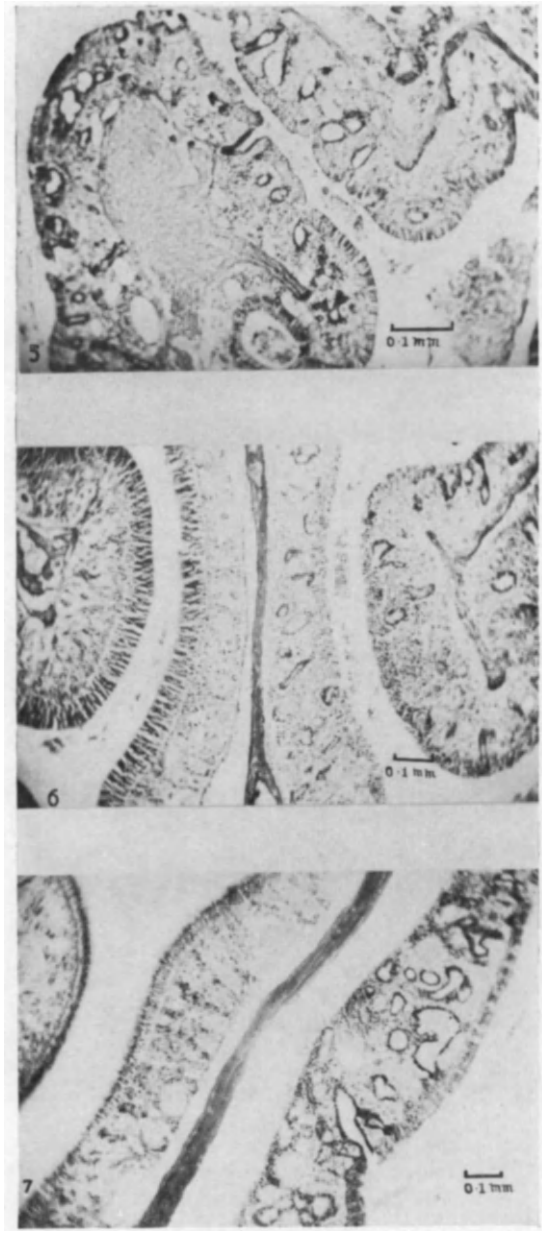


FIG. 5. Olfactory mucous membrane—after seventy-two hours of olfactory bulb destruction. Double silver impregnation of Fitzgerald.

FIG. 6. Olfactory mucous membrane—after five days of right olfactory bulb destruction. Double silver impregnation of Fitzgerald.

FIG. 7. Olfactory mucous membrane—after ten days of right olfactory bulb destruction. Double silver impregnation of Fizgerald.

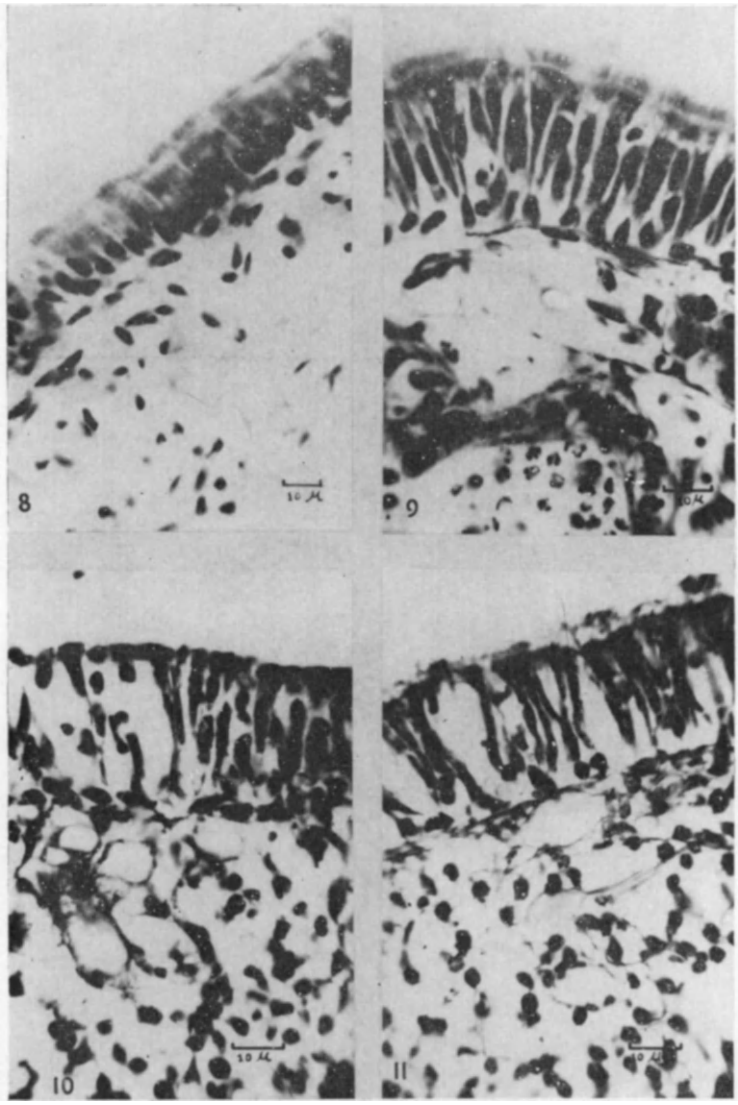


FIG. 8. Olfactory mucous membrane—after ten days of olfactory bulb destruction. Note the cilia-like processes of the sustentacular cells. Double silver impregnation of Fitzgerald.

FIG. 9. (As in Fig. 8.)

FIG. 10. Olfactory mucous membrane—after twelve days of olfactory bulb destruction. Double silver impregnation of Fitzgerald.

FIG. 11. Olfactory mucous membrane—after twenty-five days of olfactory bulb destruction. Double silver impregnation of Fitzgerald.

atrophic the basement membrane was also not intact. Glands were hypertrophied (Fig. 10).

Changes after twenty-five days. The mucous membrane as a whole was of completely different character. There was only one row of low columnar cells with a distinct basement membrane. Lamina propria contained connective tissue and some Bowman's glands with dilated acini. The olfactory receptor cells were totally absent and so also the olfactory nerve fascicles. Most of the sustentacular cells had by this time lost their cilia-like processes (Fig. 11).

DISCUSSION

Our results mainly reveal two important facts. First, the rapid and drastic degeneration of the olfactory receptor cells accompanied by the changes in the associated glandular and epithelial tissues. Second, the total loss of receptor cells after the destruction of the olfactory bulb.

Le Gros Clark (1957) reported similar findings about the rapid dissolution of the receptor cells. But his observations do not include the changes seen in the sustentacular cells and the Bowman's glands. The increased affinity for silver and the peculiar cilia-like growth at the free margins of the supporting cells are striking. The growth of the cilia-like processes appears by the seventh or eighth day and reaches its maximum by the tenth day; thereafter they diminish in size, but some persist even up to the twenty-fifth day. The increased affinity for silver stain may be due to the fragmented olfactory rods enclosed by the sustentacular cells. The significance of the cilia-like growth cannot be explained at present. In this connection it may be mentioned that De Lorenzo (1957) found that the microvilli of the sustentacular cells break off after the olfactory bulb destruction. The Bowman's gland is characterized by big dilated acini wide open into the lumen. This glandular dilatation possibly is not accompanied by hyperplasia of the acinar cells. For this a preliminary observation was carried out by injecting colchicine intraperitonially 1–2 hours before sacrificing a few of the animals (dose—0·25 mg per 100 gm of body weight—Ford and Hammerton, 1956) and staining the paraffin sections with Babe's aniline safranin; we did not find any mitotic figure in the glandular acinar cells.

The total disappearance of the receptor cells is a finding which is contrary to that of Le Gros Clark (1957) and Nagahara (1940). The central processes of the olfactory receptor cells have not been reported to have collaterals. Therefore it is rather logical to assume that all the receptors will undergo the same morphological changes at least as a reaction to

physical trauma. In our series of fifteen animals, we found a complete absence of receptor cells in twelve. In one animal that was killed twenty-four hours after the operation the degeneration of the receptors was not evident. In the remaining two cases, quite a large number of receptors were found to be healthy and intact together with the olfactory nerve bundles. When these nerves were followed towards the olfactory chamber, it was found that though apparently the major portion of the bulb was completely destroyed yet there was a thin rim of the glomerular layer of the olfactory bulb still intact on its most inferior and lateral aspect. The glomeruli were not atrophic. In this connection it may be mentioned that in rats most of the olfactory nerves enter the olfactory chamber through the inferior aspect of the cribriform plate. Simple destruction of the deeper part of the bulb without any injury to the glomerular layer will not induce degeneration in the receptor neuron itself.

The possibility that the so-called "residual receptors" are not stainable by the silver impregnation method because of some unknown change in their biochemistry is easily ruled out when the epithelium is carefully inspected. The epithelial picture is so clear and characteristic that it is difficult to believe that there could be still fifty percent unstained receptor cells lying hidden in the space between the basement membrane and the one-layered low columnar sustentacular cells.

The total absence of the receptor cells may be due to vascular injury. Our olfactory bulb lesions produced by diathermy and electrolysis are likely to cause vascular injury, but such a possibility is remote where suction had only been applied. In all these cases we did not find any change in the vascular pattern of the olfactory mucous membrane; therefore, it appears that the possibility of vascular damage is indeed very little as has also been pointed out by Le Gros Clark (1957).

SUMMARY AND CONCLUSION

Unilateral and bilateral olfactory bulb destruction have been carried out in fifteen albino rats. Serial paraffin sections of the nose were stained with Fitzgerald's technique of double silver impregnation. It was found that the olfactory mucous membrane undergoes a violent dissolution within twenty-eight to thirty-six hours of olfactory bulb lesion. Repair is attempted with a concomitant hypertrophy and cilia-like growth at the free margins of the sustentacular cells. All the receptors undergo degeneration after the olfactory bulb destruction. No "residual receptors" are seen. Since the possibilities of defective silver impregnation and vascular damage

can be ruled out, it is concluded that as a matter of fact there are two types of receptor cells, namely the rapidly degenerating ones and the residual ones.

ACKNOWLEDGEMENTS

The author wishes to express his deep sense of gratitude to Professor L. W. Chacko for the suggestion of the problem and for her healthy and constructive criticism and constant encouragement during the tenure of this work. The help rendered by Drs. M. C. Vaidya, S. DasGupta, B. K. Mukhopadhyaya and V. K. Gomathy in preparing the material is gratefully acknowledged.

REFERENCES

ADEY, W. R. 1959. Sense of smell, in *Handbook of Physiology*, Sec. I, p. 536. Amer. Physiol. Soc. Washington, D.C.

BEIDLER, L. M. 1961. The chemical senses, *Ann. Rev. Psychol.* **12**, 363–388.

CLARK, W. E. LE GROS 1957. Enquiries into the anatomical basis of olfactory discrimination. *Proc. Royal Soc.* (Lond.) Ser. B. **146**, 299–319.

CLARK, W. E. LE GROS and R. T. TURNER WARWICK, 1946. The pattern of olfactory innervation. *J. Neurol. Neurosurg. and Psychiat.* **9**, 101–111.

DE LORENZO, A. J. 1957. Electron microscopic observations in the olfactory epithelium of the rabbit. *Anat. Rec.* **127**, 284.

FITZGERALD, M. J. T. 1964. The double impregnation silver technique for nerve fibres in paraffin sections. *Quart. J. Micr. Sc.* **105**, 359–361.

FORD, C. E. and J. L. HAMMERTON. 1956. A colchicine, hypotonic citrate, squash sequence for mammalian chromosomes. *Stain Tech.* **31**, 247–251.

GESTELAND, R. C., J. Y. LETTVIN, W. H. PITTS, and ARISTIDES ROJAS. 1963. Odor specificities of the frog 'olfactory receptors, in *Olfaction and Taste*. Edit. Y. ZOTTERMAN. Pergamon Press, Oxford.

MOULTON, D. G. and D. TUCKER. 1964. Electrophysiology of olfactory system. *Ann. New York Acad. Sci.* **116**, 380–428.

NAGAHARA, Y. 1940. *Jap. J. Med. Sci.* (Sec. 5 Path) **5**, 165–199 (cited by Clark, 1957).

SEN GUPTA, P. 1964. The olfactory apparatus and its central connections in *Suncus murinus*. M. S. Thesis. Department of Anatomy, AIIMS, New Delhi.

OLFACTORY PERCEPTION IN BIRDS

BERNICE M. WENZEL

Dept. of Physiology and Brain Research Institute
University of California, Los Angeles

INTRODUCTION

It has become a truism in the past century that the subject of avian olfaction is a controversial one. Each of the occasional research articles, notes, and reviews that has appeared, ranging through the fields of ornithology, physiology, anatomy, and psychology, typically has begun with the statement that the olfactory ability of birds is open to dispute. The study of any sensory system in animals requires recognition of the tripartite nature of the functional capacity of the system. The general query —"Can birds smell?"—must be resolved into three formal questions for investigation: (1) are birds capable of perceiving olfactory stimuli; (2) do birds naturally regulate any aspects of their behavior in terms of olfactory cues; and (3) can birds learn to regulate certain aspects of their behavior in terms of olfactory cues? Some of the previous investigators may not have fully appreciated these distinctions, and have asserted that birds are "unable to smell" because negative results were obtained in experiments that were unwittingly designed to answer only questions (2) or (3) above. It is quite conceivable that question (1) could be answered affirmatively while the remaining two were answered in the negative. Just as important as the experimental design is the matter of species variation, now known to be extensive with regard to olfactory anatomy, and which may be correspondingly great for olfactory function. If so, the choice of species would be a critical determinant of the results.

The existence of both peripheral and central anatomical structures in birds that could subserve an olfactory modality has been recognized for some time. All types possess an olfactory epithelium and an olfactory bulb (Marshall, 1960). The configuration of the nasal cavity varies greatly, however, as well as the size of the olfactory bulb (Bang, 1960, 1965; Cobb, 1960). Cobb has ranked 36 different birds for the ratio of the diameters of their olfactory bulbs to their cerebral hemispheres. He reports a range

between 8 per cent for the house sparrow and 33 per cent for the kiwi. The challenge of determining the functional status of the olfactory structures has been accepted by several investigators whose results have been carefully summarized in other papers (Stager, 1964; Walter, 1943) and will not be reviewed here. Recently published electrophysiological evidence (Tucker, 1965) indicates that the avian olfactory nerve responds differentially to odorous stimuli as compared with inodorous ones. Contention now centers on the question as to whether the afferent signals are, or can be, utilized to regulate effector activity. Duncan (1964, p. 765) has expressed current opinion as follows: "It thus seems that further experiments with other species, and using a wider variety of olfactory stimuli, are necessary before we may reach any definite conclusions about the sense of smell in birds."

The experiments to be discussed here were undertaken as the first phase of a systematic investigation of avian olfaction. They were designed to evaluate the ability of birds to perceive olfactory stimuli, and to study birds with olfactory bulbs of different sizes. Perception is defined as a change in the ongoing activity of one or more effectors in response to stimulation of the olfactory receptors. No directed response is required.

As is well known, a new stimulus suddenly introduced into an animal's environment causes a group of responses collectively known as the orienting reflex, or the orienting reaction (Sokolov, 1960). If an odorous stimulus presented to a bird is capable of initiating any effector action, it should at least elicit an orienting reaction. By recording one or more of the components of the orienting reaction, it should be possible to state whether or not an odor has been perceived, according to the above definition. The work of Neuhaus (1963) followed the procedure just outlined. Neuhaus chose this technique, not on the basis of the orienting reaction, but because of anatomical evidence that the bird's olfactory bulb is connected primarily to other central sites that are concerned with vegetative, rather than associative, functions. Accordingly, he recorded respiration from greylag geese during presentations of distilled water and of scatol, and found that changes in respiration occurred during the odorous stimuli to a greater extent than during the control stimuli. Walter had followed a similar procedure earlier and reported that pigeons showed no reliable change in respiration that could be attributed solely to olfactory stimulation.

METHODS

Our basic procedure is to place the bird in a quiet, unlighted enclosure, provide it with a continuous stream of pure air to which an odor can be added whenever the experimenter wishes, and to record heart rate, respiration, and, in some cases, electrical activity of the olfactory bulbs. The majority of our work to date has been conducted on pigeons *(Columba livia)* of the Barker strain of racing homers. The pigeon is located in the middle of Cobb's list, thus representing neither extreme of anatomical olfactory endowment. In addition, we have collected data from one yellow-headed Amazon parrot *(Amazona ochrocephala)*, one turkey vulture *(Cathartes aura)*, and five shearwaters *(Puffinus puffinus opisthomelas)*. The parrot has very small olfactory bulbs, while the shearwaters and the vulture have relatively large ones.

The bird is wrapped lightly in stretch bandage so that it cannot spread its wings. Needle EKG electrodes are inserted into one pectoral muscle and into a muscle of one leg. The transducer for respiration is either a piezoelectric device or a distended balloon attached to a strain gauge. The bird is placed on a hammock suspended rather rigidly across a supporting stand and placed inside a small chamber, as shown in Fig. 1. The bird's head extends into a glass funnel which serves as the output of an olfactometer. Pure air enters the funnel continuously, flows across the bird's bill and around its head, and is exhausted from the box by means of a blower at the opposite end. This exhaust, as well as the exhaust from the olfactometer itself, are led out the window of the laboratory. The bird's EKG and respiration are recorded throughout the time it is in the box by means of a Grass polygraph or a portable Offner dynagraph. The first stimulus is presented after both heart rate and respiration have reached a steady resting level. In each session, four or five odorous stimuli are presented. Two types of control stimuli are used. One is the addition of pure air rather than odor to the continuously flowing air, while the other is a light inside the testing chamber. In the case of the pigeons, the air stimuli were presented in a number of separate sessions, but with wild birds, at least two air stimuli were presented at the start of each recording session. The final stimulus in each session with all birds was one presentation of the light. The duration of each stimulus is 10 sec, and the interstimulus interval varies from 2 to 10 min. Each recording session lasts from 30 to 45 min. Because the orienting reaction adapts quickly, any given bird can be used only in a small number of these sessions, separated by several days.

Our olfactometer follows the same general principles as that employed by Walter and by Mozell (1958), and that described by Pfaffmann, Goff, and

Bare (1958). Two streams of compressed air from the laboratory supply are blown through canisters of silica gel and activated charcoal for drying and deodorizing. One stream serves as the background, unvarying flow of pure air. The other stream bifurcates. By means of two ganged valves, one or the other branch of the second stream is added to the background flow and passes into the test chamber, while the other branch is exhausted to the street. One branch is used to provide the stimuli, and the other adds a comparable volume of air to the background flow between stimuli. Both streams flow through identical gas washing bottles placed in a constant temperature bath maintained at 25°C. A flowmeter in each stream indicates the rate of flow so that intensity of the odorous stimuli can be calculated. The total flow rate was constant at 3000 cc/min.

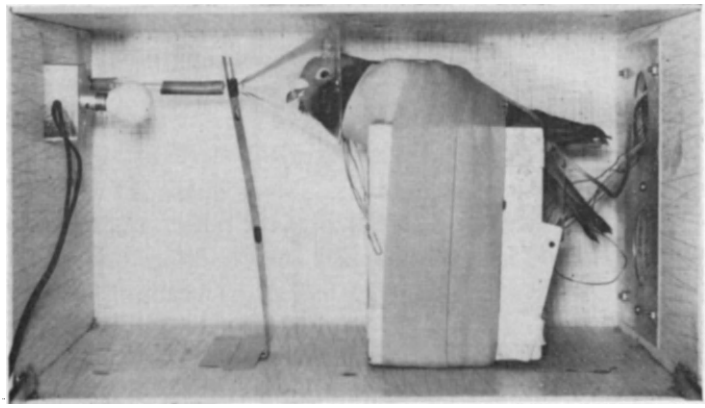


FIG. 1. A pigeon in position for stimulus presentation and recording. EKG and EEG electrodes are attached, and a respiratory transducer is in place.

Unipolar electrodes have been implanted in each olfactory bulb of four pigeons and four shearwaters. This was done by drilling a small hole in the bone over each bulb and lowering the electrodes into place using the electrical activity recorded through the electrodes as a guide to the depth of placement. The location of the bulbs was determined by means of external landmarks in the pigeon and by means of X-ray photographs in the shearwaters. The electrodes were insect pins, with 0·5 mm uninsulated tips, attached to miniature Amphenol plugs that were fastened to the skull with dental cement. All surgery was done under either pentobarbital anesthesia or Equithesin (chloral hydrate, pentobarbital, and magnesium sulfate). After several recording sessions had been completed, electrolytic lesions were made in the bulbs of four pigeons and one shearwater through the

implanted electrodes, using an intensity and a duration of current sufficient to produce coagulation of 1-mm diameter in egg albumen. Additional recording sessions were then held with the lesioned birds.

The data for heart rate and respiration have been analyzed by comparing each rate during the stimulus period with the rate during the 10 sec immediately before and after the stimulus. The stimulus period for all stimuli except the light has been subdivided into an initial section of 4 sec and a final section of 6 sec to allow time for the odorous air to reach the funnel after the two-way valve has been turned, and to be inspired. Four seconds is a generous estimate of the time needed for these events to occur.

All of the birds were tested with three different pure chemicals as odorous stimuli, viz., amyl acetate, trimethylpentane, and pyridine. Usually, only one of these odors was presented in any one session. The shearwaters and the vulture were also presented with air that had been odorized by contact with ground smelts and putrid raw veal, respectively.

RESULTS

The mean changes in heart rate and respiration to the various odorous and control stimuli are shown graphically in Figs. 2 through 5. The results are based on the responses of between eight and sixteen pigeons and five shearwaters to at least five presentations of each odorous stimulus and of the control stimuli. All of the pigeons had comparable resting heart and respiratory rates typically ranging from 150 to 180 beats per minute and 30 to 50 breaths per minute. The shearwaters' resting rates were more variable among birds but were quite constant for each individual bird. Their heart rate tended to be higher than that of the pigeons, while the respiratory rate tended to be lower. The responses to the visual stimulus, sudden presentation of a bright light in the darkened chamber, testified to the validity of the hypothesis that changes in the cardiac and respiratory rates occur in response to a new stimulus. It is clear that both groups of birds responded to the odorous stimuli to a greater degree than they did to the control stimuli of pure air. No explanation has been found for the relatively large response of the pigeons to the air stimuli.

A dose-response effect is seen in both the cardiac and respiratory changes in the pigeons, who were presented with different intensities of amyl acetate and trimethylpentane, but the direction of the effect is different for the two responses. The heart rate increased more with the stronger stimuli, while the respiratory rate increased less with the stronger stimuli. The latter result may indicate a tendency to inspire less of the odorous substance when the intensity is stronger. The inversion between the cardiac and respiratory

changes points up the absence of a reflex dependence of heart rate on respiratory rate in these response periods. Throughout the testing of the pigeons, it was noted that changes in respiration were much less consistent, either

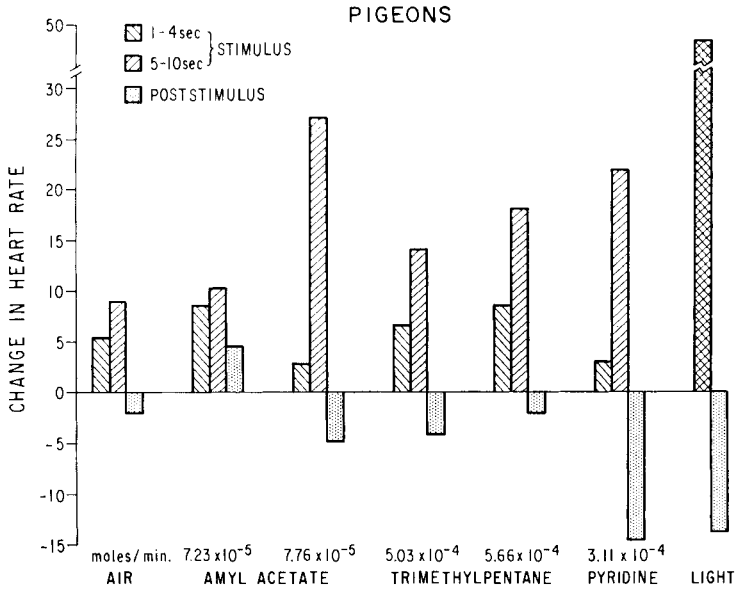


FIG. 2. Mean changes in heart rate of pigeons to various stimuli. The stimulus periods are explained in the text.

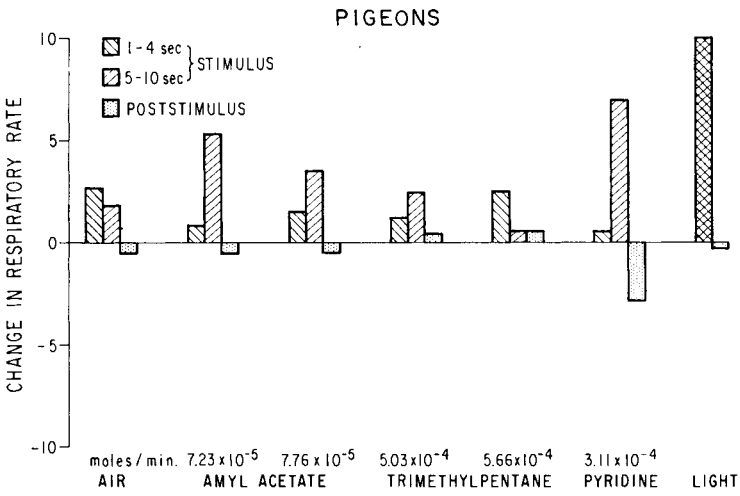


FIG. 3. Mean changes in respiratory rate of pigeons to various stimuli.

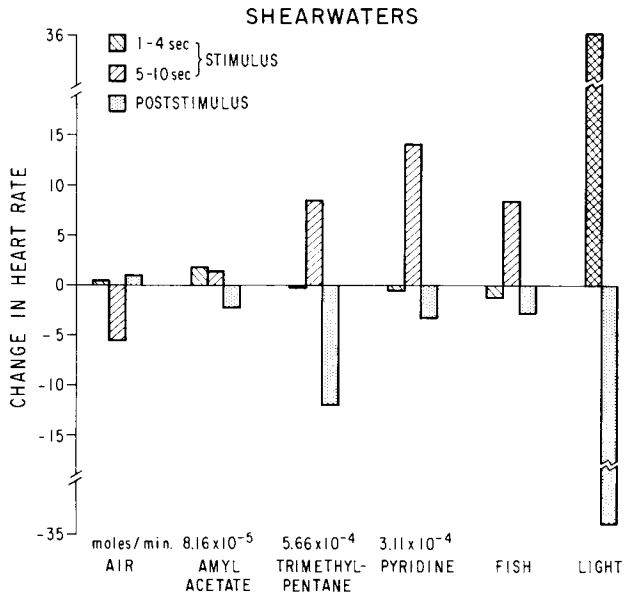


FIG. 4. Mean changes in heart rate of shearwaters to various stimuli.

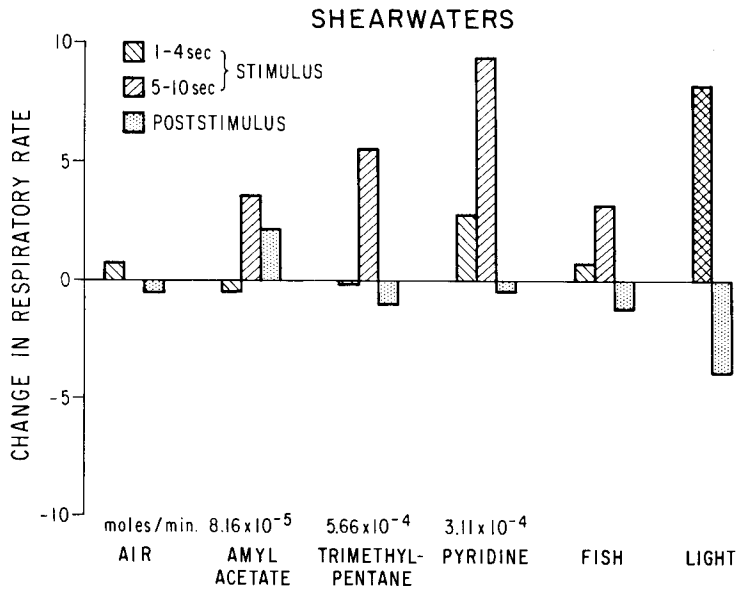


FIG. 5. Mean changes in respiratory rate of shearwaters to various stimuli.

in occurrence or in magnitude, than changes in heart rate. This difference might account for some of Walter's inability to find regular respiratory responses to odorous stimuli in pigeons. In the shearwaters, on the other hand, the opposite effect was observed, i.e. the more consistent responses occurred in respiration rather than in heart rate. Perhaps the latter pattern is characteristic of birds that have been living under wild conditions, while the former may typify birds that have lived under more sedentary conditions. If so, it might explain Neuhaus's success in obtaining regular respiratory changes to odorous stimuli in the greylag goose.

With the supraliminal stimuli used in these experiments, it is difficult to make direct comparisons of olfactory acuity among the different species tested. In Table 1, the responses of the pigeons and shearwaters are expressed as percentages of each group's response to the light, on the assumption that a visual stimulus has greater arousal value than olfactory stimuli and the response to it would serve as a suitable baseline for comparing stimulus effectiveness. The data in Table 1 do not suggest that the shearwaters, with their proportionately larger olfactory bulbs, were notably more sensitive than the pigeons, using this method of analysis. Such comparisons are further complicated by the results obtained from the turkey vulture and the parrot, shown in Table 2. It can be seen that the vulture's characteristic response to a new stimulus seems to be a reduction in both heart and respiratory rates that continues into the poststimulus period. A striking feature of the vulture's data is the relatively small response to the visual stimulus. The parrot shows a somewhat erratic pattern. Our parrot was an irascible old bird from whom a rather small amount of useable data was obtained, compared with all of the other birds. So many episodes of movement occurred during the sessions with the parrot that few stimuli could be presented because of the long delays necessitated by waiting for the heart and respiratory rates to reach an approximate resting level. Like the vulture, the parrot's responses to light were very small. Responses to the olfactory stimuli were what one might expect if the stimuli were ineffective and changes in heart rate were sampled at random from a continuous record.

In the concentrations used so far, trimethylpentane appears to be the least effective of all the stimulus substances presented. Pyridine proved to be a very potent substance for the vulture, so much so that the concentration used with the pigeons and the shearwaters produced movement every time the stimulus was presented and no satisfactory data have yet been obtained for the vulture with this substance.

The normal diet of both shearwaters and vultures consists of odorous food. When a stimulus was used that might be assumed to have some signi-

TABLE 1. RESPONSES TO OLFACTORY STIMULI (MOLES/MIN) EXPRESSED AS A PERCENTAGE OF EACH SPECIES' RESPONSE TO LIGHT

Stimulus	Pigeons		Shearwaters	
	H. R.	Resp.	H. R.	Resp.
Air	18·6	18	—15·2	0
Amyl acetate: $7{\cdot}23\times10^{-5}$	21·1	53		
$7{\cdot}76\times10^{-5}$	55·8	35		
$8{\cdot}76\times10^{-5}$			3·9	38·3
Trimethylpentane:				
$5{\cdot}03\times10^{-4}$	28·9	26		
$5{\cdot}66\times10^{-4}$	37·2	6	23·4	70·4
Pyridine: $3{\cdot}11\times10^{-4}$	45·2	70	39·4	116·0
Fish			23·1	38·3

TABLE 2. MEAN CHANGES IN HEART RATE (BEATS/MIN) AND RESPIRATORY RATE (BREATHS/MIN) OF TURKEY VULTURE AND PARROT. STIMULUS INTENSITIES ARE THE SAME AS THOSE PRESENTED TO THE SHEARWATERS

	Vulture			Parrot		
	Stimulus 1–4″	Stimulus 5–10″	Post-stimulus	Stimulus 1–4″	Stimulus 5–10″	Post-stimulus
Heart rate						
Air	−1·7	−1·9	−0·8	0·0	− 7·0	+ 6·0
Amyl acetate	−8·4	−4·8	−3·6	+10·8	+24·8	+36·8
Trimethylpentane	−4·5	−1·5	−3·0	+ 8·2	− 3·2	+28·0
Decayed meat	+0·8	−4·7	+0·1			
Light	−2·3		−7·8	+14·0		+ 3·7
Respiratory rate						
Air	−4·0	+ 1·5	−0·9	−4·7	+2·7	−2·8
Amyl acetate	−1·7	−12·1	+5·0	−2·5	+6·2	+2·6
Trimethylpentane	−2·1	− 1·3	−1·9	−2·8	−0·5	+1·2
Decayed meat	−0·5	− 0·9	−0·4			
Light	−0·9		−0·8	+5·7		−6·4

ficance in the life of the bird, viz., the smell of fish or of spoiled meat, reactions of some magnitude were elicited, although not as large as those to other stimuli. The data from the vulture were obtained following a three-day fast, while the shearwaters had been eating regularly when they were tested.

In any study of olfactory perception, attention must always be paid to the possibility that responses elicited by olfactory stimuli are actually mediated by non-olfactory sensory systems, of which the chief suspect is the array of cutaneous receptors in the nasal cavity with their trigeminal inner-

vation. Partial destruction of the olfactory bulbs by electrolytic lesions in four pigeons and one shearwater were followed by somewhat reduced responses to olfactory stimuli in two of the pigeons, unchanged responses in the other two, and lowered responsiveness in the shearwater. The data for the pigeons can be seen in Table 3 and for the shearwater in Table 4. It is important to note that the responses of all the birds to the light remained at the prelesion magnitude, indicating that their general responsiveness

TABLE 3. MEAN CHANGES IN HEART RATE AND RESPIRATORY RATE FOR EACH OF FOUR PIGEONS BEFORE AND AFTER PARTIAL DESTRUCTION OF OLFACTORY BULBS. ONE BIRD WAS TESTED AT EACH STIMULUS INTENSITY AFTER THE LESIONS WERE MADE. THE VALUES FOR THE LIGHT ARE MEANS FOR THE SAME FOUR BIRDS. STIMULUS INTENSITY IS IN MOLES/MIN

Stimulus		Heart rate			Respiratory rate		
		Stimulus 1–4″	Stimulus 5–10″	Post-stimulus	Stimulus 1–4″	Stimulus 5–10″	Post-stimulus
Amyl acetate:							
$7{\cdot}76\times10^{-5}$	Before	+ 1·8	+20·0	+10·8	+0·2	+ 2·8	+4·8
	After	− 3·0	+12·0	0·0	−2·0	+ 2·8	+3·0
$8{\cdot}76\times10^{-5}$	Before	+11·6	+40·3	−37·1	+5·7	+10·3	−4·8
	After	+10·5	+43·5	−39·0	+5·6	+ 4·1	−6·0
Trimethylpentane:							
$5{\cdot}03\times10^{-4}$	Before	+ 7·2	+ 8·4	+ 3·6	+1·6	+ 1·4	+3·0
	After	+18·6	− 0·6	− 2·4	+3·9	+ 3·4	−0·6
$5{\cdot}66\times10^{-4}$	Before	+10·8	+28·8	− 9·6	+4·5	− 0·3	−1·8
	After	+ 6·6	+27·8	−14·4	+0·8	+5·2	+1·2
Light:	Before	+42·3		− 9·1	+10·0		−2·1
	After	+54·0		−30·0	+11·0		−6·0

TABLE 4. MEAN CHANGES IN HEART RATE AND RESPIRATORY RATE FOR ONE SHEARWATER BEFORE AND AFTER PARTIAL LESIONS IN THE OLFACTORY BULBS. STIMULUS INTENSITIES AS IN FIGS. 4 AND 5

Stimulus		Heart rate			Respiratory rate		
		Stimulus 1–4″	Stimulus 5–10″	Post-stimulus	Stimulus 1–4″	Stimulus 5–10″	Post-stimulus
Air:	Before	− 4·1	−17·0	+ 5·1	0·0	−0·3	0·0
	After	+ 3·0	− 4·2	− 7·0	+2·3	−1·8	−2·2
Amyl acetate:	Before	+ 6·4	+ 3·1	− 3·5	−0·1	+7·0	+0·1
	After	+ 0·6	− 5·4	− 9·0	−0·2	+4·0	−0·6
Trimethylpentane:	Before	−11·0	+ 7·0	−14·0	+4·0	+1·0	−1·3
	After	+ 3·6	+ 1·2	− 4·8	−3·6	+7·8	−2·4
Pyridine:	Before	+ 9·0	+40·6	−11·2	+1·0	+2·0	−3·3
	After	+10·7	−21·0	− 0·2	−1·3	−1·3	0·0
Light:	Before	+31·0		−34·0	+2·6		−3·7
	After	+36·0		−42·0	+9·0		−9·0

had not declined. Further experiments are in progress to determine the contributions of the olfactory and trigeminal systems to the total response magnitude. At present, it appears likely that each contributes to the effect in some measure.

The use of stimulus substances that are assumed to have little, if any, ability to stimulate the cutaneous receptors, such as trimethylpentane, may be of some value. Tucker (1963) has presented evidence, however, showing that substances that were previously considered to be non-trigeminal stimulants actually do cause electrical activity in the mammalian and reptilian trigeminal nerve. He has also shown that the trigeminal threshold for amyl acetate is ten times higher than the olfactory threshold in the rabbit, but nothing is known of such relationships in any bird.

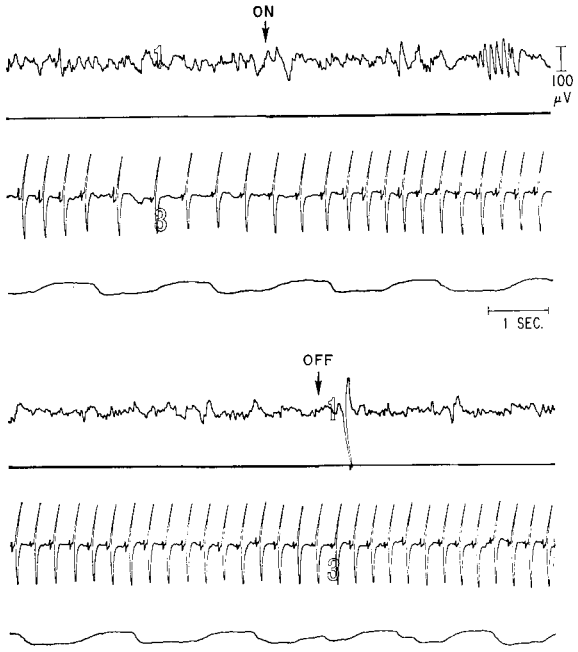


FIG. 6. EEG, EKG, and respiration from pigeon 12*s* during presentation of amyl acetate ($8{\cdot}76 \times 10^{-5}$ moles/min). The stimulus period is shown by the signal marker in the second channel. The EEG was recorded from the olfactory bulb.

The electrical activity of the olfactory bulb has been recorded from a number of these birds, and is being studied in many more. An analysis of these records will be presented at a later time. Several phenomena have been observed that are already well known in the mammalian olfactory bulb. Figure 6 shows a sample of the EEG and the EKG of two pigeons

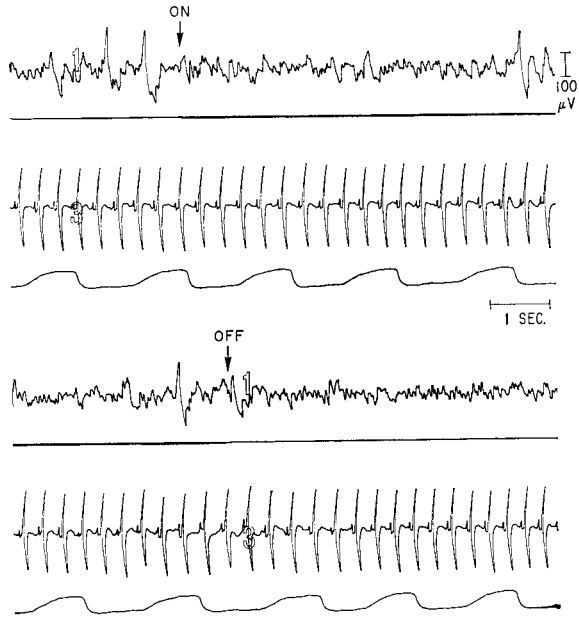


FIG. 7. EEG, EKG, and respiration from pigeon 12*s* during presentation of an air stimulus. Other conditions as for Fig. 6.

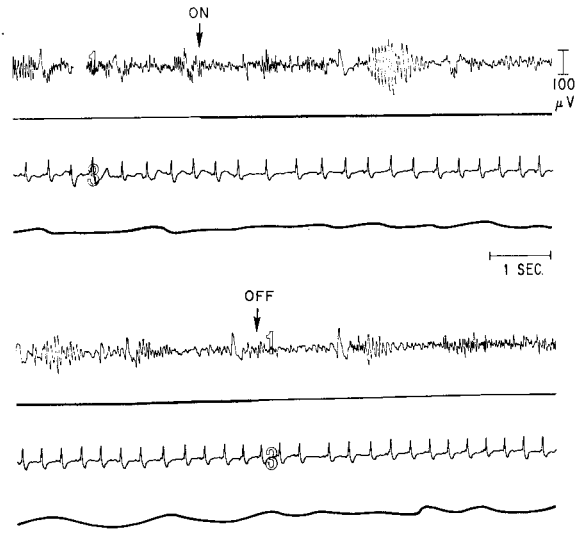


FIG. 8. EEG, EKG, and respiration from pigeon 19*s* during presentation of trimethylpentane ($5{\cdot}66 \times 10^{-4}$ moles/min). Other conditions as in Fig. 6.

before, during, and after the presentation of an odor and of pure air. The wave bursts that appear here during the presentation of amyl acetate never appeared spontaneously or during the visual stimulus in these birds. There is an obvious correlation between these bursts and the peripheral responses, but the full significance of the bursts remains to be determined.

DISCUSSION AND SUMMARY

Some writers have questioned the likelihood of birds having an effective olfactory modality on the argument that their style of life presents no need for it. In such discussions, need is usually analyzed in terms of feeding habits, but birds engage in other activities than finding food. They select and often retain mates, they breed, they return to their own nest even when it is embedded in a great colony, and they recognize their own young. The possible utility of olfaction for different species in any of these activities, including food-getting, should not be overlooked. Even if it were shown not to be involved, it would not constitute proof that odors are imperceptible. The concept of need can be a specious criterion in evaluating perceptual capacity. The only satisfactory criterion is an empirical one—the accumulation of valid evidence that perception occurs or does not occur.

The data presented here lend further support to the contention that the olfactory structures found in birds do have some functional status. All of the most recent research reports on this question have arrived at the conclusion that a degree of olfactory function does exist. Neuhaus (1963) concluded that perception, defined as in the present experiment, occurred in greylag geese. Stager (1964) collected evidence in a well-controlled field study that the turkey vulture, alone among American vultures, uses olfaction in food location; and Michelsen (1959) reported success in training two white Carneaux pigeons to discriminate between conditions of odor and no odor in an operant procedure. Affirmative answers are being obtained, in suitably designed experiments, to all three of the questions specified in the introductory section.

The use of changes in heart and respiratory rates as indices of perception of new stimuli introduced into a fairly homogeneous environment appears to be a valid procedure. Preliminary evidence has been presented to show that these changes are correlated with changes in the electrical activity of the olfactory bulbs and with electrolytic destruction of portions of the bulbs. As a further test of the method for the study of olfactory perception, two rats have served as subjects in exactly the same manner as the

birds. Their heart rates increased sharply to the olfactory stimuli and negligibly to the light. Their respiratory rates could hardly be specified, because the whole pattern changed when either type of stimulus was presented. Regular respiratory cycles were replaced by irregular sniffs. All of the birds studied, except the parrot, can be said to have shown evidence of perceiving the odorous stimuli. The problem of quantitative interspecies comparisons is complicated by the fact that different types of birds are characterized by different patterns of reaction, so that some suitable reference must be found. There is no evidence from this experiment that olfactory perception is correlated with the size of the olfactory bulbs, except in the case of the parrot, which had small bulbs and showed no regular responses to the odorous stimuli.

In the two tests in which the shearwaters and the vulture were presented with the odor of their regular food, viz., fish and decaying meat, reactions occurred but of no greater magnitude than to some of the other, less real-life, odors. Even though the vulture's regular feeding had been delayed for almost two days, it was not more highly excited by the smell of the meat than by amyl acetate. In fact, pyridine was by far the most potent stimulus for the vulture of everything that was presented.

Further work is in progress to study the electrical activity of the olfactory bulb and to correlate it with the peripheral responses, to specify the extent of trigeminal stimulation, and to increase the range of species and stimuli used.

ACKNOWLEDGEMENTS

This work was partially supported by a research grant from the National Association for Mental Health and a grant-in-aid from the Zoological Society of San Diego. The assistance of Phyllis Albritton, George Kolodner, and Michael Sieck in certain phases of this research is greatly appreciated. Some of the work was done at the Institute for Comparative Biology at the San Diego Zoo. The shearwaters were collected by an expedition from the Scripps Institution of Oceanography under the direction of Dr. Carl L. Hubbs.

REFERENCES

BANG, B. G. 1960. Anatomical evidence for olfactory function in some species of birds. *Nature*, **188,** 547–549.

BANG, B. G. 1965. Anatomical adaptations for olfaction in the snow petrel. *Nature*, **205,** 513–515.

COBB, S. 1960. Observations on the comparative anatomy of the avian brain. *Perspectives Biol. Med.*, **3**, 383–408.

DUNCAN, C. J. 1964. Smell. In *A New Dictionary of Birds*. Edited by A. L. THOMSON. McGraw-Hill Book Co. New York.

MARSHALL, A. J. 1960. *The Biology and Physiology of Birds*. Academic Press, New York.

MICHELSEN, W. J. 1959. Procedure for studying olfactory discrimination in the pigeon. *Science*, **130**, 630–631.

MOZELL, M. M. 1958. Electrophysiology of the olfactory bulb. *J. Neurophysiol.*, **21**, 183–196.

NEUHAUS, W. 1963. On the olfactory sense of birds. In *Olfaction and Taste*, pp. 111–124. Edited by Y. ZOTTERMAN. Pergamon Press, Oxford.

PFAFFMANN, C., GOFF, W. R. and BARE, J. K. 1958. An olfactometer for the rat. *Science*, **128**, 1007–1008.

SOKOLOV, E. N. 1960. Neuronal models and the orienting reflex. In *The Central Nervous System and Behavior*, pp. 187–276. Edited by M. A. B. BRAZIER. JOSIAH MACY, JR., Foundation. New York.

STAGER, K. E. 1964. The role of olfaction in food location by the turkey vulture *(Cathartes aura)*. L.A. County Museum *Contributions in Science*. Reprint No. 81, pp. 63.

TUCKER, D. 1963. Olfactory, vomeronasal and trigeminal receptor responses to odorants. In *Olfaction and Taste*, pp. 45–69. Edited by Y. ZOTTERMAN. Pergamon Press, Oxford.

TUCKER, D. 1965. Electrophysiological evidence for olfactory function in birds. *Nature*, **207**, 34–36.

WALTER, W. G. 1943. Some experiments on the sense of smell in birds. *Arch. néerl. Physiol.*, **27**, 1–73.

SINGLE UNIT RESPONSES OF OLFACTORY RECEPTORS IN VULTURES

TATSUAKI SHIBUYA* and DON TUCKER

Department of Biological Sciences, Florida State University
Tallahassee, Florida U.S.A.

IT IS well known that the olfactory mucosa consists of a large number of small olfactory receptor cells, supporting cells and basement cells over a basement membrane, under which the receptor cell axons are organized in bundles sheathed in Schwann cell membranes. Recently, several investigators led off single unit nerve impulses from the olfactory mucosa in response to odors.

Gesteland *et al.* (1963) recorded impulse discharges from the layer of olfactory nerve fibers with metal microelectrodes they designed and they studied odor specificities in olfactory receptors of frogs. Takagi and Omura (1963) also studied spike discharges of decapitated frogs with ordinary capillary microelectrodes. They mentioned that there were seven types of response in olfactory cells of frogs. On the other hand, Shibuya and Shibuya (1963) recorded simultaneously unitary response and slow potential from olfactory mucosa of gopher tortoise in Reptilia. However, single unit responses to odors in olfactory receptors of warm-blooded animals are unknown.

Bang (1964) studied anatomically and histologically the olfactory organs of several vultures and she suggested strongly that the turkey vulture at least should have a keen sense of smell. Neuhaus (1963) observed that the respiratory rhythm changed in response to odor stimulation in geese. However, recent evidence for this problem was offered by Tucker (1965). He recorded olfactory nerve twig activity in response to odors in 14 species of birds.

This work was undertaken to study single unit activity in the olfactory receptors of birds because the status of olfaction in Aves is somewhat con-

* Present Address: Department of Physiology, School of Medicine, Gunma University, Maebashi, Gunma, Japan.

troversial and because they are homoiothermal. Particularly, though, we wish to emphasize the general problem of coding in olfaction. That olfactory receptors are specific to some extent has been deduced by testing a unit with different odors, but this is an emphasis in viewpoint because more than one unit is actually sampled. The implicit assumption is that a receptor type represents a stable and simple yes-or-no situation which can be revealed by exploring a unit with odors. We shall change the emphasis in viewpoint and explore an odor with units, to find that there is much diversity among the receptors responding to a single odorant.

MATERIALS AND METHODS

The birds used in these experiments were the black vulture *(Coragyps atratus atratus)* and the turkey vulture *(Cathartes aura septentrionalis)*. Some were obtained from an animal dealer in Florida and others were collected by us in the woods near Tallahassee.

The vulture was anesthetized with ethyl urethane (1–2 gm/kg i.p.) and its head was stabilized by a holder. After incision and retraction of the skin and removal of the superficial bone, there was revealed the eminence of the olfactory cavity contoured by a thin layer of bone. Some was taken off and the olfactory mucosa was cut to make a window into the cavity. Microelectrodes were inserted through the hole into the olfactory mucosa on the septal wall. The methods described by Tucker (1963*a*, *b*, 1965) were used to get a small strand of the olfactory nerve.

A glass capillary bridge to a calomel electrode was used to record the mucosal slow potential, or EOG as it is familiarly known. Microelectrodes were filled with 10–15 per cent potassium ferricyanide or 1 M potassium chloride. However, spikes could not be held for as long a time with the KCl microelectrodes. Tip diameters were less than one micron and resistances were 40–150 megohms.

Unitary spike discharges were led to a Grass P6 amplifier via a simple cathode follower which replaced the original equipment probe. The EOG was amplified with the d.c. setting of another P6 amplifier and both signals were led to a Tektronix 565 oscilloscope. Pictures were taken with a Grass kymograph camera.

The air olfactometer made by Tucker (1963*b*) was used to introduce odorous stimuli into the cleaned air stream, which was blown through the pervious nostrils, i.e., in one and out the other since there is no septal partition. The animals breathed freely, therefore; odorous air drawn into the

naris may have been diluted with some room air drawn into the hole that admitted the electrodes. The nasal structure was left intact for the primary nerve twig preparations.

Odorants used were the best grades available from Matheson, Coleman and Bell, Eastman Kodak, etc.

RESULTS

The olfactory cavity of the black vulture was smaller than that of the turkey vulture. Their olfactory mucosae were colored brightly from yellowish to light brown. When small pieces of the mucosa were removed and observed under the microscope, the olfactory cilia were distinguished easily. They were 40–50 microns in length and hardly moved spontaneously. It was not clear whether there were two kinds. The olfactory cilium seems to be thinner than that of the gopher tortoise (Shibuya, 1964) and to be much longer than that of the chicken, which ranged about 7–10 microns in engths (Shibuya and Tucker, 1965).

Discharges of the Olfactory Nerve Twig

Neural activity in response to odor appeared during inspiration and was usually not evident during expiration. The response increased simply with concentration and then plateaued over the higher range. In most black vulture preparations at the higher odor concentrations there occurred often a partial synchronization of the neural impulses, which had the striking appearance of "waves". No doubt they were smoothed by the limited frequency response of the a.c. amplifier, but the fundamentals ranging from 35 downward to 20 in a train were within the passband used (7–500, half amplitudes). Appearance of the "waves" caused an inordinate deflection in the integrator record, which is not to be confused as a greatly enhanced neural response.

A complete response–concentration series is shown in Fig. 1 for a black vulture olfactory nerve twig preparation. Each upward deflection in the record occurred during inspiration. The averaging time (integrator time constant of about 1 sec) was short enough to resolve the patterning of response with respiration. The fairly steady background level of neural activity was about as great as the increment in response to amyl acetate at 0·1 per cent vapor saturation. The record shows that the previously mentioned "waves", which drove the recorder off scale, are easily adapted out.

But it is clear that at the higher concentrations there is some continuing adaptation of the asynchronous neural response during following inspirations.

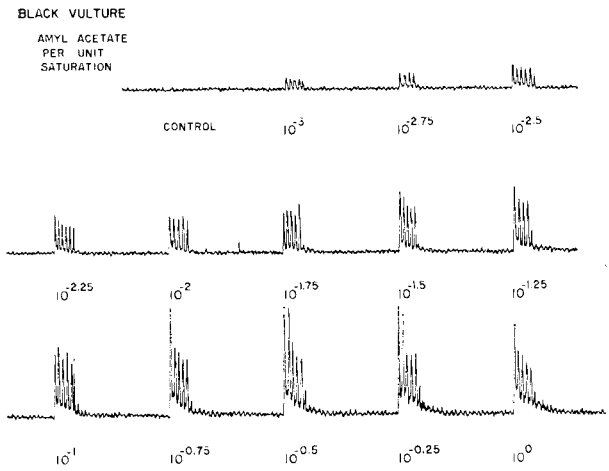


FIG. 1. Response versus concentration series of vulture olfactory nerve twig preparation. The record was obtained by processing the neural activity with a Sanborn 350–1400 amplifier on the a.c.-Linear mode of operation, for which the output indication is "full wave average". The instrument was modified to prevent over-driving of the amplifier stage before the rectifying diodes.

Slow Potentials (EOG) and Unitary Spikes

The EOG appeared during inspiration of odorous air and less obviously during expiration, but this was partly because its rising phase was sharper during inspiration. The spikes shown in Fig. 2 are in correspondence with the EOG; about 13 during inspiration and 26 during expiration. These spikes were held over 90 min and they responded well to odors. However, most units show little or no activity during expiration. Occasionally, though, one was found that responded almost exclusively during expiration. The spikes were usually diphasic with the initial deflection positive. Spike duration was 3–4 msec, which is scarcely different from those in the frog (Gesteland *et al.* 1963) and tortoise (Shibuya and Shibuya, 1963). However, the spike height was fairly well maintained at frequencies considerably higher than was observed in the tortoise.

The behavior of the unit shown in Fig. 2 for concentration variation of hexyl acetate is shown in Fig. 3. There were no spikes in the run omitted from the figure at 10^{-3} of vapor saturation. The response in terms of

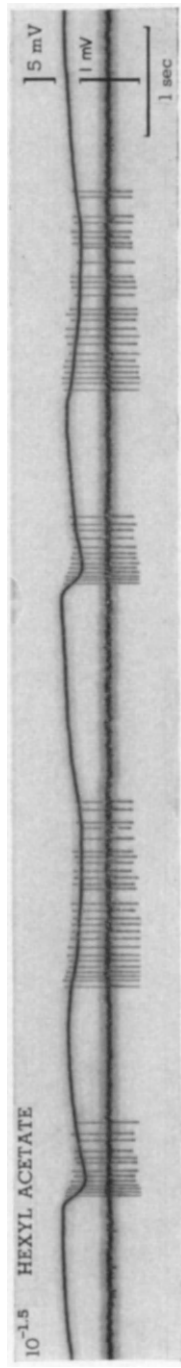


FIG. 2. EOG and microelectrode unitary spikes from black vulture. The short trains of spikes occurred during inspiration and the long trains during expiration.

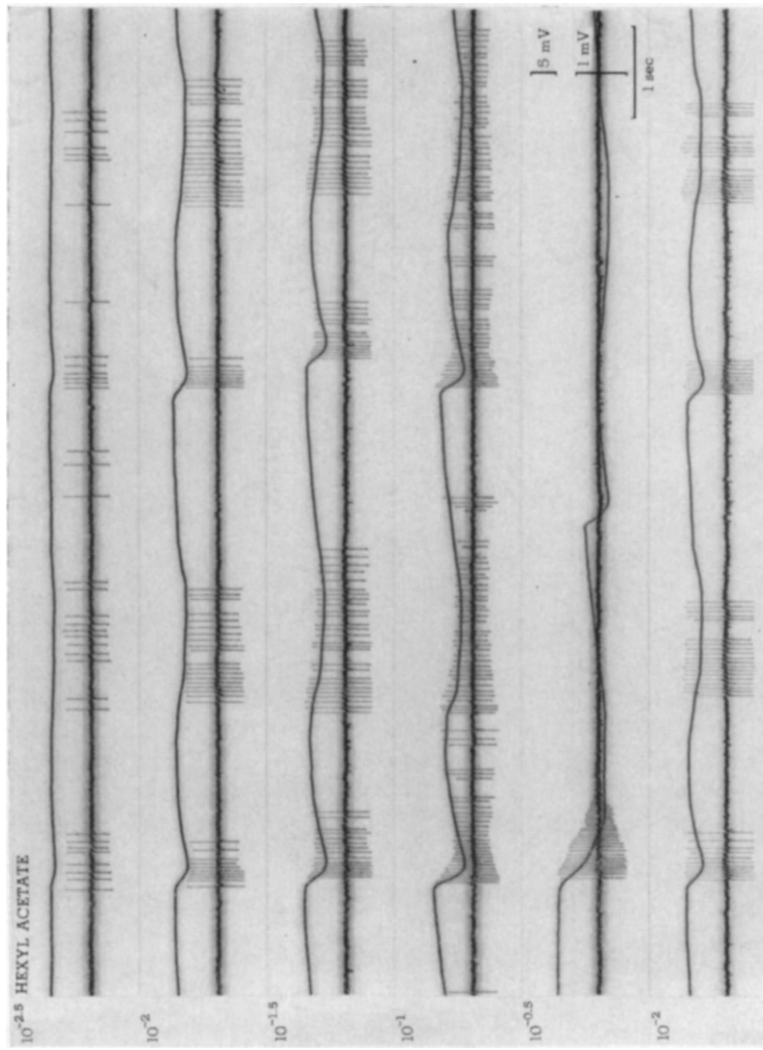


FIG. 3. Concentration variation of hexyl acetate for the same unit shown in Fig. 2.

impulses per respiratory cycle, or unit of time, grows until a block develops at 32 per cent of vapour saturation. The next run (last in Fig. 3), which replicates the earlier one at 10^{-2} hexyl acetate, demonstrates the stability of this unit. Likewise, the $10^{-1.5}$ hexyl acetate run is a replication of the one in the preceding figure. Such testing is important, because it was seen that in some units the response characteristic shifted upward on the concentration scale with passage of time. On the other hand, units that seemed to be temporally stable had thresholds distributed over a large range of concentration.

The "wave" phenomenon was observed with both the EOG and microelectrodes and differed, which is consistent with the two being in different locations (Fig. 4). The "waves" seem to be similar to oscillatory potentials

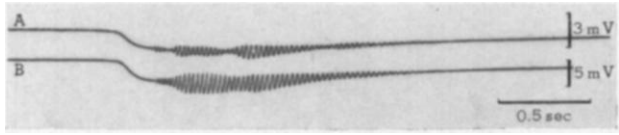


FIG. 4. "Waves" recorded from olfactory mucosa of the black vulture in response to a puff of amyl acetate with (A) the EOG electrode and (B) shallow penetration of the microelectrode.

which were obtained in the olfactory mucosa of toads (Takagi and Shibuya, 1961). Spikes were often synchronized with the waves, but we shall say no more because this subject needs further investigation.

In contrast to the type of unit whose response emerges gradually and develops with increase of concentration, Fig. 5 illustrates one from the turkey vulture with an extremely sharp turn-on characteristic. Furthermore, the number of spikes actually decreases with increase of concentration. This unit was extremely stable and we explored it repeatedly. A similar one was seen in the black vulture with a higher threshold. Although the EOG is not shown in Fig. 5, its onset is signaled by the small deflection before the spikes caused by the slight coupling through the condenser ("Lo Freq 5"). A unit of this type does not necessarily behave the same way for another odorant, as is illustrated in Fig. 6 for another turkey vulture. The response to butyl ether is of the switch-on type, but it is more like the other extreme for amyl, or pentyl, acetate. The response characteristic for hexyl acetate was similar to that for pentyl acetate, but about one half-long unit higher. The time relation between the spikes and the EOG was not so regular in this unit as in the other examples shown.

All the types of units discussed so far were those with no background activity, but from observations on the nerve twig one should expect to find

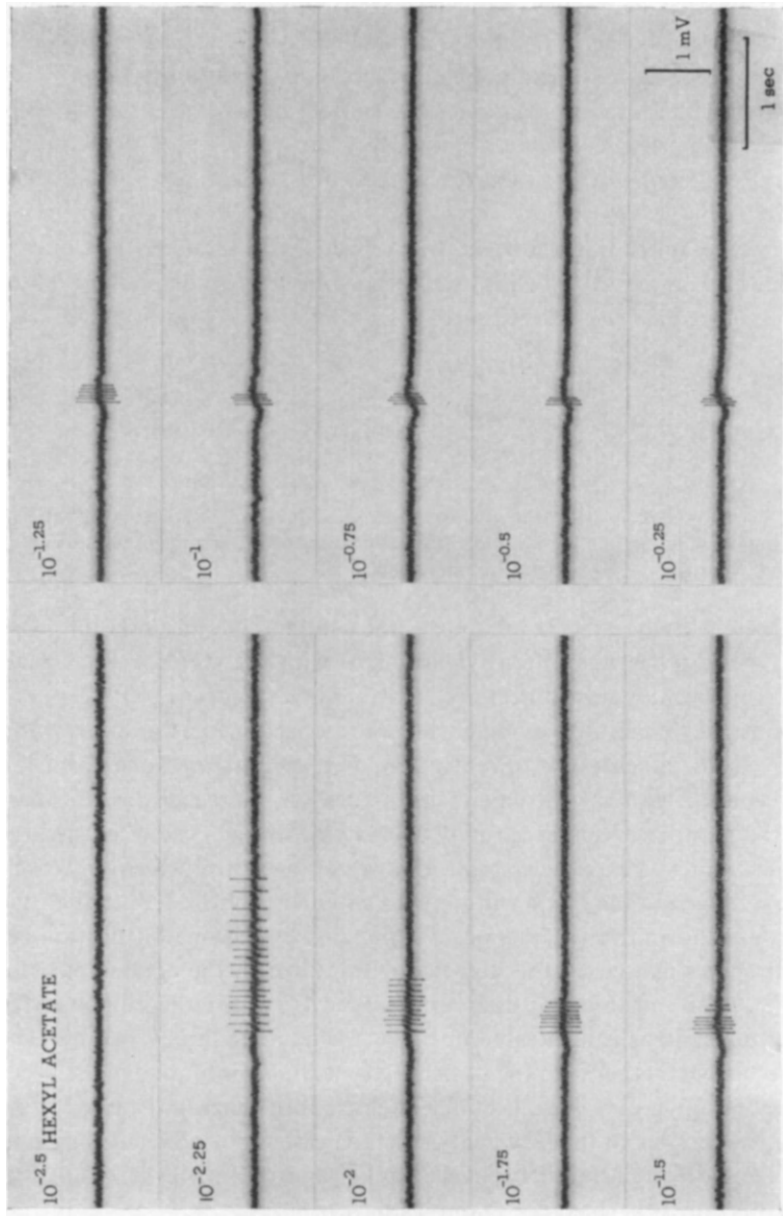


FIG. 5. An extreme example of the switch-on type of response-turkey vulture unit.

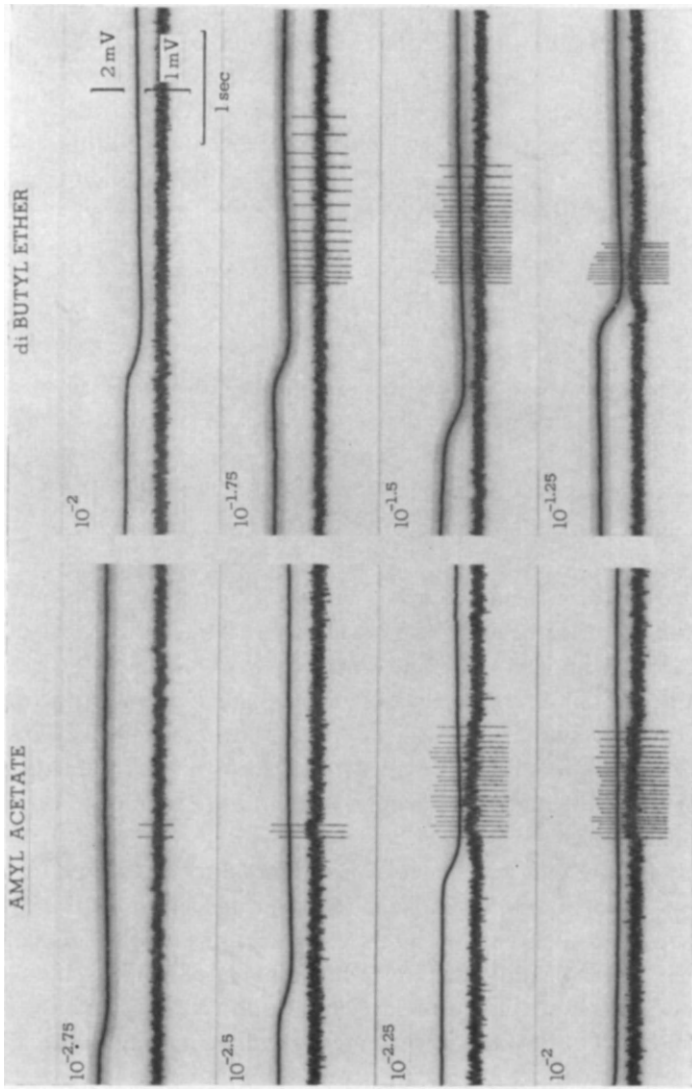


FIG. 6. Variation in type of response of a turkey vulture unit to amyl acetate and butyl ether.

the other class and in fact it appears to contain most of the units that have been described in the literature. Such a "spontaneous" unit may respond to a suitably strong stimulus with increase or with suppression of the firing rate. It seems that the higher the rate of firing the higher must be the concentration of odorant to alter it perceptibly. For a unit that responds with a positive acceleration, it may be that the threshold corresponds to a hypothetical response rate that would have obtained had there been no background activity. Again, for a unit that is suppressed as illustrated in Fig. 7, there may be an analogy with the switch-on (phasic) type in the no-background class that was shown in Fig. 5. Some units that fired at a high rate could hardly be affected with the odorants tested.

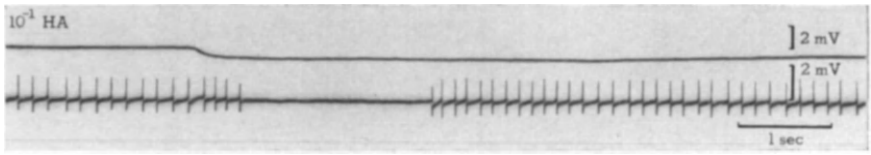


FIG. 7. Inhibition of background activity in a turkey vulture unit with hexyl acetate stimulation.

Units are commonly picked up by virtue of their "spontaneous" firing and the rate can often be affected by small movements of the electrode manipulator. We are not in close agreement between ourselves on how prominent is the possibility that observed background activity is artifactual, but it is true that many "spontaneous" units are somewhat unstable. For example, we held a unit for more than an hour which was repeatedly varied from zero to various levels of firing by moving the electrode carriage in and out small distances.

A turkey vulture unit was tested with several odors by the puff technique for the records in Fig. 8. Most of the irregular deflections in the baselines were caused by inadvertently rapid movements of the teflon squeeze bottles and tubing. Teflon readily acquires a high charge of electrostatic electricity. The odorants were not diluted. There is no way of predicting the results had they been. But tests with some arbitrary extent of dilution would be no more informative. An uncontrolled variable in the experiments reported in this paper, which is likely of importance in this instance, is the timing of the appearance of odorant at the nares relative to the respiratory cycle. The same puff applied during inspiration versus expiration would hardly represent equivalent stimuli, unless it were rather forceful. Neverthe-

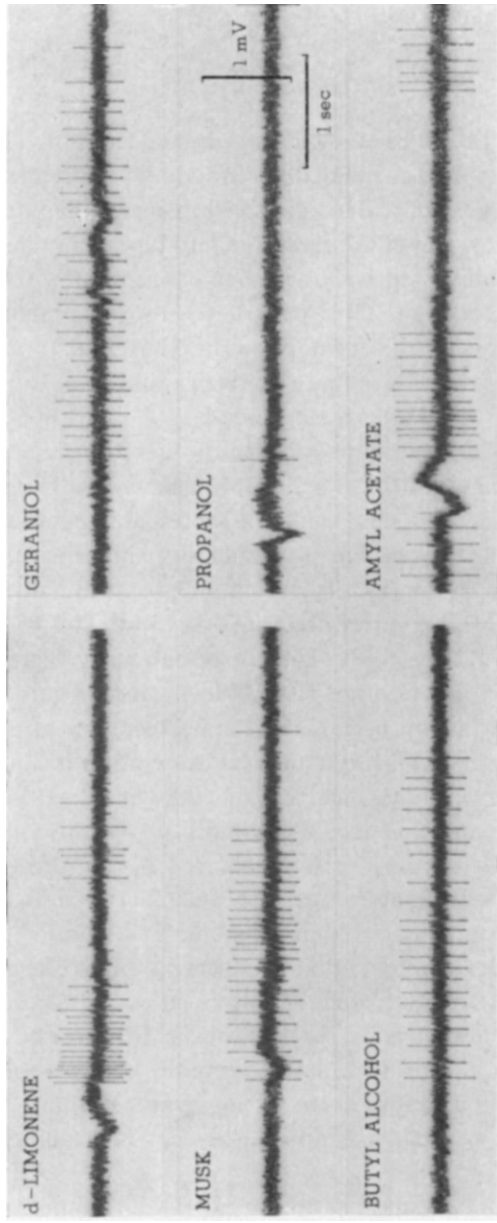


FIG. 8. Responses of a turkey vulture unit to various odors. Puff technique with liquid odorants in teflon squeeze bottles.

less, as has been found in other investigations, a unit tested repeatedly this way with a group of odorants clearly responds differently to at least some members.

DISCUSSION

An olfactory unit can be studied for a limited time only. Thus it is natural to desire to test with as many different odors as possible, typically each at one concentration only. This approach reveals differences which allow at least a loose grouping of the receptors into types. On the other hand, we chose to study a unit intensively with one odorant, or with two or three if it were held long enough. Thus we saw the response characteristics of a number of receptors for the one odorant, hexyl acetate. If the other method reveals differences, it follows that this one should also. But did we see the kind of differences that might be expected?

The response of a random population of olfactory receptors sampled in a nerve twig preparation follows a simple time course bound to the respiratory cycle. The simplest case would be represented by firing of a unit during inspiration and increase in the number of spikes per inspiration with increase in concentration. This is just simple proportionality with the integrator record shown in Fig. 1. But, more realistically, we know that various units have different thresholds. Therefore; one must hypothesize that the response curves of units do not rise as steeply as the curve for the whole bundle of receptor axons, because new units keep appearing to contribute to the whole response. This hypothetical description is not true for many of the units observed, for which that of Fig. 3 may serve as an example. However, those units that increase rapidly in response with increase in concentration may be more or less balanced out by those whose output decreases in the same range (Figs. 5, 6 and 7). But we have already left behind the vague predictions that might be made about the behavior of individual receptors as a function of concentration, to come to favor a picture of units that respond preferentially over various ranges at different locations on the concentration scale. If this held true for odorants in general, the method of exploring a unit with many odors would not yield invariant results with a change in the testing concentrations. And, of course, this is significant because an animal does not normally encounter fixed concentrations.

The observation of units that fire on expiration would not be predicted from the integrator record. Perhaps they are countered by those spontaneously firing units which are suppressed by the stimulus. However, we

have observed a response during expiration from the rattlesnake olfactory nerve preparation. The frequency with which spontaneously firing units are picked up seems abnormally high in comparison with the integrator record in Fig. 1. One of us (D. T.) feels that this may be a sampling problem. Certainly, the two of us tended to be specialists at getting quiet units versus active units. Recall, also, that units may be made to fire because of pressure or some other effect from the presence of the electrode. The number of units studied may have to be extended considerably before the integrator record can be reconstructed, in analogy with taste nerve studies.

Although the time available for study of a unit is limited, we emphasize the desirability of replication. Spontaneously firing units were frequently unstable. In one instance we observed a progressive increase in the threshold of a unit. In another the decrementing of spike height (e.g. Fig. 3) occurred at lower concentrations with the passage of time. One of us (T. S.) feels that decrease in spike heights might explain some observations on the olfactory nerve twig preparation. However, there is no direct evidence that this occurs in the axons far away from the mucosa.

How the various psychological attributes of an olfactory stimulus are encoded in the neural message to the olfactory bulb is a problem that appears to be growing more complex as study progresses. Units vary greatly in the time course of their response relative to stimulation of the organ (EOG and integrator record from nerve) and in their concentration sensitivity. Units more sensitive to one odorant than to another would likely be relatively specific for signaling quality characterizing the former. On the other hand, the appearance of new units with increase of concentration could just as likely signal intensity. An apt description is that all the units studied appear to differ from one another (Gesteland, 1965; Boeckh, 1965). However, when enough have been studied (recall that the rabbit's organ contains 50–100 million receptors), they must appear almost to form a continuum, likely with some modes in frequency of distribution. Perhaps the across-fiber pattern theory put forward by Erickson (1963) to account for the results of taste studies is applicable here. However, one should keep in mind the possibility that the units clustered at a mode may not be equivalent. Some might be signaling intensity, some quality, and others might not contribute to sensation at all, but instead be the afferents in vegetative reflex arcs (Neuhaus, 1963). This concept was expressed as allocation of fibers by the early workers in electrophysiology, such as Matthews and Adrian, and has not received much attention by the workers in chemosensory physiology. It is difficult to attack experimentally, of course.

We feel that our results establish the desirability of investigating the concentration variable in studies of olfaction. It is only a small detraction from this desirability that the absolute concentration at the level of the receptor is usually unknown (Tucker, 1963*b*). Furthermore, odorant concentration is a more fundamental measure of the stimulus than is the rate of arrival of the molecules. That is, flow rate is not equivalent to concentration, as is obvious for the limiting case of zero concentration. It is often possible to virtually eliminate the attenuation due to the chromatographic effect by increasing the nasal flow rate until the olfactory neural response plateaus as a function of further increase, at a level determined by the concentration of odorant. Thus the point is made again, which is not to deny the relative importance of the rate of arrival of odorant molecules. We must not forget the possibility that the patterning of the stimulus over the organ also contributes information utilized by the central nervous system in assessing an olfactory experience. The chromatographic effect has been clearly demonstrated in simultaneous recordings from two olfactory nerve twigs (Tucker, 1963*a*, Fig. 12). The dependence of the olfactory response on nasal flow rate makes it important in free breathing experiments, e.g. Fig. 1, to compare the responses at various concentrations for uniform rate and depth of respiration. Particularly is this true for the range of concentration over which the response magnitude increases.

ACKNOWLEDGMENTS

We wish to thank Dr. L. M. Beidler for his encouragement and support of this work. This research was supported by U. S. Public Health Service grant TI NB 5258-05 and the Division of Biology and Medicine, U. S. Atomic Energy Commision contract AT-(40-1)-2690.

REFERENCES

BANG, B. G. 1964. The nasal organs of the black and turkey vultures. A comparative study of the cathartid species *Coragyps atratus atratus* and *Cathartes aura septentrionalis* (with notes on *Cathartes aura Falklandica*, *Pseudogyps bengalensis* and *Neophron percnopterus*). *J. Morph.*, **115**, 153–184.

BOECKH, J. 1965. Peripheral inhibition as a code in insect olfaction. Abstracts of Papers presented at XXIII International Congress of Physiological Sciences, p. 379.

ERICKSON, R. P. 1963. Sensory neural patterns and gustation. In *Olfaction and Taste*. Y. ZOTTERMAN, (Ed.) 205–213. Pergamon Press, N. Y.

GESTELAND, R. C., J. Y. LETTVIN, W. H. PITTS and A. ROJAS. 1963. Odor specificities of frog's olfactory receptors. In *Olfaction and Taste*. Y. ZOTTERMAN, (Ed.) 19–44. Pergamon Press, N. Y.

GESTELAND, R. C. 1965. Patterns of olfactory receptor response. Abstracts of Papers presented at XXIII International Congress of Physiological Sciences, p. 379.

NEUHAUS, W. 1963. On the olfactory sense of birds. In *Olfaction and Taste*. Y. ZOTTERMAN, (Ed.) 111–123. Pergamon Press, N. Y.

SHIBUYA, T. and S. SHIBUYA. 1963. Olfactory epithelium: Unitary responses in the tortoise. *Science*, **140,** 495–496.

SHIBUYA, T. 1964. Dissociation of olfactory neural response and mucosal potential. *Science*, **143,** 1338–1340.

SHIBUYA, T. and D. TUCKER. 1965. In preparation.

TAKAGI, S. F. and K. OMURA. 1963. Responses of the olfactory receptor cells to odours. *Proc. Japan Acad.*, **39,** 253–255.

TAKAGI, S. F. and T. SHIBUYA. 1961. Studies on the potential oscillation appearing in the olfactory epithelium of the toad. *Jap. J. Physiol.*, **11,** 23–37.

TUCKER, D. 1963*a*. Olfactory, vomeronasal and trigeminal receptor responses to odorants. In *Olfaction and Taste*. Y. ZOTTERMAN, (Ed.) 45–69. Pergamon Press, N. Y.

TUCKER, D. 1963*b*. Physical variables in the olfactory stimulation process. *J. Gen. Physiol.*, **46,** 453–489.

TUCKER, D. 1965. Electrophysiological evidence for olfactory function in birds. *Nature*, **207,** 34–36.

THE DEFINITION OF PRIMARY AND ACCESSORY AREAS OF OLFACTION AS THE BASIS FOR A CLASSIFICATION OF DECREASED OLFACTORY ACUITY

ROBERT I. HENKIN

Clinical Endocrinology Branch,
National Heart Institute, Bethesda, Maryland

IN OUR recent studies we have found that patients with adrenal insufficiency have a much greater sensitivity for taste and smell than do normal individuals.[1, 2] This sensitivity is particularly striking for the sense of smell. Because of these observations we were interested in studying the olfactory epithelium as a tissue. We hoped that anatomical and biochemical evaluation of this tissue might lead us to a clearer understanding of this problem. As little information about normal human olfactory epithelium was available, we decided to obtain this tissue from living patients. This type of investigation could be undertaken, since patients with invasive tumors of the paranasal sinuses have been treated by radical excision of the tumors and the olfactory epithelium as well at the National Cancer Institute of the National Institutes of Health.[3] Because we were interested in evaluating this tissue anatomically and biochemically, the status of the sense of smell in each patient was measured by obtaining detection and recognition thresholds for various odors in the patients prior to operation.

Detection threshold, defined as the lowest concentration of a substance consistently detected as different from two comparison solutions, was obtained by requesting the patient to detect by sniff of a vapor the different solution from among three solutions. Two of the solutions were water or mineral oil.

Recognition threshold, defined as the lowest concentration of a substance consistently recognized as that substance, was evaluated by requiring the patient to characterize the substance which was detected. These techniques have been previously described in detail.[1, 4] The patients were also required to locate the site of detection and/or of characterization. For

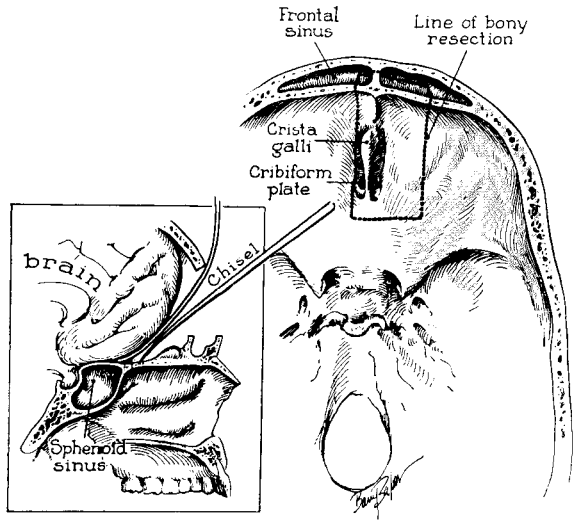


FIG. 1. A combined resection of malignant tumor involving the paranasal sinuses. The left insert is an artist's representation of the elevation of the bone flap and separation of the dura from the cranial floor. The central picture is an artist's representation of this intracranial site of surgical exploration. (Courtesy of A. S. Ketcham, National Cancer Institute, Bethesda, Maryland.)

FIG. 2. A photograph taken at operation illustrating the intracranial site of exploration. The crista galli and cribriform plate are easily seen in the center of the picture. (Courtesy of A. S. Ketcham, National Cancer Institute, Bethesda, Maryland.)

smell, solutions of pyridine, nitrobenzene, thiophene, hydrochloric acid, ammonium hydroxide and camphor were used.

At operation, the tumors and surrounding tissue of these patients were excised. The general operative procedure is illustrated in Fig. 1. Initially, a frontal bone flap was elevated and the dura separated from the anterior cranial floor (Fig. 2). During this maneuver, all the olfactory nerve fibers were transected at the cribriform plate. The floor of the anterior cranial fossa was resected from the frontal bone to the anterior sphenoidal ridge bilaterally, to include the medial portion of the supraorbital plate. The entire cribriform plate, the ethmoid sinues, the posterior half of the medial nasal septum, and the involved maxilla and adjacent hard palate were removed en-bloc. This procedure insured the total surgical removal of the olfactory epithelium in each case (Fig. 3).

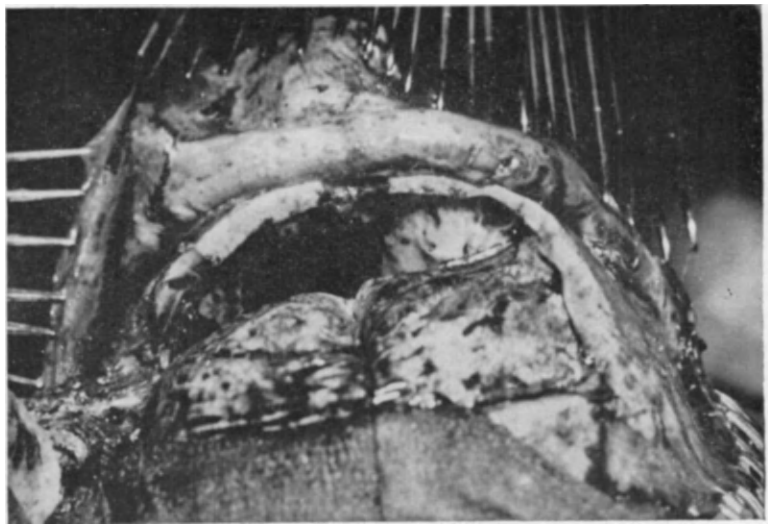


FIG. 3. A photograph taken at operation illustrating the extent of the intracranial defect after removal of the tumor. (Courtesy of A. S. Ketcham, National Cancer Institute, Bethesda, Maryland.)

Following operation, we evaluated the status of the olfactory epithelium physiologically by measuring thresholds for smell in each patient. Immediately after operation, detection thresholds for various substances, including pyridine in water and thiophene in mineral oil could not be obtained. However, much to our surprise, approximately two to three weeks after the procedure, detection thresholds for various odors could again be

obtained (Fig. 4). These thresholds gradually decreased over a period of weeks until they became stable approximately three months after operation. The detection thresholds after operation were approximately 10 times higher than the thresholds before operation. The patients stated that they did not detect vapors as they had prior to operation. In general, the patients stated that the different solution could be detected as different from

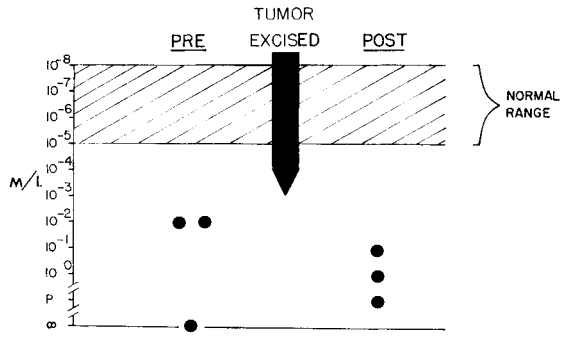


FIG. 4. Detection thresholds for the odor of pyridine in water in patients with paranasal sinus tumors. The ordinate is plotted in M/l., the abscissa in time. The black dots represent the individual detection thresholds expressed in M/l. The shaded areas represent the normal range. The three dots to the left of the arrow are the detection thresholds obtained in these patients prior to operation. The dot at ∞ indicates that one of the patients could not detect pure pyridine as different from water. The three dots to the right of the arrow are the detection thresholds obtained in these same patients six months after operation. The dot at *P* indicates that one of the patients could detect only pure pyridine as different from water. Preoperatively, detection thresholds were obtained at levels which were 10^5 times those in normal subjects. The median detection threshold before operation was 10^{-2} M/l. whereas that in normals was 10^{-7} M/l. On the right the detection threshold obtained six months after operation is shown as 10 times the threshold obtained before operation.

the two comparison stimuli because it felt cool, warm, irritating or simply different, without further description. However, one patient described the vapor of pyridine in the same fashion as did normal subjects, stating that the recognitions were made in her upper and lower pharynx and not in the area of the olfactory epithelium where they had been made prior to surgery. In general, the patients localized these sensations of olfaction in various regions of the nose and pharynx, as occurring in one, two, or all of three of the following anatomic areas: (1) the anterior and lateral portions of the nasal cavity, including the external nares, (2) the upper pharynx in the area of the soft palate, and (3) the lower pharynx in the area

of the epiglottis (Fig. 5). The sensory innervation of the area of the nasal cavity which the patients described as responsive to vapors is through branches of the ophthalmic and maxillary divisions of the trigeminal nerve; the sensory innervation of the area of the upper pharynx, another area where the patients described responsiveness to vapors, is through the third

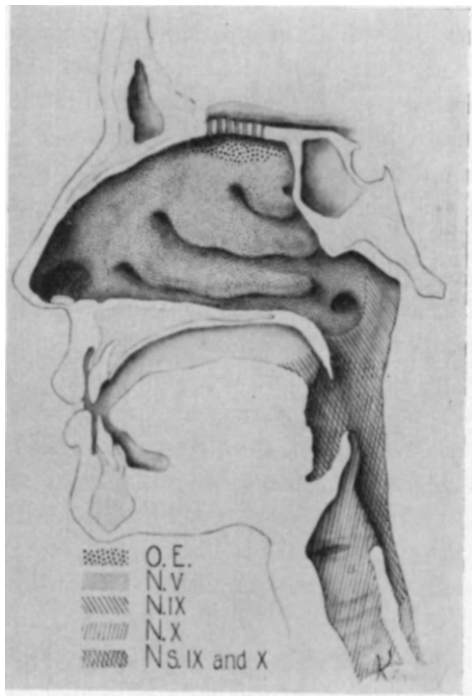


FIG. 5. An artist's representation of the primary and accessory areas of olfaction. O. E. represents the area of olfactory epithelium, an area innervated by the olfactory nerve (I). N. V. represents the area of the anterior and lateral portions of the nasal cavity which subserves olfaction, innervated by branches of the trigeminal nerve (V). N. IX represents the area of the upper pharynx which subserves olfaction, innervated by branches of the glossopharyngeal nerve (IX). N. X represents the area of the lower pharynx which subserves olfaction, innervated by branches of the vagus nerve (X). The cross hatched area represents the area of sensory overlap between nerves IX and X.

branch of the glossopharyngeal nerve via the pharyngeal plexus; the sensory innervation of the area of the lower pharynx referred to by these patients as sensitive to vapors is through the 4th and 6th branches of the vagus nerve, via the vagus' contribution to the pharyngeal plexus through

its pharyngeal branches. There is significant overlap in the contribution of the 9th and 10th nerves to the sensory innervation of the upper and lower pharynx. The major sensory innervation to this area is through the glossopharyngeal nerve. However, pharyngeal branches of the glossopharyngeal nerve join with pharyngeal branches of the vagus nerve and branches of the superior cervical ganglion to form the pharyngeal plexus, which provides ancillary sensory innervation to this area.

There can be little doubt that these patients responded to vapors through the use of areas other than those which involve the olfactory epithelium. Since this is a response to a stimulus administered in the vapor phase it is by definition olfaction, although somewhat different from what we usually designate by this term. Because of this difference in meaning, we need words to describe this type of response. For convenience we can speak of the olfactory nerve-olfactory epithelium area as the *primary olfactory area*, the area of olfactory nerve distribution. The other areas that we have discussed, the nasal cavity and the upper and the lower pharynx, can be described as *accessory areas of olfaction.*

With this concept of primary and accessory areas of olfaction in mind, it was of interest to study a wide variety of patients who have difficulty in their ability to smell. It would be of interest to classify patients with smell disabili ies into a consistent system based on this anatomical and physiological approach. Over the past two years we have studied a broad spectrum of patients with various disorders of olfaction.[5] In some of these patients there was an obvious genetic abnormality, as the smell abnormality was present in successive generations. In some, the smell abnormality was the result of a biochemical deficiency. Some of the patients had hypogonadism associated with hyposmia, some did not. Some had smell abnormalities together with anatomic abnormalities of the facial area, such as cleft palates or facial hypoplasia. In spite of the diversity within the group of patients it seems possible to classify them by the presence or absence of response at primary or accessory areas of olfaction. In what follows, such a classification will be presented along with a brief description of some of the patients who make up the various categories.

By definition *anosmia*, or complete anosmia, is taken to be the absence of response at both primary and accessory areas of olfaction. We have searched the literature for references to, or descriptions of, this type of patient, but have found none. In our screening of many patients with impaired olfaction, however, we have found five patients who would fall into this category. Figure 6 illustrates the characteristics of these patients with complete anosmia or simply anosmia.

The median detection threshold (MDT) for pyridine in water for normal subjects (Fig. 6) is 10^{-7} M/l. with a range from 10^{-5} to 10^{-8} M/l. In contrast, the MDT for all patients with complete anosmia is designated ∞, as none of the patients could detect pure pyridine as different from water even when 100 per cent pyridine was used. These patients could not detect substances as noxious as 37 per cent formalin as different from water or

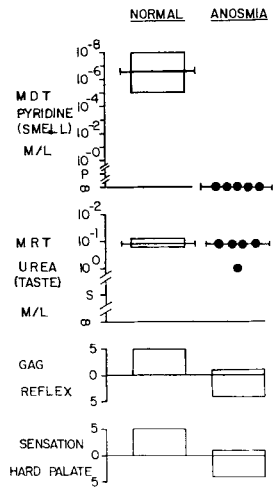


FIG. 6. A description of some of the characteristics of complete anosmia. This syndrome is defined by absence of response at either the primary or accessory areas of olfaction. In the upper row, MDT pyridine refers to the median detection threshold for the odor of pyridine in water as expressed in moles per liter. *P* refers to 100 per cent concentration, or pure pyridine. A black dot at this level signifies the patient can detect only a pure solution of pyridine as different from water. A dot level with the infinity sign signifies that the patient could not detect any difference between the two comparison stimuli and pure pyridine. A black dot at any other level signifies that the threshold of the individual patient is at that concentration. The box represents the range of response of normal subjects; the line through the box or through the dots signifies the median threshold response for the group of patients or normal subjects. Since many determinations have been carried out in normal subjects, only the range and median response line are shown, without illustrating each individual response. In the next panel row, the MRT urea refers to median recognition threshold for the taste of urea, expressed in M/l. In the next panel below the results of testing five patients and five matched normal subjects for the presence or absence of a gag reflex is plotted. The ordinate is scaled in number of patients or subjects in whom a gag reflex could be elicited (area above the line) and the number of patients of subjects in whom a gag reflex could not be elicited (area below the line). The lowest panel illustrates the presence or absence of normal sensation on the hard palate. The ordinate is scaled in number of patients or subjects in whom normal sensation is elicited (area above the line) and the number of patients or subjects in whom normal sensation cannot be elicited (area below the line).

pure thiophene as different from mineral oil. There was not only an absence of response of olfaction in the usual sense, that is, at the primary olfactory area, but also an absence of response at the accessory areas of olfaction. Thus, the patients did not detect a vapor as noxious as pure pyridine in the nose or in the throat. They showed no signs of withdrawal upon inhalation of such noxious vapors. The median recognition threshold (MRT) for the taste of urea in normal subjects is 0·12 M/l. (Fig. 6). This is about the MRT observed in the patients with anosmia (Fig. 6). Detection and recognition thresholds for sodium chloride for salt, hydrochloric acid for sour, and sucrose for sweet are within normal limits for four of the patients tested. Gag reflexes could not be elicited in four of these patients, even with a tongue blade or soft probe placed on the posterior pharyngeal wall. Control patients or normal subjects respond to this maneuver with at least a gag reflex. Sensation on the hard palate in four of these patients was grossly deficient (Fig. 6) in comparison to the normal response obtained from control patients or from normal subjects. Sensation was estimated in part through the measurement of light touch by the application of a graded series of nylon fibers to the hard palate.[6] Sensation was also estimated by measurement of two-point discrimination on the hard palate, which was approximately half to a fourth as sensitive in these patients as in normal subjects.

The absence of any response to vapors as noxious as pure pyridine in these patients was so surprising that it was necessary to verify it by a more objective technique. Figure 7 illustrates the results. The subject was placed on a table in a room from which odors had been carefully excluded. A pneumotachometer was placed around his chest to allow continuous measurement of rate and character of respiration, and a Cournand needle was placed in the right brachial artery to allow continuous measurement of heart rate and mean arterial blood pressure. The upper panel of Fig. 7 shows the respiratory pattern, the lower panel the blood pressure and heart rate. The upper panel shows an initial full inspiration of water vapor followed by a full expiration. There is then a second full inspiration of water vapor followed by a full expiration. This is followed by a full inspiration of the vapor from a sample of concentrated NH_4OH. It is clearly shown that the inspiratory pattern breaks abruptly with the subject's withdrawal from the noxious stimulus with a reflex expiration followed by an irregular pattern of respiration for some time.

The response to the same procedure of a patient with complete anosmia is shown in Fig. 8. The patient inhaled the first stimulus, water vapor, vigorously. A full inspiration is shown, followed by a full expiration. The

patient then inhaled water vapor again. There was another full inspiration followed by a full expiration. Finally, she inhaled a similar volume of the vapor of pure pyridine. Here again a full inspiration is followed by a full expiration. Comparison of the three respiratory patterns shows no differ-

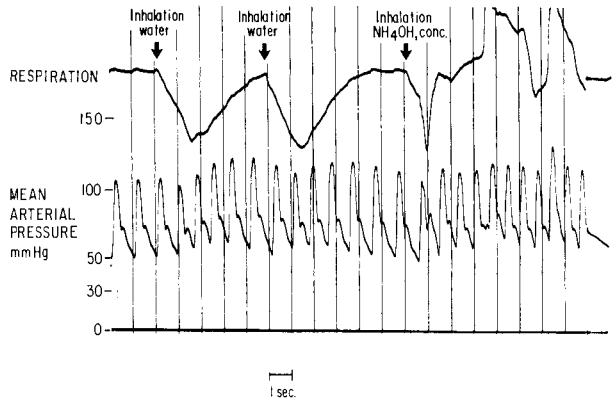


FIG. 7. The normal response of a subject to the inhalation of the vapor of concentrated ammonium hydroxide (NH_4OH). The top horizontal line indicates the subject's respiration measured continuously by a pneumotachometer. The lower horizontal line indicates the subject's mean arterial pressure measured continuously in mmHg by an indwelling Cournand needle in the right brachial artery. The respiratory responses to inhalation of concentrated NH_4OH is normal as indicated by the abrupt expiration after initial inspiration, followed by an irregular respiratory pattern.

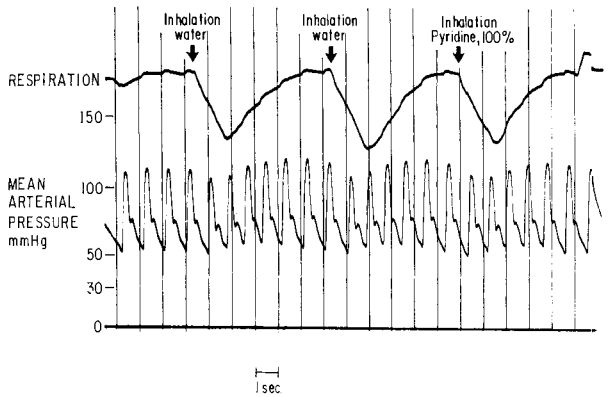


FIG. 8. The response of a patient with complete anosmia to the inhalation of the vapor of pure pyridine. The top horizontal line indicates the patient's respiration, the lower horizontal line the patient's heart rate and mean arterial blood pressure. There is no change in the respiration or blood pressure whether the vapor of water or of pure pyridine is inhaled.

ence in rate or in character of response with the three stimuli. There is no withdrawal, no break in inspiration, and no forced expiration. There is no change in heart rate or mean arterial pressure with any of the three stimuli. This response is in sharp contrast to the normal response shown previously and to observations made by Allen in 1929 who reported that a patient who denied recognition of a vapor "responded" to that vapor by alteration of his respiratory rate and character as well as by a slight decrease in blood pressure.[7]

Thus, patients with anosmia do not respond to the vapor of substances as noxious as pure pyridine either subjectively or physiologically, through the use of either primary or accessory areas of olfaction. A similar absence of response has been observed in these patients after inhalation of substances as noxious as 10N HCl and, under special conditions, to substances as noxious as concentrated NH_4OH.

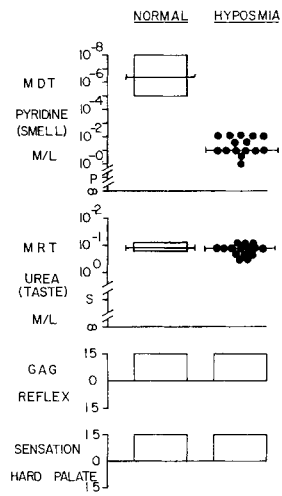


FIG. 9. A description of some of the characteristics of type I hyposmia. This syndrome is defined by absence of response at the primary area of olfaction but the presence of responsiveness at all accessory areas of olfaction. The figure is organized in the same manner as Fig. 6.

Figure 9 illustrates another category of patient with a decrease in olfactory acuity which may be classified through the presence or absence of responsiveness to primary and accessory areas of olfaction. We have designated their disorder as type I hyposmia. *Hyposmia* is defined as the *relative inability to smell.* The patients with type I hyposmia do not respond to vapors at the primary area of olfaction, but do respond at accessory

areas of olfaction. Those patients whose olfactory epithelium was excised with tumors of the paranasal sinuses fall into this category. Careful review of the literature describing patients who have been called "anosmic" suggests that with the present classification these patients would fall into the category of type I hyposmia rather than anosmia. They differ from patients with anosmia in that they use one or more of the accessory areas of olfaction to detect vapors.

Figure 9 shows the MDT for pyridine in water in patients with type I hyposmia as 10° or 1 M pyridine. This is significantly higher than the normal threshold for pyridine which is 10^{-7} M. The range of response for the patients with type I hyposmia is narrow, from pure solutions, to solutions as dilute as 10^{-1} M. This is in contrast to the rather wide range of response to dilute solutions in the normal populations. There is no overlap between the normal and patient populations. The patients denied detection or recognition of any odor through the use of primary olfactory areas. Most of them denied recognition of any odor through the accessory areas of olfaction. However, all described detection as occurring in the accessory olfactory areas. MRT for the taste of urea for the patients and for the normal subjects was the same, 0·12 M/l. (Fig. 9). Detection and recognition thresholds for representatives of each of the modalities of taste were within normal limits in the patients. Gag reflexes were elicited on the posterior pharynx in the patients as in the normal subjects. Tactile perception and two point discrimination on the hard palate was as sensitive in the patients as in the normal subjects.

Figure 10 illustrates a third category of patient with impaired olfaction. We have designated the disorder in these patients as type II hyposmia. These patients respond at both primary and accessory areas of olfaction, but quantitatively less well than normal subjects. They resemble normal subjects in that they can detect and even recognize vapors in a normal fashion. Most of them have no awareness that they have any difficulty in olfaction, since many odors in the real world are quite concentrated and a relative impairment of olfaction is not a severe handicap. We find these patients mainly through quantitative smell testing. The MDT for pyridine for the patients with type II hyposmia is 10^{-2} M (Fig. 10) 10^5 times the MDT of the normal subjects, 10^{-7}. Similar to type I hyposmia there is no overlap in response between the normal and the patient population. However, these patients can recognize as well as detect odors. They do so through the use of the primary olfactory area. However, their acuity is markedly impaired; the most sensitive patient with type II hyposmia being at least 1000 times less sensitive than the average normal subject.

The MRT for urea for the patients was the same as that for normal subjects, 0·12 M/l. Gag reflexes are present in the patients; tactile perception and two point discrimination on the palate is normal.

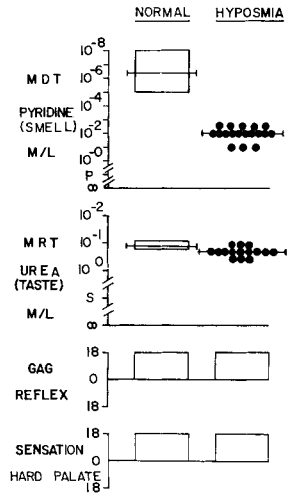


FIG. 10. A description of some of the characteristics of type II hyposmia. The syndrome is defined by the presence of responsiveness at both primary and accesory areas of olfaction but responsiveness at the primary area is less than that in normal subjects. The figure is organized in the same manner as Figs. 6 and 9.

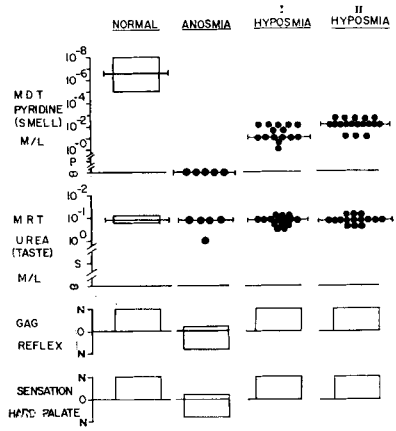


FIG. 11. A composite showing the characteristics of each of the syndromes of anosmia and hyposmia compared to normal subjects. The figure is organized in general in the same manner as Figs. 6, 9 and 10. N refers to the number of patients in each group tested in whom either a gag reflex was or was not elicited or who did or did not show normal sensation on the hard palate.

Figure 11 is a composite illustrating the characteristics of normal subjects as well as those of each of the three major classifications of patients with decreased olfactory acuity.

Patients with anosmia do not respond to vapors at either primary or accessory areas of olfaction; they totally lack the ability to either detect or recognize vapors as noxious as pyridine. Their recognition of the taste of urea is essentially normal, as is their detection of each of the other modalities of taste. In general, gag reflexes cannot be elicited in them and they have marked hypesthesia over the hard palate. These patients comprise a group whose characteristics have not been previously described; they represent a new syndrome. The factors responsible for this syndrome are not known. Logically the syndrome could be related to either a metabolic or an anatomic abnormality. Patients who have been called "anosmic" have been described as showing agenesis of the olfactory lobes at post-mortem examination. This anatomical abnormality has been taken as the *raison d'etre* for the anosmia. However, as noted above, those patients previously described as "anosmic" would be classified as hyposmic by present definitions, since they report responses to vapors through the use of accessory olfactory areas. Other investigators have also observed this phenomenon. Stirnimann reported that an arhinencephalic infant responded to Chenopodium oil and ammonium carbonate even though post-mortem examination revealed that he had complete absence of the olfactory nerves and of the lamina cribrosa.[8] In the only one of our patients with complete anosmia studied by pneumoencephalography the presence of olfactory sulci could not be demonstrated.[9] Both the anosmia and the structural brain defects of these patients could be accounted for if an intrauterine abnormality occurred at about six weeks gestation. These conflicting observations make it impossible to specify whether an anatomic abnormality is responsible for the many aspects of this syndrome. We have no definite evidence of a metabolic abnormality in these patients. Nevertheless, it is interesting that one of our five patients had hypogonadotropic hypogonadism and another had hyperglycinuria in addition to anosmia and sensory abnormalities of the palate and pharynx.

Patients with type I hyposmia cannot detect or recognize any vapor through the use of the primary area of olfaction. However, they can detect vapors through the use of the accessory areas of olfaction—the MDT for pyridine being approximately 10^6 times normal but significantly more sensitive than that in patients with anosmia. Some of these patients could recognize the odor of vapors through the use of receptors in the accessory areas. As in patients with complete anosmia, the MRT for urea is normal,

as are the MDT and MRT for the other taste modalities. However, gag reflexes and sensation on the hard palate both absent in patients with anosmia are normal. Thus, these patients exhibit some characteristics which distinguish them from patients with complete anosmia, yet they also cannot recognize vapors in the normal manner. The patients who comprise this general category fall into several types (Fig. 12): first, those whose

HYPOSMIA TYPE I

1. Acquired
   a. Excision of olfactory epithelium
   b. Trauma to olfactory area
   c. Infection of olfactory area
2. Hypogonadotropic hypogonadism
3. Idiopathic

FIG. 12. Some of the patient groups who exhibit characteristics of hyposmia, type I.

hyposmia has been acquired, such as the patients we have studied after excision of the olfactory epithelium, patients with trauma to the olfactory nerves, or patients with infectious processes involving this area; secondly, patients whose hyposmia has been associated with hypogonadotropic hypogonadism, and, third, a group whose hyposmia seems to be without other associated characteristics, "idiopathic". Type I hyposmia which is associated with hypogonadotropic hypogonadism is familial and appears to be a sex-linked recessive trait. A group of these patients has been described previously by other investigators although the familial relationship has not been consistently specified.[10, 11] However, the association of hypogonadism with hyposmia of this type may be an extremely important one for future research. Certainly the relationship between olfaction and the ability to bear young has been well demonstrated in lower species. Bruce has shown that pregnant female mice will abort subsequent to the introduction of the odor of male mice of another genetic strain.[12] This effect is prevented by the injection of prolactin in the pregnant female mice. In these animals the hypothalamic-pituitary-gonadal axis seems to be particularly sensitive to olfactory stimuli. How important this stimulus–response type of relationship is for higher species is not known. However, quantitative smell testing has already proven to be a very useful tool in specifying the relationship between hypogonadism and hyposmia in our patients. We have presented these results at a previous meeting and will not dwell on them at this time.[13]

It is of interest that detection thresholds for vapors in patients within this category of type I hyposmia are similar regardless of whether the patients had hypogonadotropic hypogonadism or complete excision of the olfactory epithelium. It is also curious that detection thresholds in patients who have had their olfactory epithelium excised are lower than those in patients with complete anosmia who have had no surgical intervention or trauma to the olfactory area.

Patients with type II hyposmia are clearly differentiated from those with anosmia or type I hyposmia. They are responsive at both primary and accessory areas of olfaction, although this response is less than normal. The MDT for pyridine for this group is approximately 100 times more sensitive than that of patients with type I hyposmia. However, the MDT for pyridine in patients with type II hyposmia is still 10^5 times less sensitive than that in normal subjects. There is some overlap in detection thresholds for pyridine between patients with type I and those with type II hyposmia. However, they can be easily differentiated since only those with type II hyposmia can recognize odors through the use of the primary olfactory area. In other characteristics, they are similar to patients with type I hyposmia, for the MRT for urea is within normal limits, and they have

HYPOSMIA TYPE II

1. Acquired
   a. Tumor involving olfactory area
   b. Trauma to olfactory area
2. Hypovitaminosis A
   a. Acanthocytosis
   b. Malabsorption syndromes
3. Hypogonadism
4. Facial Hypoplasia
5. Idiopathic

FIG. 13. Some of the patient groups who exhibit the characteristics of hyposmia, type II.

normal gag reflexes and normal sensation over the hard palate. Since these patients can easily recognize most undiluted vapors, the only way that they can be differentiated from the normal population is through the use of quantitative smell testing.

We can designate several types of patients who fall into the category of type II hyposmia (Fig. 13). Those whose hyposmia has been acquired, as exemplified by studies of our tumor patients carried out prior to surgery, fall into this category. Presumably, the tumor, or the tissue response

associated with the tumor, produced the moderate decrease in olfactory acuity observed. Other patients with trauma to the olfactory area less severe than that described in type I hyposmia would probably have hyposmia of this type. Patients with hypovitaminosis A fall into this category. We have observed two major causes for the hyposmia associated with this syndrome: (1) patients with acanthocytosis or abetalipoproteinemia, and (2) patients with malabsorption syndromes of various types.[14] There is a group of patients with this syndrome without any other associated abnormality. We have called these patients "idiopathic". In addition, there are two major subcategories of this type of hyposmia: one related to hypogonadism of specific types, the other to facial hypoplasia. While these patients fall into the category of type II hyposmia, they exhibit differences from some of the major characteristics described. These patients will be described in detail at a later date.

We have attempted to provide a classification for patients with decreased olfactory acuity. This classification will be useful if it will allow the inclusion of the major categories of patients who possess this disability. As we are still in the process of collecting data concerning the various categories of smell disabilities the value of this classification will await future testing. However, this classification, through the use of responsiveness at primary and accessory areas of olfaction, may have value in and of itself. There may be some elements of organization which will allow formulation of a unifying principle in the concept of primary and accessory olfactory areas.

All patients with hyposmia described responses to vapors localized in what I have called the accessory areas of olfaction. It is almost gratuitous to state that in this area there must be receptors or free nerve endings which are responsive to vapors. How specific these receptors are, and whether they possess any anatomical structure specialized to appreciate vapors is not yet known. What is surprising is not that such a system of accessory areas exists but that it is as widespread and as specific as it appears to be. Using these accessory areas alone, after excision of the olfactory epithelium, patients have been able to recognize some vapors by their odors. These areas are innervated by the 5th, 9th and 10th nerves, a rather diffuse and varied innervation. In the organism living in the primeval ooze of early existence, one of the first drives may well have been the drive towards oxygen; a second may have been the drive of the organism to maintain a constant pH. Wright anecdotally referred to this concept when he paraphrased Descartes' *Cogito, ergo sum* to *olfacio, ergo cogito*.[15] Perhaps one of the most primitive and basic systems for the maintenance of life in pri-

mitive organisms was their chemoreceptive system. Undoubtedly, as the organism developed over the ages, this chemoreceptive system has become more diversified, more specialized. It is of interest to speculate that the sensitivity and widespread innervation of the accessory areas of olfaction might be the indication for the presence in man of a very complex but somewhat primitive chemoreceptor system which underlies the specialized sense of smell. Support for this speculation is not completely lacking. Negus has shown that the entire nasopharynx subserves olfaction in lower species.[16] He reasons teleologically that this is necessary since these animals need to maintain their olfactory sense while feeding. In some mammalian species, such as the deer and the seal, anatomical structures such as the epiglottis developed which allowed separation of the functions of respiration, and its associated olfactory capabilities, from deglutition. Dethier and others have referred to chemoreceptive mechanisms in insects which have separate receptors and which are responsive to vapors.[17]

The studies presented in this paper have allowed us the opportunity to begin to intertwine these homological threads. Perhaps our future studies will allow us to shape the fabric of chemoreception in man.

REFERENCES

1. HENKIN, R. I., GILL, J. R., JR. and BARTTER, F. C.: Studies on taste thresholds in normal man and in patients with adrenal cortical insufficiency: the role of adrenal cortical steroids and of serum sodium concentration. *J. Clin. Invest.* **42,** 727, 1963.
2. HENKIN, R. I., GILL, J. R., JR. and BARTTER, F. C.: Effect of adrenal insufficiency and of corticosteroids on smell threshold. *Clin. Res.* **10,** 400, 1962.
3. KETCHAM, A. S., WILKINS, R. H., VAN BUREN, J. M. and SMITH, R. P.: A combined intracranial facial approach to the paranasal sinuses. *Am. J. Surg.* **106,** 698, 1963.
4. HENKIN, R. I. and KOPIN, I. J.: Abnormalities of taste and smell thresholds in familial dysautonomia: improvement with methacholine. *Life Sciences*, **3,** 1319, 1964.
5. The author wishes to thank DR. SAUL ROSEN, National Institute of Arthritis and Metabolic Diseases, for referring many of these patients to the National Heart Institute.
6. SEMMES, J., WEINSTEIN, S., GHENT, L. and TEUBER, H. L.: *Somatosensory Changes After Penetrating Brain Wounds In Man.* Harvard Univ. Press. Cambridge, Mass. 1960.
7. ALLEN, W. F.: Effect of various inhaled vapors on respiration and blood pressure in anesthetized, unanesthetized, sleeping and anosmic subjects. *Am. J. Physiol.* **88,** 620, 1929.
8. STIRNIMANN, F.: Le goût et l'odorat du nouveau-né; une contribution à la connaissance des réactions du nouveau-né. *Rev. Franc Pediatr.* **12,** 453, 1936.
9. Personal communication. G. di CHIRIO.
10. KALLMANN, F. J., SCHOENFELD, W. A. and BERRERA, S. E.: The genetic aspects of primary eunuchoidism. *Am. J. Mental Deficiency*, **48,** 203, 1944.

11. NOWAKOWSKI, H. and LENZ, W.: Genetic aspects in male hypogonadism. *Rec. Prog. in Horm. Res.* **7,** 53, 1961.
12. PARKES, A. S. and BRUCE, H. M.: Olfactory stimuli in mammalian reproduction, *Science* **134,** 1049, 1961.
13. HENKIN, R. I.: Hypogonadism associated with familial hyposmia. *Clin. Res.* **13,** 244, 1965.
14. These patients have been studied in association with Dr. LEONARD LASTER, National Institute of Arthritis and Metabolic Diseases, Bethesda, Maryland.
15. WRIGHT, R. H.: *The Science of Smell.* Basic Books, Inc., New York, 1964.
16. NEGUS, V. E.: *The Comparative Anatomy and Physiology of the Larynx.* Grune and Stratton, Inc., New York, 1849.
17. DETHIER, V. G.: *The Physiology of Insect Senses.* Wiley, New York, 1963.

A COMPARATIVE STUDY OF NEURAL AND PSYCHOPHYSICAL RESPONSES TO GUSTATORY STIMULI

G. BORG, H. DIAMANT, B. OAKLEY, L. STRÖM and Y. ZOTTERMAN

Department of Otorhinolaryngology Umeå and the Department of Physiology, Veterinärhögskolan, Stockholm.

IN A previous communication to the First International Symposium on Olfaction and Taste in Stockholm 1962, Diamant *et al.* (1963) gave a report on a study of the summated integrated electrical response from the chorda tympani to the application of various sapid solutions to the tongue of patients. These experiments were carried out in a screened operation room at the Ear Clinic of Karolinska Sjukhuset during operations undertaken in order to mobilize the stapes. These earlier investigations gave valuable information about the relation between the strength of the gustatory stimulus and the recorded summated response from the human chorda tympani to various taste solutions.

Working on human subjects, however, offers the possibility of collecting information not only of the relationship between the gustatory stimulus and the peripheral neural response, but also about the relation between the neural and the psychophysical responses.

This report presents the results of such combined investigations made at the Department of Otorhinolaryngology at Umeå University since 1963, when one of us (H.D.) was appointed the head of that clinic. Most of the results were recently printed in Acta Physiologica Scandinavica (Diamant *et al.* 1965) and will be discussed here together with some more recent data.

METHODS

Subjects. In all, 12 cases of otosclerosis were examined at Umeå in January, April and October 1964 and in February 1965. Chemical solutions used were described recently (Diamant *et al.* 1965).

Neural recording. The special precautions to be taken in an unscreened operation room in a big hospital and a description of the summator circuit used have also been previously described (Diamant *et al.* 1965).

Psychophysical tests. The tests applied for the first 6 patients (in 1964) were described in a previous report (Diamant *et al.* 1965). In the more recent experiments ratio scaling methods were used (Ekman 1961). These methods require that the subjects can handle figures and make ratio estimations. They also had to make estimations of surfaces of different size so that we could screen out those patients who obviously could not make ratio estimations. Two patients of six were thus left for psychophysical experiments.

Two days before the operation a psychophysical taste experiment with NaCl was carried out on both patients. The same stimuli and the same order of presentation were used in the electrophysiological experiments. As a psychophysical method the "magnitude estimation" method was used. In the first series 0·12 M and in the second 0·08 M were used as the standard and called "10". The stimuli were presented in pairs, the standard with one comparative stimulus.

RESULTS

Of 12 patients tested in these experiments satisfactory neural responses from the chorda tympani were obtained from 8 cases. In two cases we did not obtain any response at all from the nerve, and in the other two cases the neural responses were too weak or there were too many disturbances of various kinds, preventing a proper analysis of the records.

The summated chorda tympani nerve response to a 0·2 M NaCl solution will be seen in Fig. 1. The decline in neural activity in response to a continuous flow of salt solution over the tongue for 3 minutes may be seen in the records *ABC* from 3 different subjects. The initial large response to the application of the salt solution is indicated by an arrow, while the application of distilled water is indicated by a dot above the records. The responses to water here were due to cooling and mechanical stimulation. In patient *A* the response was 95 per cent adapted within 50 sec. In addition to the three records shown here, the adaptation of the salt response in patient 4 and 6 was very similar to that shown in *A* and *C*. There is a very distinct contrast between the records of human neural adaptation and that of record *D* which is taken from the rat's chorda tympani. In the latter record the response declines very slowly over a 3 min period and little or

no further decline in amplitude is seen (Beidler 1953, Zotterman 1956). Patient no. 3, whose neural response is seen in record *C*, indicated in the psychophysical test that the salt taste disappeared after 90 sec. Patient no. 4 indicated that he could no longer taste salt after 79 sec, which corresponded to a 95 per cent reduction in the magnitude of his neural response.

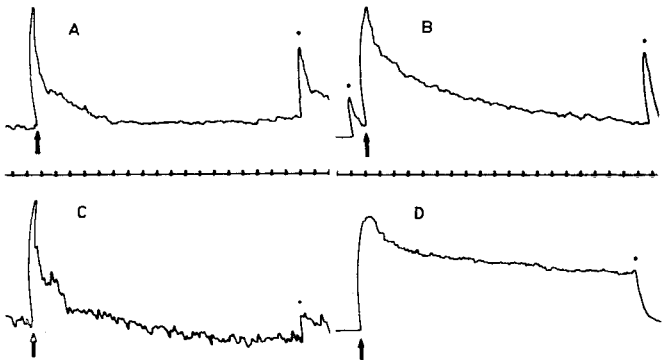


FIG. 1. The summated chorda tympani response to a continuous 3 min flow of 0·2 M NaCl. *A*, *B*, *C* are human responses for patients no. 1, 2, 3 respectively and *D* is a rat response. Dots indicate response during application of distilled water: arrows onset of salt. Tape recorded data processed under identical conditions with rise and fall time constants of 1·5 sec. The tape recorder was off at beginning of *B*. Time base in 10 sec intervals. (From Diamant *et al.* 1965.)

Cameron (1947) has shown that there are rather substantial individual differences in the gustatory sensitivity to sweet tasting substances. Thus it is of little value to compare the nerve responses of individual patients with the psychophysical responses obtained from other subjects. In our studies we were able to obtain both psychophysical magnitude estimates of the relative sweetness of different chemicals and summated electrical chorda tympani responses to these same chemicals (Table 1). The values in each column were rounded off to the nearest 5 per cent and are relative to the response to 0·5 M sucrose, which has been set at 100. The psychophysical reports are means of two determinations, while the neural values are based upon a single determination. The correspondance between the psychophysical and the neural data seems quite good, especially for the sugars. The artificial sweeteners, saccharin and cyclamate have qualitatively different tastes from the sugars and this may have affected the judgements of the sweetness. The psychophysical method used with patient no. 4 produced the better agreement.

TABLE 1. COMPARISON OF PSYCHOPHYSICAL AND NEURAL RESPONSE TO SWEET TASTING SUBSTANCES. THE VALUES IN EACH COLUMN ARE RELATIVE TO 0·5 M SUCROSE SET AT 100. THE MAXIMUM HEIGHT OF THE SUMMATOR RECORD WAS MEASURED

Stimulus	Patient 3		Patient 4	
	Psy.	Neur.	Psy.	Neur.
0·5 M sucrose	100	100	100	100
0·5 M fructose	100	100	80	80
0·5 M maltose	—	40	75	60
0·5 M galactose	40	45	45	40
0·5 M lactose	45	45	30	30
0·5 M glucose	25	45	35	40
0·004 M Na saccharin	100	65	125	105
0·03 M Na cyclamate	55	80	115	100

TABLE 2. INDIVIDUAL DIFFERENCES IN THE CHORDA TYMPANI DISCHARGE. THE VALUES IN EACH COLUMN ARE RELATIVE TO 0·5 M SUCROSE SET AT 100. THE MAXIMUM HEIGHT OF THE SUMMATOR RECORD WAS MEASURED

Stimulus	Patient no.			
	1	2	3	4
0·5 M sucrose	100	100	100	100
0·2 M NaCl	105	100	150	50
0·02 M citric acid	155	195	60	100
0·004 M Na saccharin	50	70	65	105
0·03 M Na cyclamate	90	120	80	100

It was also found that there is significant individual variation in the response of the chorda tympani to different sapid substances. A comparison of the neural response of patients 3 and 4 in Table 1 gives an indication of the individual variation for the sugars and artificial sweeteners. Diamant *et al.* (1963) obtained still another order of the neural responses in one patient. In Table 2 individual differences for other chemicals are presented relative to the response to a 0·5 M sucrose solution which has been given the value 100. It is quite obvious that there is a wide variation in responsiveness to the different chemicals. For example, patient no. 4 had a poor response to 0·2 M NaCl, and patient no. 2 had a very good response to 0·02 M citric acid.

The result of the group experiment with 16 normal subjects is seen in Fig. 2. A straight line may be very nicely adjusted to the psychophysical responses to citric acid when plotted in log–log coordinates. A simple power function, $R=cM^n$, with $n=0{\cdot}67$ describes the relation. The adjustment to

the salt values is not so good, but the relation may also be described with a power function of the form $R=\alpha+cM^n$, with a rather high α-value and $n=1{\cdot}0$.

The psychophysical salt experiment on the two patients gave about the same result as the group experiment. The dispersion of the values was, however, rather great and the individual functions rather uncertain. The

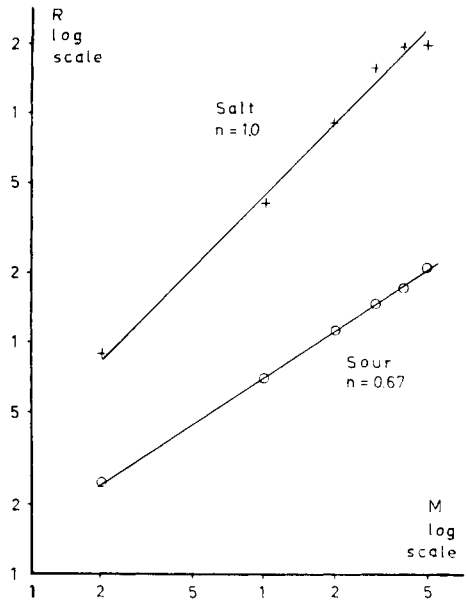


FIG. 2. Result of a group psychophysical experiment. Relative subjective intensity plotted against molarity of stimulus in log–log scale.

agreement between the patients' functions for the psychophysical and the neural responses for salt stimuli is not so good. The neural activity may rather well be described by a Fechnerian log-function.

In Fig. 3 both the relative psychophysical and neural responses to citric acid are plotted in a log–log diagram. Straight lines may be adjusted to the values, i.e. a power function may describe the relations. (A Fechnerian log-function does, however, better describe the variation in neural activity.) If as a first rough approximation we describe both the neural and the psychophysical responses with power functions of the simple form: $R=cM^n$, we find an astonishingly good agreement. The good correspondence is also seen in Fig. 4, where the psychophysical estimates are plotted against the neural responses. The exponent of the psychophysical function is $n_R=0{\cdot}5$ and of the neurophysical exactly the same, $n_N=0{\cdot}5$. This very

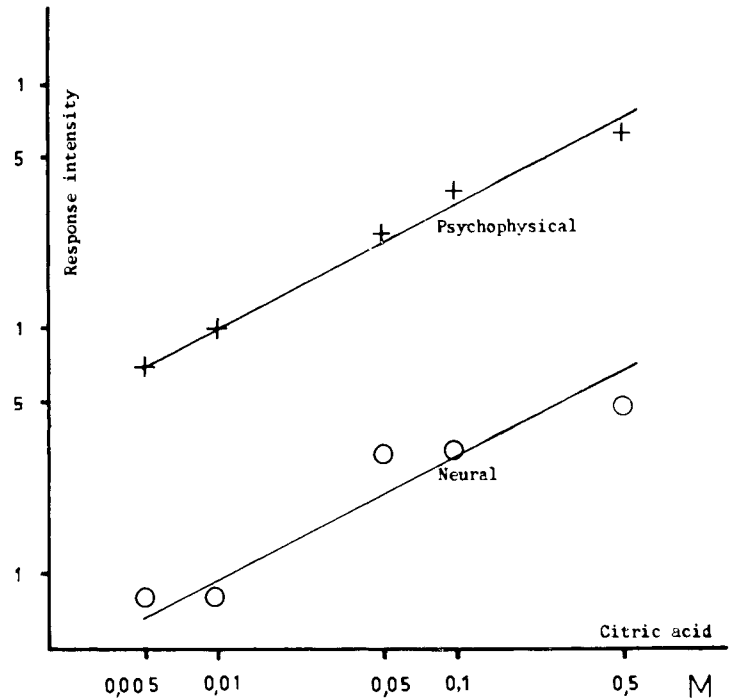


FIG. 3. Graph for one patient showing subjective intensity and neural response plotted against molarity of citric acid in log–log scale.

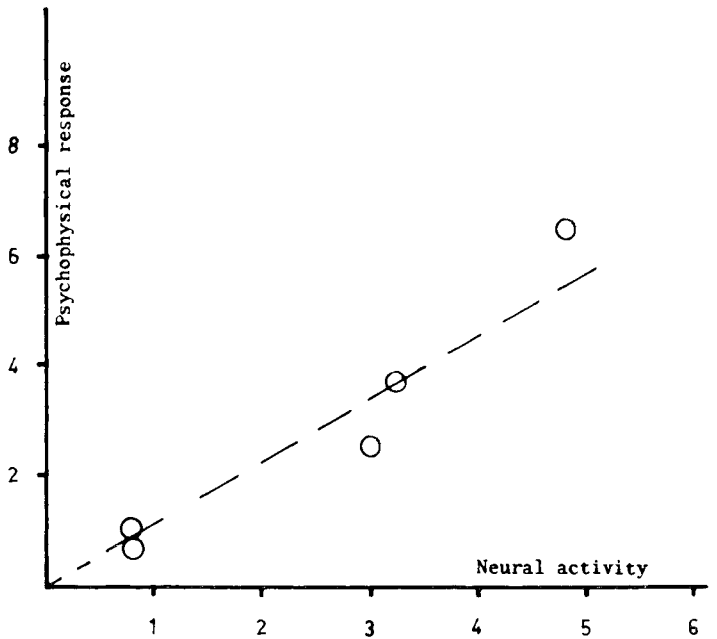


FIG. 4. Relative subjective intensity in relation to relative neural activity for one patient. Stimulus: citric acid.

good correspondance must of course be interpreted with great caution but indicates that further research on this line may be of interest.

In textbooks it is maintained that extract of the Indian herb *Gymnema sylvestre* painted on the tongue abolishes the sweet and bitter taste. Figure 5 shows the responses from the human chorda tympani to salt, sucrose, citric acid, quinine and saccharin before and after treatment with gymnema extracts. We tested five patients in this fashion and found that only the response to sweet tasting substances was affected; viz., sucrose,

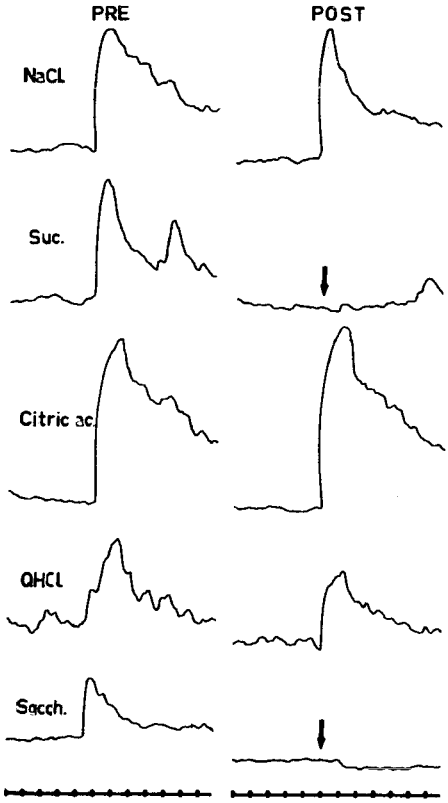


FIG. 5. Elimination of the neural response to sweeteners by *Gymnema* extract. The stimuli were presented in the order shown, before and after treatment of the tongue for 90 sec with 1 per cent *Gymnema* extract. The arrows show the time at which saccharin and sucrose were applied. The dot in the sucrose record indicates a temperature response to cool distilled water. Time marks in seconds. The responses to quinine are displayed at a somewhat higher amplification. The "pre" response to saccharin was recorded earlier in the experiment. The concentrations were 0·2 M NaCl, 0·5 M sucrose, 0·02 M citric acid, 0·002 M quinine hydrochloride and 0·004 M sodium saccharin. Data from patient no. 1. (From Diamant *et al.* 1965.)

fructose, saccharin and cyclamate. There were no significant effects (i.e. greater than 10 per cent change) upon responses to 0·2 M NaCl, 0·002 M citric acid, or quinine (0·002 M and 0·005 M quinine hydrochloride or 0·008 M quinine sulphate). The main result from the psychophysical experiments was the same; namely, that the sweet taste was eliminated. No subject reported a decrease in bitter taste (quinine).

Ethyl alcohol needs concentrations above 1·0 M to elicit good nerve responses (Fig. 6). These neural responses were positively accelerating func-

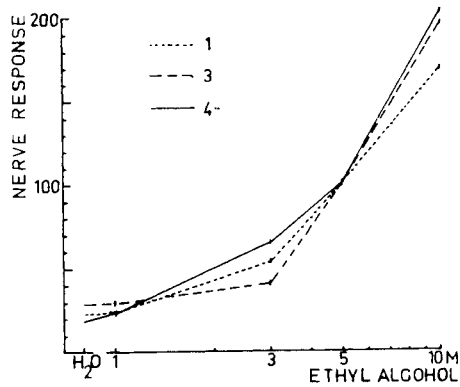


FIG. 6. The maximum amplitude of the summated chorda tympani response as a function of ethyl alcohol concentration for 3 patients. The ordinate gives the relative nerve response, where the response of each patient to 5·0 M alcohol has been set at 100. (From Diamant *et al.* 1965).

tions of $\log_{10}$ molar concentration, very similar to the curves of the summated responses from the chorda tympani of the cat and the dog recently presented by Hellekant (1965). These curves show a course differing completely from the usual linear trend with other taste solutions (Diamant *et al.* 1963), which is readily explained by the fact that alcohol at higher concentrations penetrating the mucous membrane of the tongue stimulates all kinds of sensory nerve endings (Hellekant 1965). Use of the strong 5 M and 10 M solutions in 5 out of 6 cases resulted in a slowly rising negatively accelerated, summated response which took 10 or 20 sec to reach its maximum height. Concentrations greater than 3 M produce a smarting or burning sensation (Diamant *et al.* 1963).

DISCUSSION

Our records seen in Fig. 1 suggest that complete adaptation to zero of the neural response to sodium chloride takes place in man in contrast to the rat, where the gustatory receptors are in continuous activity in response to the low salt concentration of the saliva. In this animal you have to wash the tongue with distilled water to abolish the spontaneous activity of the gustatory fibres (Zotterman 1956).

Bujas (1953) has studied psychophysical salt adaptation in two subjects. Using 0·15 M NaCl to stimulate a tongue area 1 cm in diameter, he found complete adaptation in 50 and 54 sec. These values are of the same order of magnitude as those given by our patients in psychophysical tests when the whole tongue was stimulated with 0·2 M NaCl (79, 90 and 122 sec). The shape of Bujas's psychophysical adaptation curves to 0·15 M NaCl are very similar in form to our neural adaptation records in Fig. 2, but there are too many unknown factors to permit a precise and quantitative comparison of the two sets of curves.

As we found a reasonable correspondence between the neural and psychophysical records for the time necessary for complete adaptation we may conclude that the rapid and complete salt adaptation reported by our subjects can be accounted for by the declined impulse traffic in the gustatory nerve fibres. There is no need to postulate the existence of central adaptation mechanisms.

That when applied to the tongue extracts of *Gymnema sylvestre* will temporarily eliminate the ability to taste sweet substances was reported by Shore as early as 1892. Andersson *et al.* (1950) working on dogs and Hangstrom (1959) using hamsters, demonstrated that this drug will suppress the chorda tympani response to sucrose while leaving the response to NaCl unaffected. In our recent research we found that the same *Gymnema* extract eliminates both the neural and the psychophysical responses to sweet tasting substances in the same human subject. Shore and all textbooks have stated that *Gymnema* extracts depressed the sensitivity to bitter substances. However, in our experiments this extract had no clear effect on the sensitivity to suprathreshold concentrations of quinine either in psychophysical tests and neural recording sessions with the patients, or in psychophysical tests with three other normal subjects. We are not able to explain this discrepancy.

The existence in the monkey of gustatory fibres responding strictly specifically to (1) sweet-tasting substances, (2) salt and (3) acid, was reported by Gordon *et al.* (1959). Gustatory fibres responding to sugars were also found

to be excited by saccharin in contrast to the behaviour of the "sweet" fibres of the dog (Andersson *et al.* 1950) in which animal saccharin elicited a response only from quinine sensitive fibres. In Fig. 7, from the paper by

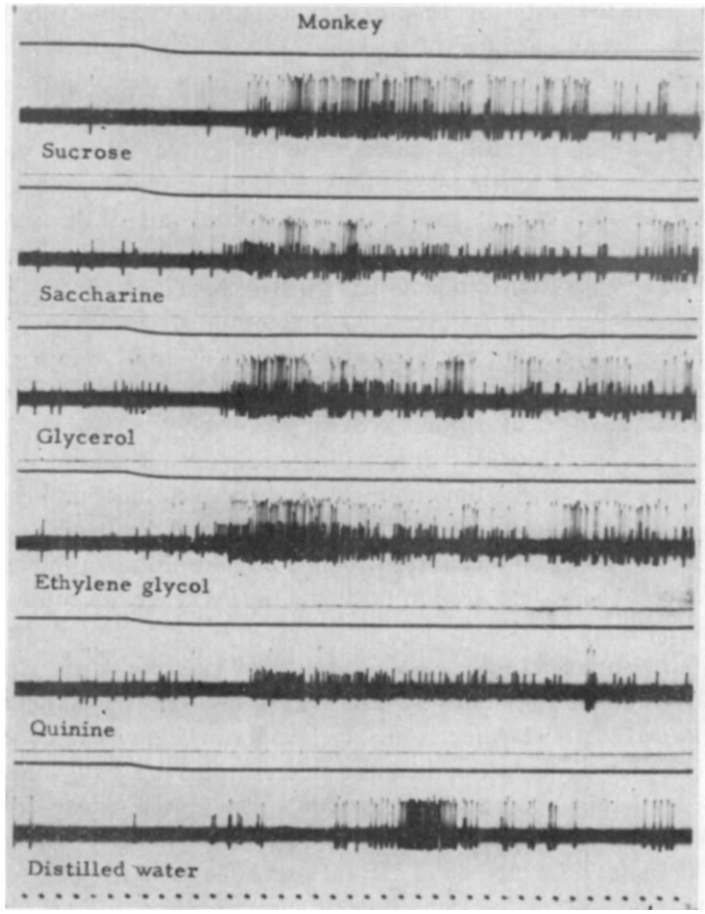


FIG. 7. Records from a small strend of the chorda tympani of the rhesus monkey. The preparation contained few active fibres. Note the large spikes, produced by all sweet tasting substances (and dist. water) and also the smaller spikes appearing in response to quinine and to saccharin, glycerol and ethylene glycol but very sparsely or not at all to sucrose and to water. All solutions were made up in Ringer's solution in order to abolish the response to water. Time 10 per sec. (From Gordon *et al.* 1959.)

Gordon *et al.*, it will be seen that in the monkey saccharin, glycerol and ethylene glycol stimulate, in addition to the "sweet" fibre (large spikes),

also the fibres responding to quinine (small spikes), while sucrose produces a response of the "sweet" fibre alone with only a very few small spikes.

In our previous report (Diamant *et al.* 1965) it was emphasized that the correspondence for the sugars between the summated neural response and the psychophysical report suggests that sweetness is determined by the discharge from all of the active gustatory fibres in the nerve, and not only from specific "sweet" fibres. The fact that *Gymnema* extract totally abolishes the effect of sucrose does not solve the problem of specificity. It may as well wipe out the sensitivity of the sweet receptive sites of the multi-quality fibres as that of the specific "sweet" fibres.

It seems reasonable, however, to assume that man, like monkey, possesses a certain number of strictly specific gustatory fibres which could serve as specific switchers in the central nervous system, directing the impulse traffic arriving from the multi-quality fibres into specific central channels inhibiting or depressing the information sent via other pathways. It is, however, futile to enter into more detailed discussions of this problem until we have obtained recordings from individual gustatory nerve fibres in man and from cortical gustatory units.

SUMMARY

The neural response to the application of sapid solutions has been recorded from the middle ear portion of the chorda tympani nerve of otosclerotic patients from whom psychophysical responses to taste stimuli were obtained prior to the operation.

The impulse traffic in the human chorda tympani produced by a constant flow of 0·2 M NaCl over the tongue adapts to zero. The time required agrees with the psychophysical reports. Considerable individual variations were observed in the neural response to different sugars as well as in the patients' estimates of the sweetness of different sugars. The psychophysical measurements corresponded closely with the magnitude of the nerve response to sugars and citric acid.

Gymnema extract abolished both the sweet sensation and the nerve response to sweet tasting solutions but did not affect to any marked degree the responses to quinine or other gustatory stimuli. There were large individual differences in the relative size of the neural responses to different taste stimuli.

This investigation has been supported by grants from Svenska Maltdrycksforskningsinstitutet, the Swedish Medical Research Council and the Swedish Council for Social Science Research.

REFERENCES

ANDERSSON, B., LANDGREN, S., OLSSON, L. and ZOTTERMAN, Y. 1950. The sweet taste fibres of the dog. *Acta physiol. scand.* **21,** 105–119.

BEIDLER, L. M. 1953. Properties of chemoreceptors of tongue of rat. *J. Neurophysiol.* **16,** 595–607.

BUJAS, Z. 1953. L'adaptation gustative et son mécanisme. *Acta Inst. Psychol.* Univ. Zagreb. **17,** 1–10.

CAMERON, A. T. 1947. The taste sense and the relative sweetness of sugars and other sweet substances. Scientific Rep. Ser. No. 9. New York, Sugar Research Foundation.

DIAMANT, H., FUNAKOSHI, M., STRÖM, L. and ZOTTERMAN, Y. 1963. Electrophysiological studies on human taste nerves. *Olfaction and Taste.* Editor Y. ZOTTERMAN, Pergamon Press, Oxford. 193–203.

DIAMANT, H., OAKLEY, B., STRÖM, L., WELLS, C. and ZOTTERMAN, Y. 1965. A comparison of neural and psychophysical responses to taste stimuli in man. *Acta physiol. scand.* **64,** 67–74.

EKMAN, G. 1961. Methodological note on scales of gustatory intensity. *Scand. J. Psychol.* **2,** 185–190.

GORDON, G., KITCHELL, R., STRÖM, L. and ZOTTERMAN, Y. 1959. The response pattern of taste fibres in the chorda tympani of the monkey. *Acta physiol scand.* **46,** 119–132.

HANGSTROM, E. C., quoted in PFAFFMANN, C. 1959. The sense of taste. In FIELD, *J. Ed. Handbook of Physiology*, Section 1: Neurophysiology, Vol. 1. Washington, D.C. *Amer. Physiol. Soc.* p. 520.

HELLEKANT, G. 1965. The effect of ethyl alcohol on non-gustatory receptors of the tongue of the cat. *Acta physiol. scand.* **65,** 243–250.

SHORE, L. E. 1892. A contribution to our knowledge of taste sensations. *J. Physiol. (Lond.).* **13,** 191–217.

ZOTTERMAN, Y. 1956. Species differences in the water taste. *Acta physiol. scand.* **37,** 60–70.

MEASUREMENT OF HUMAN GUSTATORY PERCEPTION USING THE PAROTID GLAND SECRETION RATE

HOWARD H. CHAUNCEY*, RALPH P. FELLER and IRA L. SHANNON

Department of Oral Pathology,
Tufts University School of Dental Medicine, Boston, Mass., and
Department of Experimental Dentistry, School of Aerospace Medicine, USAF,
Brooks Air Force Base, Texas

THE unconditioned reflex secretion of fluid by the parotid salivary gland, subsequent to the application of taste stimuli, provides a means for the quantitative assay of human response to gustatory stimuli. Prior observations have indicated the existence of a correlation between secretory response, stimulation frequency, and stimulus concentration.[1, 2] The graphics of this relationship, however, appear to be dependent on the molecular configuration and physical properties of the test substance.[3]

Material herein presented was gathered from a series of investigations designed to study both gustation and the neuromechanisms which control human salivary gland function. The gustatory-salivary reflex was employed to evaluate the interrelationship between structure and stimulation efficacy of acids; to determine some of the kinetics characteristic of acid action; and to delineate the effect of salt and sucrose on acid stimulation of gustatory chemoreceptors.

Parotid saliva samples were obtained by means of vacuum caps.[4] The secretion was collected in 15 ml centrifuge tubes graduated to 0·1 ml. The rate of secretion was determined by measuring the volume secreted in a standard 10-minute time period.

After initial placement of the vacuum cap, a 5-minute acquaintance interval of stimulation was given to each subject. This secretion was considered to be a clearance sample and was discarded. Upon completion of the acquaintance sample, a 10-minute experimental sample was collected. This was followed by a 5-minute rest interval, during which time each subject

* Current address: Dept. Medicine and Surgery Veterans Administration Central Office Washington, D.C.

was given a drink of water. Using this procedure a total of not more than three experimental samples were obtained during a single collection period. All samples were collected preprandial. With each group tested a control series using water was conducted to determine the secretory response due to tactile stimulation. The water, or control, values were subtracted from the experimental data so that only the gustatory response was used in the evaluation.

The nine subjects used in the first study were divided into three groups of three, and the acid solutions were presented in groups of three. The first subject in each group received the acids in the order *ABC*, the second in the order *BCA* and the third in the order *CAB*. Test solutions were applied using wetted cotton applicator sticks. One complete application consisted of running the wetted swab around the lateral edges of the tongue followed by swabbing the entire dorsum of the tongue, from the area delineated by the circumvallate papillae to the tip. Applications were at the rate of 4 times per minute.

Subjective evaluation of the degree of sourness was determined by having the participants swab their tongues in the manner described, using groups of three acids. The subjects were allowed to sample the acids as often as necessary and in any sequence so as to thoroughly evaluate which acid was the most sour, which was intermediate in sourness and which was the least sour. Statistical analysis revealed that the subjective evaluation and the objective parotid flow measurement were significantly correlated for any group of three acids tested.

TABLE 1. RESULT OF 3 SUCCESSIVE APPLICATIONS OF CITRIC ACID*

Sample	A	B	C
Mean flow rate (ml/10 min/gland)	4·78	4·78	4·79

* Acid concentration = 0·067 M.

Initial findings (Table 1) revealed that variations in the group mean flow rate, for samples collected during a single collection period—using a single stimulus—were negligible. Response to five concentrations of citric and malic acids (Table 2) showed that week to week variations in response to test solutions were not statistically significant. Thus, the data indicated that the measurement of secretory response could be used to rank the effect of a large group of acids.

TABLE 2. DUPLICATION OF FLOW RATES FROM PAROTID SALIVA SAMPLES COLLECTED ON DIFFERENT DAYS

Citric acid (M)	Mean flow rate (ml/10 min/gland)		Malic acid (M)	Mean flow rate (ml/10 min/gland)	
	Week I	Week II		Week I	Week II
0·025	2·31	2·40	0·0375	2·95	2·90
0·050	3·89	4·44	0·0750	4·97	5·04
0·075	5·03	5·03	0·1250	6·27	6·26
0·100	6·27	5·65	0·1500	6·80	6·60
0·125	7·65	7·98	0·1875	7·72	8·09

Figure 1 illustrates the result obtained with increasing solution concentration for four organic acids. A plot of concentration *vs.* secretion rate produced curves which were different for each acid. When the hydrogen ion concentration of each solution was plotted against the secretion rate

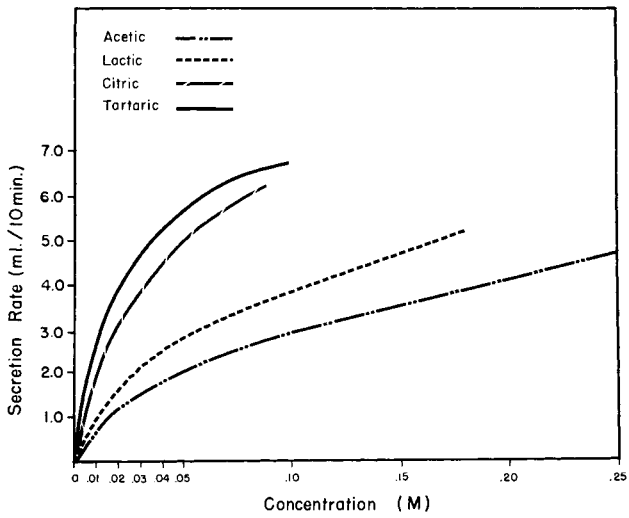


FIG. 1.

(Fig. 2) it was observed that there was a direct linear relationship between these variables. Four distinct lines were obtained, indicating that each acid evoked a definite response pattern. Thus, it was seen that the hydrogen ion is one of the principal factors responsible for triggering the gustatory-salivary reflex. As shown in Fig. 2 a ten-fold increase in hydrogen ion con-

centration, for any of the four acid solutions, produced a seven to twenty-fold increase in the secretion rate.

To determine more accurately the effect of the anion, a series of experiments was carried out using 17 organic acids. The first phase consisted of measuring the parotid secretion rate response when a constant solution pH of 2·60±0·10 was maintained. The results (Table 3) were ranked according to the secretion rate that each acid produced. It was noted that most

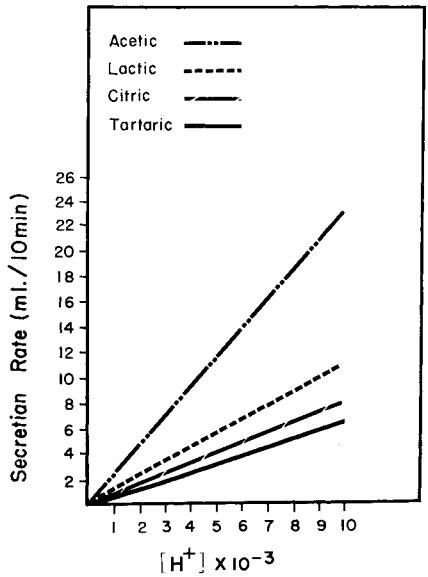


FIG. 2.

of the acids elicited different responses, indicating variation in the degree of chemoreceptor stimulation.

When the data obtained with constant pH were categorized by molecular configuration (Table 4) it became possible to evaluate the effect of carbon chain length, carbon to carbon double bonds, number of carboxyl groups, and polar groupings. With the monocarboxylic acids increasing, the carbon chain length of the anion decreased the stimulating action of the acid. The opposite was noted for dicarboxylic acids. The reason for this became apparent after comparing the effect produced by mono, di, and tricarbocylic acids. Acetic acid was more effective than all the dicarboxylic acids, except for glutaric acid. The presence of a second carboxyl group apparently reduced the stimulatory effect of the acids, and only when a sufficient number of intervening methyl groups were present was the stimulatory action returned to maximum activity. Thus, acetic and propionic acids were

TABLE 3. EFFECT OF CONSTANT HYDROGEN ION CONCENTRATION*

Acid	Total concentration† (M)	Mean flow rate (ml/ 10 min/gland)
Acetic	0·20	4·86
Glutaric	0·10	4·85
Succinic	0·10	4·05
Crotonic	0·20	3·13
Tricarballylic	0·113	3·07
Isobutyric	0·20	3·04
Acrylic	0·113	3·03
Propionic	0·20	2·87
Butyric	0·20	2·24
Glycolic	0·0429	1·72
Malic	0·0163	1·23
Citric	0·00756	1·00
Malonic	0·00426	0·45
Lactic	0·020	0·39
Pyruvic	0·00604	0·37
Oxalic	0·0027	0·35
Tartaric	0·0058	0·21

* $[H^+] = 2{\cdot}54 \times 10^{-3} \pm 0{\cdot}25 \times 10^{-3}$ M.
† Undissociated acid concentrations = total — $[H^+]$.

more effective than their dicarboxylic counterparts — oxalic and malonic acids. Only in the case of butyric and succinic acids was the dicarboxylic acid more effective than the monocarboxylic acid. This apparently was due to the inhibitory action of carbon chain length in monocarboxylic acids, while carbon chain length had an activator effect in the dicarboxylic acids.

The presence of a third carboxyl group also had an inhibitory effect. This was noted when glutaric and tricarballylic acids were compared. In the second column of Table 4 it can be seen that the presence of polar groups such as hydroxyl and carbonyl groups appear to have an inhibitory effect.

The hypothesis that these differences in effect are indeed due to structural differences is supported when Table 4 is re-examined for concentration effect. There are four acid solutions — acetic, propionic, butyric, and crotonic — which have a concentration of 0·2 M, are at the same pH, and yet they evoke different gustatory responses. Similarly, there are 4 acids — succinic, glutaric, tricarballylic and acrylic — which were used at a concentration of 0·1 M, $\pm$0·01 M, yet three of these evoked markedly different gustatory responses.

TABLE 4. EFFECT OF STRUCTURE RELATIVE TO CONSTANT HYDROGEN ION CONCENTRATION

Acid	Structure	Conc. (M)	Flow rate
	Carbon length chain		
Acetic	CH_3COOH	0·20	4·86
Propionic	CH_3CH_2COOH	0·20	2·87
Butyric	$CH_3CH_2CH_2COOH$	0·20	2·24
Oxalic	$HOOCCOOH$	0·0027	0·35
Malonic	$HOOCCH_2COOH$	0·0043	0·45
Succinic	$HOOCCH_2CH_2COOH$	0·10	4·05
Glutaric	$HOOCCH_2CH_2CH_2COOH$	0·10	4·85
	Carboxyl groups		
Acetic	CH_3COOH	0·20	4·86
Oxalic	$HOOCCOOH$	0·0027	0·35
Propionic	CH_3CH_2COOH	0·20	2·87
Malonic	$HOOCCH_2COOH$	0·0043	0·45
Butyric	$CH_3CH_2CH_2COOH$	0·20	2·24
Succinic	$HOOCCH_2CH_2COOH$	0·10	4·05
Glutaric	$HOOCCH_2CH_2CH_2COOH$	0·10	4·85
Tricarballyic	$HOOCCH_2CH(COOH)CH_2COOH$	0·113	3·07
	Carbon–carbon double bond		
Propionic	CH_3CH_2COOH	0·20	2·87
Acrylic	$CH_2{=}CHCOOH$	0·113	3·03
Butyric	$CH_3CH_2CH_2COOH$	0·20	2·24
Crotonic	$CH_3CH{=}CHCOOH$	0·20	3·13
	Polar groups		
Propionic	CH_3CH_2COOH	0·20	2·87
Lactic	$CH_3CHOHCOOH$	0·020	0·39
Pyruvic	$CH_3COCOOH$	0·0060	0·37
Acetic	CH_3COOH	0·20	4·86
Glycolic	$HOCH_2COOH$	0·0429	1·72
Succinic	$HOOCCH_2CH_2COOH$	0·10	4·05
Malic	$HOOCCHOHCH_2COOH$	0·0163	1·23
Tartaric	$HOOCCHOHCHOHCOOH$	0·0058	0·21
Tricarballylic	$HOOCCH_2CH(COOH)CH_2COOH$	0·113	3·07
Citric	$HOOCCH_2HOC(COOH)CH_2COOH$	0·0076	1·00

To further ascertain that these effects were actually due to the structure of the anions and not a result of the concentration of undissociated acid in solution, the same series of acids was tested maintaining a constant concentration of 0·1 M ±0·01 M undissociated acid. The rank order (Table 5) was different from that found when constant hydrogen ion solutions were tested. The order was apparently due to two affecting variables, hydrogen ion concentration and structure.

TABLE 5. EFFECT OF CONSTANT UNDISSOCIATED ACID CONCENTRATION*

Acid	Hydrogen ion concentration	Mean flow rate (ml/ 10 min/gland)
Oxalic	$5{\cdot}45\times10^{-2}$	8·00
Malonic	$1{\cdot}58\times10^{-2}$	7·13
Citric	$1{\cdot}00\times10^{-2}$	6·70
Tricarballylic	$6{\cdot}03\times10^{-3}$	5·72
Succinic	$3{\cdot}16\times10^{-3}$	5·31
Tartaric	$1{\cdot}01\times10^{-2}$	4·88
Pyruvic	$2{\cdot}04\times10^{-2}$	4·23
Malic	$6{\cdot}61\times10^{-3}$	4·18
Glycolic	$4{\cdot}47\times10^{-3}$	3·92
Glutaric	$2{\cdot}40\times10^{-3}$	3·84
Acetic	$1{\cdot}48\times10^{-3}$	3·12
Lactic	$5{\cdot}25\times10^{-3}$	2·51
Acrylic	$3{\cdot}02\times10^{-3}$	2·31
Isobutyric	$1{\cdot}32\times10^{-3}$	1·93
Propionic	$1{\cdot}02\times10^{-3}$	1·83
Crotonic	$1{\cdot}66\times10^{-3}$	1·33
Butyric	$1{\cdot}41\times10^{-3}$	1·28

* Undissociated acid concentration = 0·100±0·010 M.

When the data was charted into the categories previously used (Table 6), it was noted that in almost all categories the hydrogen ion concentration was the governing factor, while anion structure was of secondary importance. For the monocarboxylic acids the pH or hydrogen ion concentrations were almost identical, but increasing carbon chain length decreased the effectiveness of the acid. For the dicarboxylic acid series — oxalic through glutaric acid—the hydrogen ion concentration was important and was the governing factor. However, even though the hydrogen ion concentration of the oxalic acid solution was more than ten-fold greater than the glutaric acid solution there was only slightly more than a two-fold differ-

TABLE 6. EFFECT OF STRUCTURE—CONSTANT UNDISSOCIATED ACID CONCENTRATION*

Acid	$[H^+]$	Flow rate
Carbon chain length		
Acetic	$1{\cdot}48\times10^{-2}$ M	3·12
Propionic	$1{\cdot}02\times10^{-3}$	1·83
Butyric	$1{\cdot}41\times10^{-3}$	1·28
Oxalic	$5{\cdot}45\times10^{-2}$	8·00
Malonic	$1{\cdot}58\times10^{-2}$	7·13
Succinic	$3{\cdot}16\times10^{-3}$	5·31
Glutaric	$2{\cdot}40\times10^{-3}$	3·84
Carbon—carbon double bond		
Propionic	$1{\cdot}02\times10^{-3}$	1·83
Acrylic	$3{\cdot}02\times10^{-3}$	2·31
Butyric	$1{\cdot}41\times10^{-3}$	1·28
Crotonic	$1{\cdot}66\times10^{-3}$	1·33
Carboxyl groups		
Acetic	$1{\cdot}48\times10^{-3}$	3·12
Oxalic	$5{\cdot}45\times10^{-3}$	8·00
Propionic	$1{\cdot}02\times10^{-3}$	1·83
Malonic	$1{\cdot}58\times10^{-2}$	3·84
Butyric	$1{\cdot}41\times10^{-3}$	1·28
Succinic	$3{\cdot}16\times10^{-3}$	5·31
Glutaric	$2{\cdot}40\times10^{-3}$	3·84
Tricarballylic	$6{\cdot}03\times10^{-3}$	5·72
Polar groups		
Propionic	$1{\cdot}02\times10^{-3}$	1·83
Lactic	$5{\cdot}25\times10^{-3}$	2·51
Pyruvic	$2{\cdot}04\times10^{-2}$	4·23
Acetic	$1{\cdot}48\times10^{-3}$	3·12
Glycolic	$4{\cdot}47\times10^{-3}$	3·92
Succinic	$3{\cdot}16\times10^{-3}$	5·31
Malic	$6{\cdot}61\times10^{-3}$	4·18
Tartaric	$1{\cdot}01\times10^{-2}$	4·88
Tricarballylic	$6{\cdot}03\times10^{-3}$	5·72
Citric	$1{\cdot}00\times10^{-2}$	6·70

ence in secretion rate, instead of the large increase which would be obtained if a single acid were employed.

Similar results were noted when acetic and oxalic acids, propionic and pyruvic acids, and succinic and tartaric acids were compared. This again

appeared to indicate that the effect was due to the structural configuration of the anion.

Thus, it would seem that the hydrogen ion concentration and the molecular structure of the anion both play an important role in determining the degree of response elicited.

A study was subsequently initiated to obtain a partial kinetic characterization of acid action on the gustatory chemoreceptors. Solutions of citric and malic acids were employed. A constant application rate was maintained during each weekly — five-day — experimental period, while the solution concentrations increased daily from 0·5 to 2·5 g/100 ml. The application rates used during the six weekly experimental periods were 1, 2, 4, 6, 8 and 12 swabs/min. The eight human subjects utilized in this study were divided into two groups. Group one had an initial application frequency of 1 swab/min and changed their application rate in ascending order. Group two started with 12 swabs/min and changed their swabbing rate in descending order.

This study was also concerned with the effect of varying solution temperatures on the reflex secretion of parotid saliva. Citric and malic acids, at a concentration of 1·5 g/100 ml, were applied at a rate of 4 swabs/min in

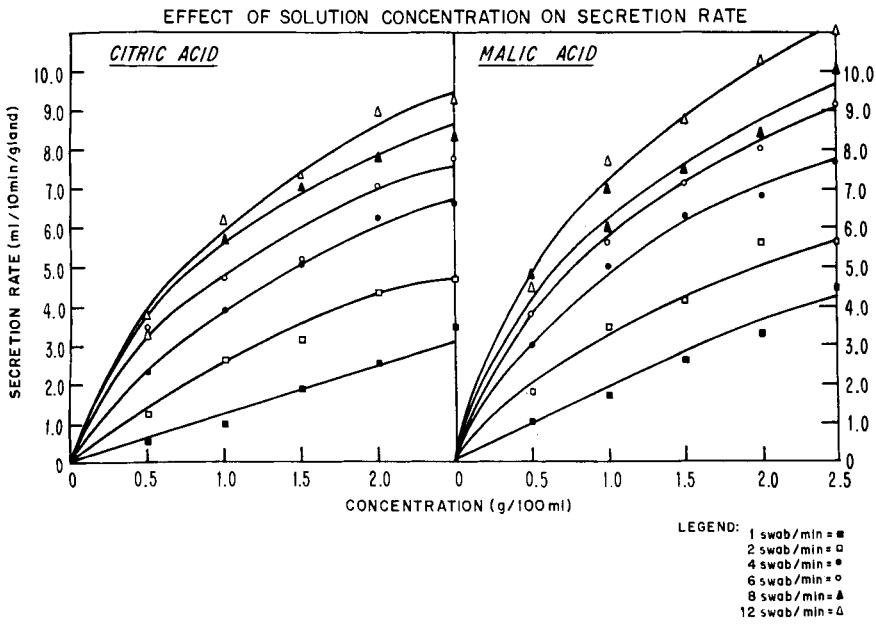


FIG. 3.

the manner previously described. The temperatures studies were 5, 15, 25, 35 and 45°C.

Concentrations of the acid solutions employed were selected to fall in a range between the threshold values and levels compatible for continuous use without damaging the oral mucosa. The maximal rates of application

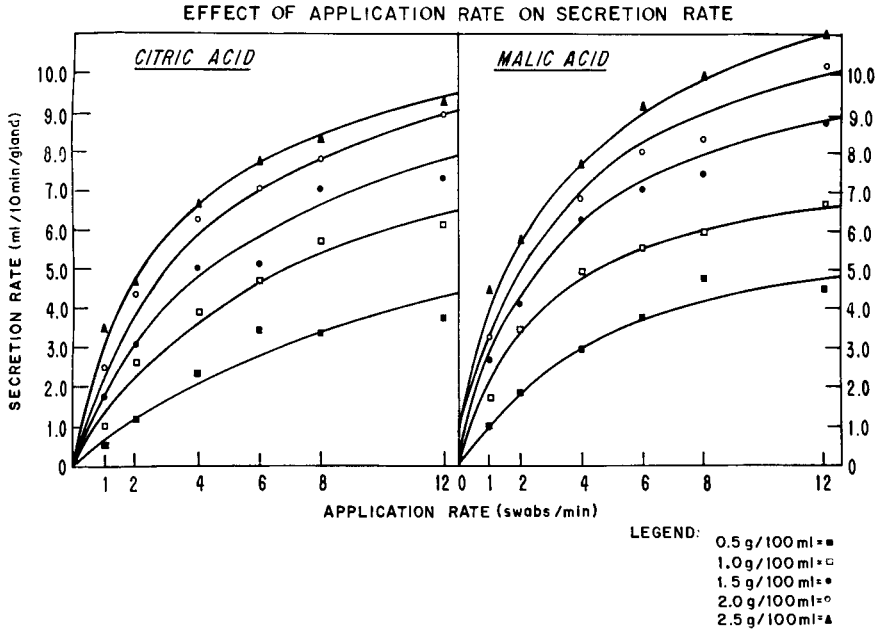


FIG. 4.

were limited by the inability of the subject to efficiently perform the swabbing technique.

Figure 3, a plot of the acid concentration vs. the secretion rates, illustrates that the parotid gland response has a curvilinear relationship to solution concentration. Similarly, a plot of application rate vs. secretion rate showed a curvilinear relationship between gland response and application rate (Fig. 4). When the hydrogen ion concentration for each acid solution was plotted against secretion rate a direct linear relationship was observed (Fig. 5).

Thus, this data confirmed that application rate has a curvilinear relationship to parotid gland secretion rate. In addition, it corroborated the work showing that there was a curvilinear relationship between acid concentration and secretion rate. The latter was true for five of the six appli-

cation rates. Only at the lowest rate (1 swab/min) was there an indication of a linear relationship between concentration and secretion rate. The results also confirm that there was a direct linear relationship between hydrogen ion concentration and secretion rate.

The results of the temperature study (Table 7) showed that the response elicited at 5°C was significantly lower ($P<0{\cdot}05$) than the other values. The rates obtained at the four other temperatures were not significantly different.

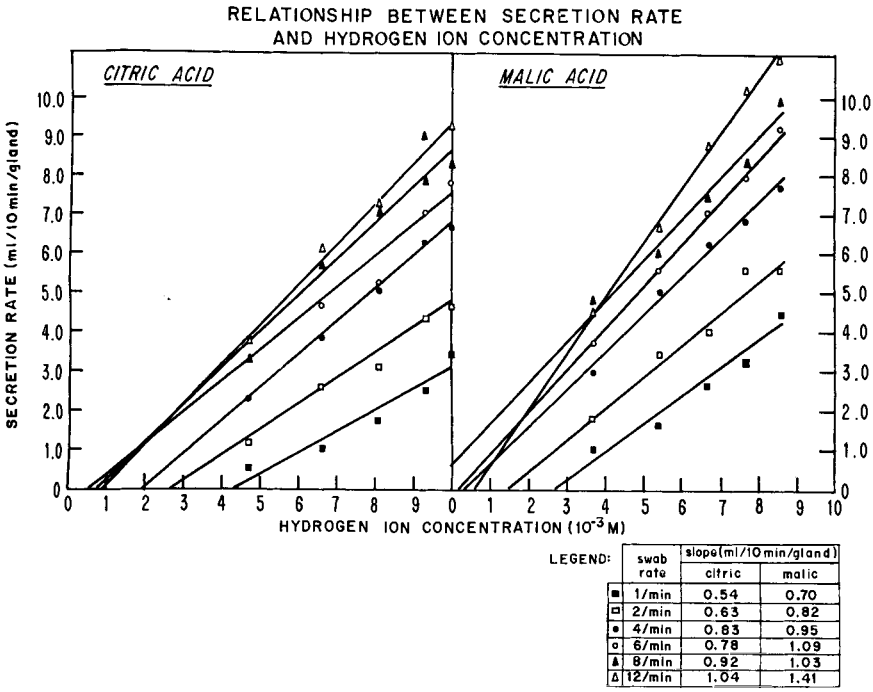


FIG. 5.

TABLE 7. EFFECT OF TEMPERATURE UPON THE GUSTATORY-SALIVARY REFLEX INDICATED BY PAROTID GLAND SECRETION RATE

Acid (1·5 g/100 ml)	Secretion rate (ml/10 min/gland)				
	5°C	15°C	25°C	35°C	45°C
Citric	6·33*	7·10	7·20	7·00	7·39
Malic	6·98*	7·73	7·93	7·50	7·78

* $P<0{\cdot}05$ (significance of 5°C values as compared to other temperatures)

Finally a third study was initiated to evaluate the effect of mixtures of sour, sweet and salt solutions. The test solutions consisted of 0·5, 1·0, 1·5, 2·0 and 2·5 per cent citric acid, 5·0, 10·0, 15·0, 20·0 and 25·0 per cent sodium citrate and 5·0, 10·0, 15·0, 20·0 and 25·0 per cent sucrose. To compare relative responses elicited by each modality, solutions containing one compound were tested at each concentration. To study the effect of mixtures

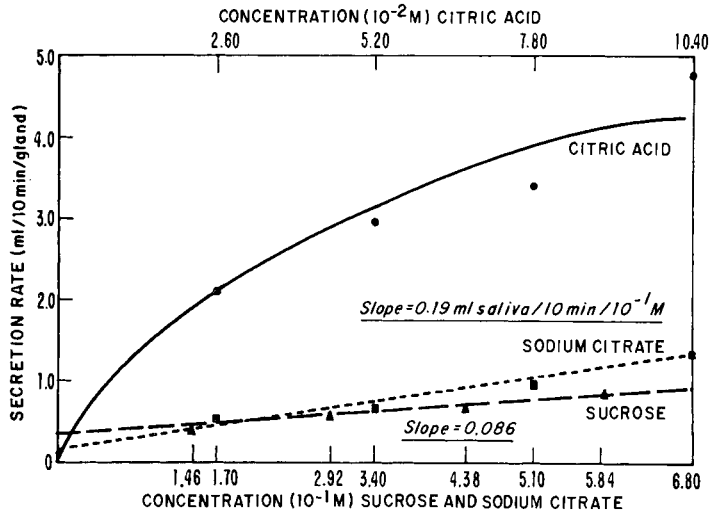


FIG. 6.

of these modalities one compound was combined with another at each concentration.

The nine male subjects used were divided into three groups of three, and the solutions were presented in groups of three. The first group received the solutions in the order *ABC*, the second in the order *BCA* and the third in the order *CAB*.

Collection of parotid saliva and calculation of secretion rate were done in the same manner as in the prior studies.

The relationship between secretory response and solution concentration of sucrose and sodium citrate in solution appeared to produce a linear response, whereas citric acid produced the expected curvilinear response (Fig. 6). When the hydrogen ion concentration was plotted against the parotid gland secretion rate a direct linear relationship was obtained.

The intensity of response resulting from mixtures of sucrose, sodium citrate and citric acid was recorded and compared with the sum of the components tested separately (Table 8). Comparison of the actual and calcu-

TABLE 8. PAROTID SALIVA SECRETION RATES (ml/10 min/GLAND) FROM MIXTURES OF CITRIC ACID AND SUCROSE

Citric acid (M)	0·026		0·052		0·078		0·104		Sum	
Sucrose (M)	Actual	Calc.	Actual	Calc.	Actual	Calc.	Actual	Calc.	Actual	Calc.
0·146	2·6	2·5	3·3	3·4	3·8	3·8	6·3	5·2	16·0	14·9
0·292	2·9	2·7	4·2	3·5	4·9	4·0	5·8	5·3	17·8	15·5
0·438	2·6	2·8	3·6	3·6	4·2	4·1	5·5	5·4	15·9	15·9
0·584	2·5	2·9	3·7	3·8	4·7	4·2	4·8	5·6	15·7	16·5
Sum	10·6	10·9	14·8	14·3	17·6	16·1	22·4	21·5	65·4	62·8

TABLE 9. PAROTID SALIVA SECRETION RATES (ml/10 min/GLAND) FROM MIXTURES OF SUCROSE AND SODIUM CITRATE

Sucrose (M)	0·146		0·292		0·438		0·584		Sum	
Sodium citrate (M)	Actual	Calc.	Actual	Calc.	Actual	Calc.	Actual	Calc.	Actual	Calc.
0·170	0·3	0·9	0·8	1·1	0·9	1·2	1·4	1·3	3·4	4·5
0·340	0·7	1·1	0·8	1·2	1·1	1·3	1·5	1·5	4·1	5·1
0·510	1·1	1·4	1·4	1·5	1·4	1·6	1·8	1·8	5·7	6·3
0·680	1·5	1·8	1·7	1·9	1·8	2·0	2·1	2·2	7·1	7·9
Sum	3·6	5·2	4·7	5·7	5·2	6·1	6·8	6·8	20·3	23·8

lated responses indicated that citric acid and sucrose have a direct additive effect.

Results obtained with mixtures of sodium citrate and sucrose (Table 9) show a close correlation between the actual and calculated responses, except at low solution concentrations.

Comparison of actual parotid gland secretion rates, obtained from mixtures of citric acid and sodium citrate, with the calculated results indicated

TABLE 10. PAROTID SALIVA SECRETION RATES (ml/10 min/GLAND) FROM MIXTURES OF CITRIC ACID AND SODIUM CITRATE

Citric acid (M)	0·026		0·052		0·078		0·104		Sum	
Sodium citrate (M)	Actual	Calc.	Actual	Calc.	Actual	Calc.	Actual	Calc.	Actual	Calc.
0·170	1·3	2·6	2·0	3·4	2·8	3·9	5·1	5·3	11·2	15·2
0·340	0·9	2·7	1·7	3·6	2·4	4·0	4·0	5·4	9·0	15·7
0·510	1·1	3·0	1·4	3·9	2·0	4·3	3·5	5·7	8·0	16·9
0·680	1·3	3·4	1·6	4·3	2·3	4·7	3·4	6·1	8·6	18·5
Sum	4·6	11·7	6·7	15·2	9·5	16·9	16·0	22·5	36·8	66·3

that the stimulatory effect of this mixture had been significantly reduced below what was expected (Table 10).

A plot of secretion rate vs. sodium citrate concentration, with constant citric acid concentration, illustrates the buffering effect of the mixture (Fig. 7). When the ratio of citric acid to sodium citrate was high, the sodium citrate concentration and the secretion rate were inversely rated. As this ratio was reduced, the secretion rate tended to increase as the sodium citrate concentration was increased.

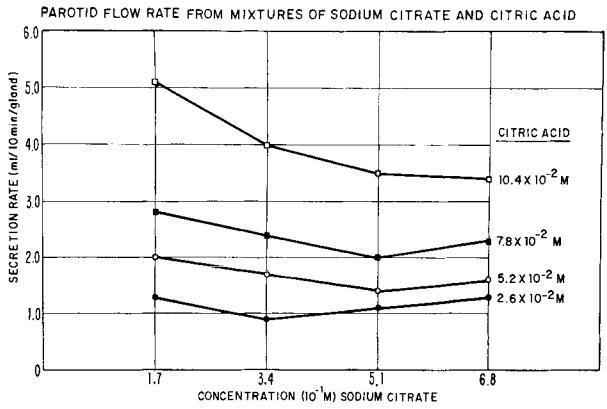


FIG. 7.

When the hydrogen ion concentration of the citric acid – sodium citrate mixtures was determined, the hydrogen ion level was found to be considerably lower than the response threshold (10^{-3} M) in the pure citric acid solution. Despite this, a plot of hydrogen ion concentration against parotid gland secretion rate showed that with all four concentrations of sodium citrate relatively small increases in the hydrogen ion concentration of the mixture — increases that would not be discerned in a pure citric acid solution — produced notable increases in the secretory rate (Fig. 8).

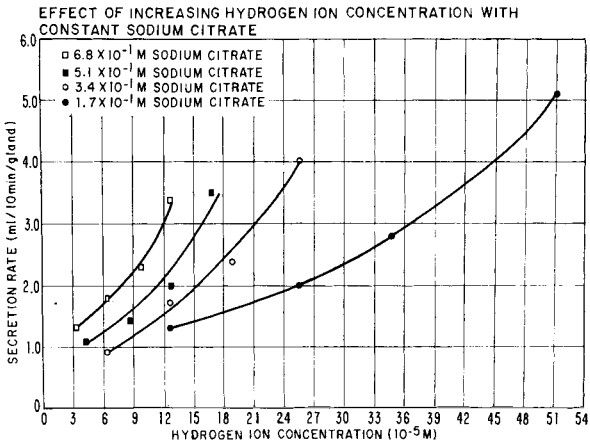


FIG. 8.

Utilizing mixtures of sodium citrate and citric acid which contained the same hydrogen ion concentration, the concentration of sodium citrate was plotted against the secretion rate (Fig. 9). This indicated that when a substantial amount of hydrogen ion was present small increments of sodium citrate resulted in a large increase in secretion rate.

This study has shown that salt-sweet and sweet-sour mixtures in solution evoke additive responses. Mixtures of acid and salt, which produced a buffering effect with a reduced hydrogen ion concentration, evoked diminished responses. However, analysis of the data indicated that the increase in response associated with increasing hydrogen ion was greater than that obtained when sodium citrate was not present. Similarly, the increase in response to increasing concentration of sodium citrate was greater than that obtained in the absence of citric acid. Thus, it would appear that there was a potentiation effect when hydrogen and sodium ions were present in the same solution.

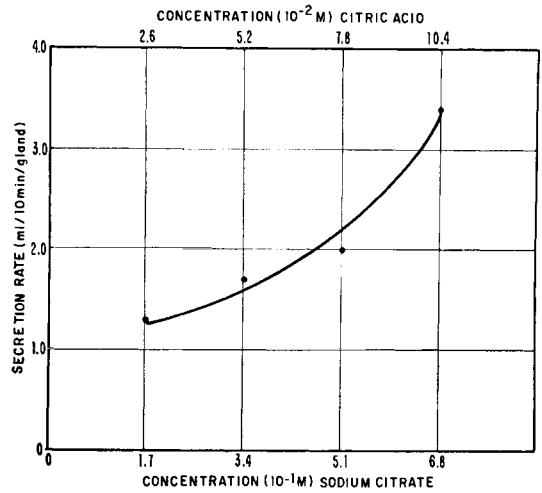


FIG. 9. Effect of increasing sodium citrate and citric acid with constant hydrogen ion concentration ($[H^+] = 1.27 \times 10^{-4}$ m)

ACKNOWLEDGEMENTS

These investigations were supported by National Institute of Dental Research Grants DE-00402 and DE-K_3-4991 and National Science Foundation Grants G-21375.

The authors would like to thank the Society for Experimental Biology and Medicine (Tables 1 – 6) and the *Journal of Applied Physiology* (Figs. 6 – 7; Tables 8 – 10) for permission to reprint selected material from previously published manuscripts.

REFERENCES

1. CHAUNCEY, H. H. and I. L. SHANNON. 1960. Parotid gland secretion rate as method for measuring response to gustatory stimuli in humans. *Proc. Soc. Exptl. Biol. and Med.* **103,** 459–463.
2. CHAUNCEY, H. H., R. P. FELLER and I. L. SHANNON . 1963. Effect of acid solutions on human gustatory chemoreceptors as determined by parotid gland secretions rate. *Proc. Soc. Exptl. Biol. and Med.* **112,** 917–923.
3. FELLER, R. P., I. M. SHARON, H. H. CHAUNCEY and I. L. SHANNON. 1965. Gustatory perception of sour, sweet and salt mixtures using parotid gland flow rate. *J. Applied Physiol.* **20,** 1341–1344.
4. SHANNON, I. L., J. R. PRIGMORE and H. H. CHAUNCEY. 1962. Modified Carlson-Crittendon device for the collection of parotid fluid. *J. Dental Res.* **41,** 778–783.

RELATIONS BETWEEN TASTE QUALITIES AND PAROTID GLAND SECRETION RATE

MASAYA FUNAKOSHI and YOJIRO KAWAMURA

Department of Oral Physiology, Dental School, Osaka University
Osaka, Japan

SINCE Pavlov (1910) stated that salivary response in dog was adapted teleologically to the nature of the stimulus, there have been reported many works of quantitative measurements of salivary secretion to different stimuli (Lashley, 1916; Allen, 1929; Baxter, 1933; Gantt, 1937; Schneyer and Levin, 1955; Chauncey and Shannon, 1960; Kerr, 1961). It is also well known that the volumes of saliva produced in response to gustatory stimuli are markedly different with different submodalities of the taste sensation. However, neural mechanism of this phenomenon of taste effectiveness on salivation still remains obscure.

When we try to compare the effectiveness of different kinds of taste stimuli on the salivary reflex, the same intensity of stimuli must be applied, otherwise we cannot discuss them on the same level.

In the present experiment, four basic taste stimuli (sour, salt, sweet and bitter) with similar intensity were used to produce salivary secretion. As criteria of the intensity, psychological intensity of taste sensation in human and also electrical activity of taste nerve in dog were chosen.

METHODS

In the first experiment, twenty male and female subjects between the age of 22 and 34 years old were selected for measurement of the volume of salivary secretion responding to gustatory stimuli. As stimulants, the solutions of sucrose, sodium chloride, tartaric acid and quinine hydrochloride were employed. According to the modified method of Beebe-Center and Waddell (1948), concentrations of the four basic taste solutions were determined to elicit the same psychological intensity of sensation. The 3 per cent sodium chloride was used as the standard solution and the concentrations of comparison solutions extended in four submodalities

over a range of 3·25 log units in quarter log unit steps. Each subject tasted the standard solution first, then rinsed his mouth and tasted a comparison solution, and stated whether the comparison solution was stronger or weaker than the standard solution. The procedure was repeated with standard solution and another comparison solution. For each comparison stimulation, the per cent frequency of subjects who judged the comparison solution was stronger than the standard one plotted in ordinate and concentration of the comparison solutions was plotted in abscissa.

The concentrations of each taste solutions which had similar intensity to the standard solution were determined by the level of 50 per cent of per cent frequency.

The test solution of 5 ml was delivered from a syringe into the subject's mouth and parotid saliva responding to the solution was collected by means of the modified Lashley's vacuum cup for three minutes.

In the second experiment, five mongrel dogs with parotid fistula were used. A taste solution of 5 ml was poured onto the tongue surface from a syringe and unilateral parotid saliva responding to gustatory stimulation was collected into the graded small glass tube attached to the opening of the parotid fistula. The concentrations of taste solution extended in four submodalities over a range from 0·0005 to 2 M. After the measurements of the volume of saliva, the integrated responses of the chorda tympani and glossopharyngeal nerve to the used four basic taste solutions were recorded. The magnitude of integrated response to the taste stimuli was estimated by an area encircled with integrated response curve and base-line. Thus, the ratio between volume of saliva and taste nerve activity was calculated.

RESULTS AND DISCUSSIONS

I. Experiment in Human

A graphical determination of concentrations of quinine hydrochloride, tartaric acid and sucrose which have similar psychological intensity of taste sensation to the 3 per cent standard sodium chloride solution was made as shown in Fig. 1. Concentrations of equi-intensive solutions are indicated on the figure corresponding to 50 per cent level of per cent frequency in ordinate, namely they were 0·02 per cent for quinine hydrochloride, 0·28 per cent for tartaric acid and 18·8 per cent for sucrose solution respectively.

The volume of the unilateral parotid saliva elicited by the above described four basic taste solutions for 3 min is shown in Table 1. Although there were bonsiderable individual variations in the volume of parotid saliva, the mean values of salivary flow to each solution were 0.46 ml to

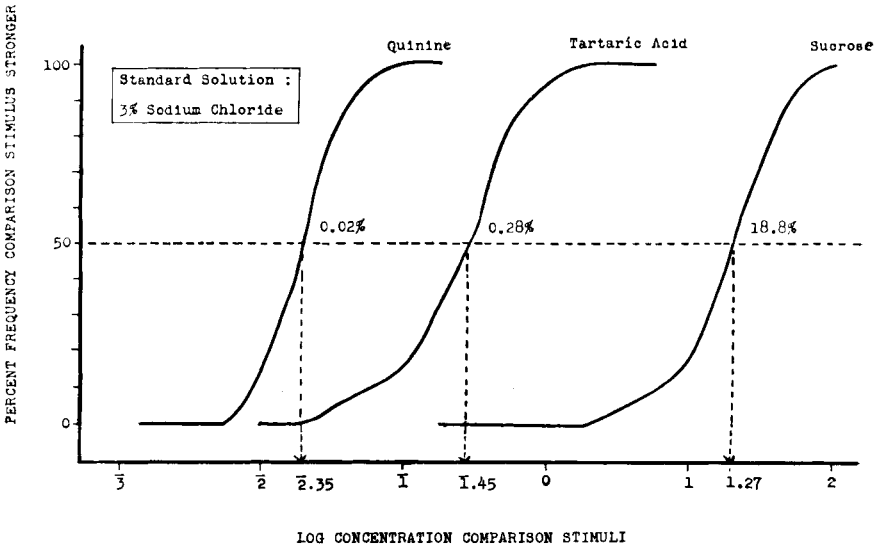


FIG. 1. Graphical determination of equi-intensive test solutions to the standard solution.

TABLE 1. VOLUME OF HUMAN PAROTID SALIVA INDUCED BY FOUR BASIC TASTE SOLUTIONS

Sub.	Sucrose 18·8%	Sodium chloride 3%	Tartaric acid 0·28%	Quinine 0·02%
N.T.	0·78	0·66	1·36	1·26
A.K.	0·12	0·34	1·90	0·18
I.N.	0·30	0·28	0·54	0·22
E.K.	0·45	0·70	1·56	0·68
I.T.	0·34	0·64	1·50	0·35
F.M.	0·41	0·44	3·32	0·20
K.I.	0·97	0·84	1·10	0·58
M.F.	0·78	0·56	3·61	0·90
N.T.	0·28	0·37	1·30	0·37
M.T.	0·14	1·84	2·15	0·98
MEAN	0·46	0·67	1·83	0·57

18·8 per cent sucrose, 0·67 ml to 3 per cent sodium chloride, 1·83 ml to 0·28 per cent tartaric acid and 0·57 ml to 0·02 per cent quinine hydrochloride respectively. In spite of the similar psychological intensity of the used taste stimuli, tartaric acid was the most effective stimulant among the four basic taste solutions, and produced salivary secretion to tartaric acid was nearly four times more than sucrose.

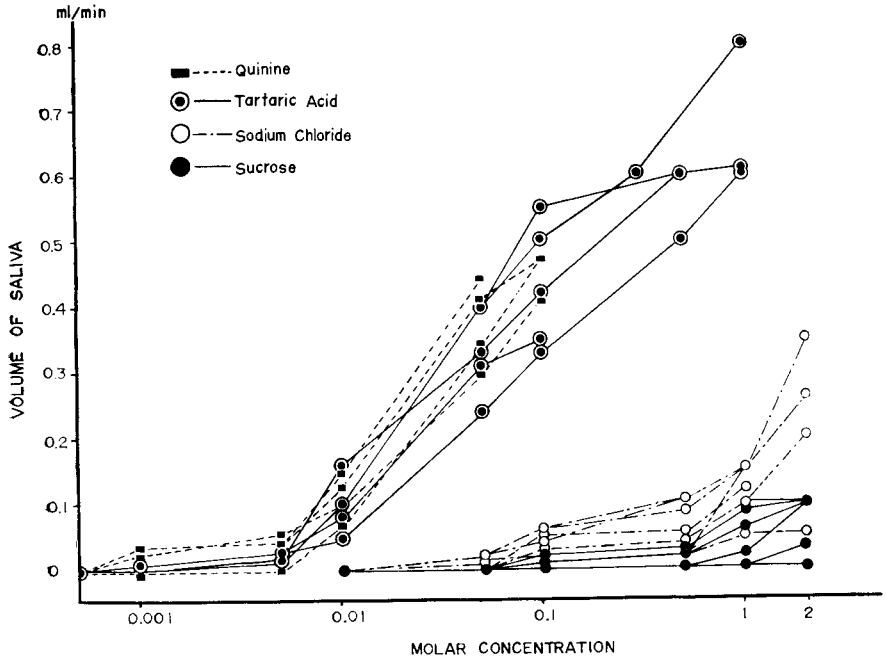


FIG. 2. Parotid secretion rate to four basic taste submodalities in dog.

II. Experiment in Dog

The volumes of parotid saliva responding to four basic taste solutions in various concentrations is shown in Fig. 2. Patterns of parotid secretion to each taste stimulant were divided into the two groups. One was the response type to quinine hydrochloride and tartaric acid, the other type was the response to sodium chloride and sucrose. Quinine hydrochloride or tartaric acid solution elicited conspicuous salivary secretion and also rate of salivary flow increased remarkably following the increase of the concentration of the test solution, while the saliva response to sodium chloride or sucrose solution did not show such rapid increasing of secretion, even when the concentration of the solution increased.

After measurements of the salivary secretion, electrical activities of the taste nerves to the taste solutions were recorded in the same dogs.

Figure 3 shows integrated responses of the chorda tympani and glossopharyngeal nerves to four basic taste solutions with various concentrations. Quinine hydrochloride induced much more responses in the glossopharyngeal nerve than in the chorda tympani. Tartaric acid induced weak and long-lasting responses in the glossopharyngeal nerve, while strong and short responses to tartaric acid were recorded from the chorda tympani. Responses to sucrose solution were stronger in the chorda tympani than in the glossopharyngeal nerve. Responses to sodium chloride were also different in the two nerves and the chorda tympani showed stronger and more long-lasting responses than the glossopharyngeal nerve.

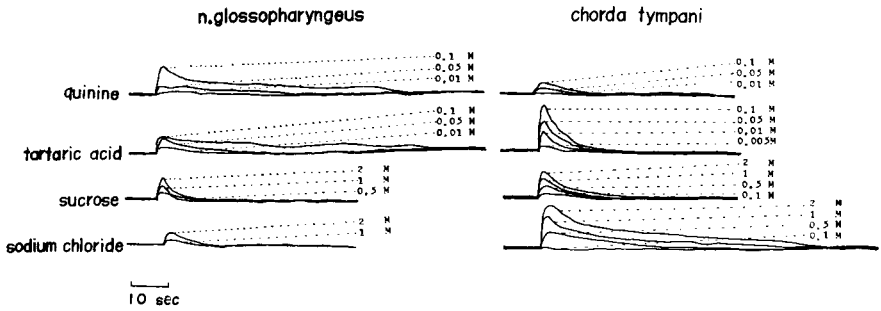


Fig. 3. Integrated responses of the chorda tympani and glossopharyngeal nerves of dog to the four basic taste submodalities.

From these records, magnitudes of the responses to each taste solution recorded from the two nerves were estimated as a sum of areas encircled with response curve and base-line.

The volumes of saliva which were measured before and quantitative representations of the integrated responses of the taste nerves to four basic taste solutions were summarized in Table 2. From the table, ratio between volume of saliva and taste nerve activity was calculated, that is, the volume of saliva per the unitary taste nerve activity was estimated. As shown in Fig. 4. the ratio was 0·37 for quinine hydrochloride, 0·18 for tartaric acid, 0·04 for sodium chloride and 0·02 for sucrose. Quinine hydrochloride induced the most dominant parotid secretion, tartaric acid was the next and sodium chloride and sucrose followed in turn.

Although, there are many reports of the quantitative measurement of salivary gland responses to gustatory stimuli, no attempt has been made to compare the effectiveness of four basic taste stimuli with the same inten-

TABLE 2. PAROTID SECRETION RATE AND QUANTITATIVE REPRESENTATION OF THE INTEGRATED RESPONSES OF THE TASTE NERVES TO FOUR BASIC TASTE SUBMODALITIES IN DOG

	Glosso-pharyngeal nerve	Chorda tympani	Total	Volume of Saliva (ml/min)
Quinine	(cm^2)	(cm^2)	(cm^2)	
0·1 M	3·396	0·566	3·962	0·44
0·05 M	0·943	0·015	0·958	0·37
0·01 M	0·021	0	0·021	0·09
Tartaric acid				
0·1 M	3·208	1·698	4·906	0·37
0·05 M	0·642	0·943	1·585	0·29
0·01 M	0·021	0·509	0·530	0·08
0·005 M	0	0·020	0·020	0·02
Sucrose				
2 M	0·566	1·415	1·981	0·06
1 M	0·015	0·623	0·638	0·04
0·5 M	0·011	0·472	0·483	0·01
0·1 M	0	0·013	0·013	0·01
Sodium chloride				
2 M	2·642	6·311	8·953	0·22
1 M	0·075	2·755	2·830	0·11
0·5 M	0	1·113	1·113	0·05
0·1 M	0	0·623	0·623	0·03

sity. Beebe-Center and Waddell (1948) developed a method to determine the general psychological scale of hetero-qualitative taste strength on the base of subjective strength. Neurophysiologists showed that integrated response of the taste nerve to chemical stimuli also represents the strength of taste sensation.

In the present experiment, we applied the four taste stimuli of the same psychological intensity to the human tongue and acid was detected to induce the largest parotid secretion among four basic taste stimuli. In addition, experiment in the dog also indicated a similar result although quinine hydrochloride was the most effective stimulant. These results suggest that the volumes of saliva induced by four basic taste stimuli are neither proportional to the electrical activity of the primary neurones nor the psychological intensity of the taste sensations. Therefore, we may assume

that the taste nerve pathway of sour and bitter information might connect more closely with cells in the salivary center in the medulla than that of salt and sugar.

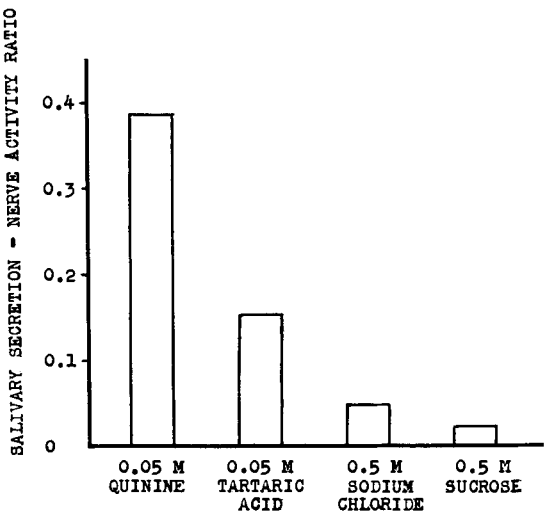


FIG. 4. Salivary secretion—nerve activity ratios of four basic taste submodalities.

REFERENCES

ALLEN, F. 1929. The secretory activity of the parotid gland. *Quart. J. Exp. Physiol.* **19,** 337–362.

BAXTER, H. 1933. Variations in the inorganic constituents of mixed and parotid gland saliva activated by reflex stimulation in the dog. *J. Biol. Chem.* **102,** 203–217.

BEEBE-CENTER, J. G. and WADDELL, D. 1948. A general psychological scale of taste. *J. Psychol.* **26,** 517–524.

CHAUNCEY, H. H. and SHANNON, I. L. 1960. Parotid gland secretion rate as method for measuring response to gustatory stimuli in humans. *Proc. Soc. Exp. Biol. Med.* **103,** 459–463.

GANTT, W. H. 1937. The nervous secretion of saliva: Quantitative studies in the natural unconditioned reflex secretion of parotid saliva. *Am. J. Physiol.* **119,** 493–507.

KERR, A. C. 1961. *The Physiological Regulation of Salivary Secretions in Man.* **VII,** The effect of gustatory stimulation on the production of salivary secretions, pp. 61–73, Pergamon Press, London.

LASHLEY, K. S. 1916. Reflex secretion of the human parotid gland. *J. Exp. Psychol.* **1,** 461–493.

PAVLOV, I. P. 1910. *The Work of Digestive Glands.* 2nd English Ed. London, Cited by DAWES, C. and JENKINS, G. N. *J. Physiol.* **170,** 86–100, 1964.

SCHNEYER, L. H. and LEVIN, L. K. 1955. Rate of secretion by exogenously stimulated salivary gland pairs of man. *J. Appl. Physiol.* **7,** 609–613.

GUSTATORY RESPONSE TO L-AMINO ACIDS IN MAN

HERBERT STONE

Life Sciences Research, Stanford Research Institute,
Menlo Park, California 94025

THE unique taste properties of amino acids have been recognized by many investigators (Kaneko, 1938, 1939, in *Chem. Abs.* 1938, 1942; Moncrieff, 1951, and Berg, 1953). For example, L-forms are reported as eliciting a bitter or sweet response, while their associated D-forms are reported to be primarily sweet. However, not all investigators agree about the taste of the amino acids Pfaffmann, 1959 and Lawrence and Ferguson, 1959). These inconsistencies are probably attributable to differences in concentration, racemic mixtures, or impurities. Recent studies by Schutz and Pilgrim (1957) have corroborated earlier reports that glycine (up to 3·2 M) and D, L-alanine (up to 1·8 M) are sweet. In studies with the rat, Halpern *et al.* (1962) reported that for glycine and racemic mixtures of valine, methionine, and alanine, increasing concentration resulted in decreased acceptance which ultimately reached complete rejection. Also, higher concentration of glycine and D, L-alanine resulted in sharp increases of chorda tympani response magnitude and time to maximum response. Although the data for glycine and alanine were not in agreement with the results reported by Schutz and Pilgrim (1957), the authors indicated that there was no real basis for comparison since animal and human responses were not well correlated.

The fact that amino acids possess diverse sensory properties and great chemical specificity suggests that much could be learned about gustation through study of this class of chemical compounds. As Beidler (1961) noted, the differences in response to D- and L-amino acids indicate steric effects of surface adsorption. Thus, one could conceivably use the amino acids as a model system to study the mechanisms of action in gustation and the effect on the behavioral response as a function of changes in their physico-chemical properties through the addition of various reactive groupings. Therefore, this research was undertaken to study the sensory

properties of some L-amino acids on a quantitative basis. By studying each amino acid over a series of concentrations, it was also possible to apply these data to current theories of gustation.

METHODS AND PROCEDURE

Eight L-amino acids were studied over an 8-month period by a panel familiar with psychophysical procedures. The panel consisted of 5 subjects, 2 males and 3 females, between the ages of 22 and 40. All subjects took part in each experiment unless otherwise noted.

Test stimuli included glycine, L-valine, -leucine, -isoleucine, -phenylalanine, -arginine, and -aspartic and -glutamic acids. All the amino acids were obtained from commercial sources (Table 1) with purity specifications $\geq$ 98 per cent. Additional evidence of purity was obtained by analysis with the amino acid analyzer (Beckman Model 120).

Stimulus concentrations were selected by the experimenter to yield a test series which would cover a major portion of the response continuum and minimize sample interaction. A four-step geometric series with a factor of 2 was used to prepare stimulus concentrations. For each stimulus, a reference was also prepared at the geometric mean of the series. Sufficient solution was prepared to complete each experiment. Solutions were kept in cold storage, and only that amount required for testing was removed daily. All samples were served at $22° \pm 0.5°$C. The pH of all solutions was checked at regular intervals. Water for sample preparation and for oral rinsing was deionized, glass distilled, and percolated through charcoal to remove all odor and taste.

TEST PROCEDURE

Testing was carried out at the same time each day, to minimize diurnal variations. Subjects were served individually in partitioned, air-conditioned booths with controlled lighting. A single-sample order of presentation was used: each subject received the identified reference followed by five samples—four test and one distilled water blank in a randomized sequence. The procedure was repeated once, thus 20 responses were obtained at each concentration over the 10-day test period. At the conclusion of each test, subjects were informed of their results.

The actual test procedure required completion of a three-part questionnaire: rating intensity on a semi-anchored, 100-point scale (0—no discernable taste, 50—moderate intensity, 100—extreme intensity); indication of

TABLE 1. MAGNITUDE OF RESPONSE TO THE TEST CONCENTRATIONS FOR THE PANEL, pH, AND PURITY

Amino acid	Concentration (M)	Response (R)	pH	Purity (%)
Glycine	1·0	0·867	6·3	⩾98
	0·50	0·536	6·4	
	0·35 (ref.)	0·500	6·4	
	0·25	0·233	6·5	
	0·125	0·149	6·5	
Valine	0·25	0·842	4·92	⩾98
	0·125	0·604	4·92	
	0·089 (ref.)	0·500	4·92	
	0·0625	0·229	4·95	
	0·0313	0·112	5·00	
Leucine	0·125	0·888	6·07	⩾98
	0·0625	0·637	6·02	
	0·044 (ref.)	0·500	6·0	
	0·0313	0·327	5·98	
	0·0156	0·171	5·95	
Isoleucine	0·125	0·942	6·08	⩾98
	0·0625	0·673	6·00	
	0·044 (ref.)	0·500	6·00	
	0·0313	0·281	6·02	
	0·0156	0·144	6·00	
Phenylalanine	0·0625	0·913	5·82	⩾99·8
	0·0313	0·667	5·82	
	0·022 (ref.)	0·500	5·82	
	0·0157	0·319	5·82	
	0·0078	0·125	5·85	
Arginine	0·10	0·785	10·31	⩾99·95
	0·05	0·675	10·00	
	0·036 (ref.)	0·500	10·00	
	0·025	0·442	9·90	
	0·0125	0·306	9·79	
Aspartic acid	0·025	0·851	2·98	⩾98
	0·0125	0·684	3·07	
	0·0088 (ref.)	0·500	3·12	
	0·00625	0·441	3·15	
	0·00313	0·236	3·23	
Glutamic acid	0·0125	0·861	3·48	⩾99·8
	0·00625	0·686	3·58	
	0·0044 (ref.)	0·500	3·60	
	0·00313	0·385	3·79	
	0·00156	0·208	3·95	

Responses are the sum of 15 replications per subject.

Glycine was supplied by Schwarz Bioresearch, Inc., New York; arginine and glutamic acid by Scientific Products, Calif.; leucine and aspartic acid by Henley and Co., Inc., New York; phenylalanine by Daiichi Seiyaka Co. Ltd., Tokyo; and isoleucine and valine by Kyowa Hakko Kogyo Co., Tokyo.

extreme like/dislike on a nine-point hedonic scale (Jones *et al.*, 1955); and recording of which of the four basic tastes were perceived and the degree that each contributed to the over-all taste. The latter task required subjects to taste the sample again and, when appropriate, to refer to reference samples (dilute sweet, sour, salty, and bitter solutions) supplied by the experimenter. The reference sample, with an intensity of 50 noted on the score sheet, was presented to the subject before each of the two daily series. It served the dual functions of panel orientation and control of the validity of the scaling procedure.

Additional experiments were carried out to determine the inter-sample intensities of each stimulus compared with each other, and to determine individual detection thresholds to 6, n-propyl thiouracil. In the first test, subjects ranked all eight amino acids on a 100-point scale (0—least intense taste, 100—most intense taste). Stimulus concentrations selected for this experiment were the intensities rated moderate by the panel (the second highest concentration in the series).

The thresholds to 6, n-propyl thiouracil were determined by a triangle test after selection of the appropriate concentration range by means of a randomized single sample test. The purpose of this experiment was to determine if all subjects had essentially the same sensitivity to bitterness. The experiment was necessitated by the preponderance of bitter-tasting amino acids.

DATA ANALYSIS

Only the final 15 scores from each subject were used for analysis of the data, since subjects required about 2–3 days to become familiar with each stimulus. In all experiments, data from each subject were evaluated individually. Mean scores were obtained from all three responses; hedonic ratings were assigned numerical values (1—dislike extremely, 5—neither like nor dislike, 9—like extremely), and then averaged. The rankings and detection threshold experiments were evaluated separately.

Where appropriate, the method of least squares was used to determine the equation of a line (Goulden, 1952).

RESULTS

Intensity Ratings

An increase in subjective response was noted with increasing stimulus concentration for all of the amino acids (Table 1 and Fig. 1). These data follow the typical stimulus–response relationship which, according to

Beidler (1961), reaches a plateau as concentration is maximized. The differences in stimulus concentrations were adjusted by dividing them by the reference concentration, permitting direct comparison of response. All subjects appeared to rate the stimuli on the same scale, although subject 1 tended to avoid the upper limits, a not uncommon situation in subjective rating procedures.

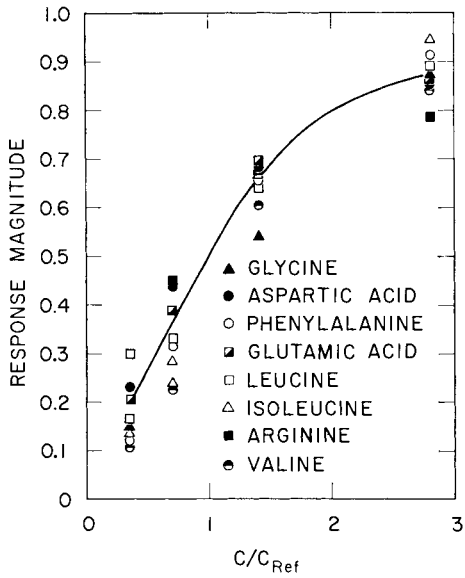


FIG. 1. Response magnitude as a function of the stimulus concentration. Test concentrations were made equivalent by dividing by the reference concentration. Each point is the average of 75 responses by the panel.

Preference Tests

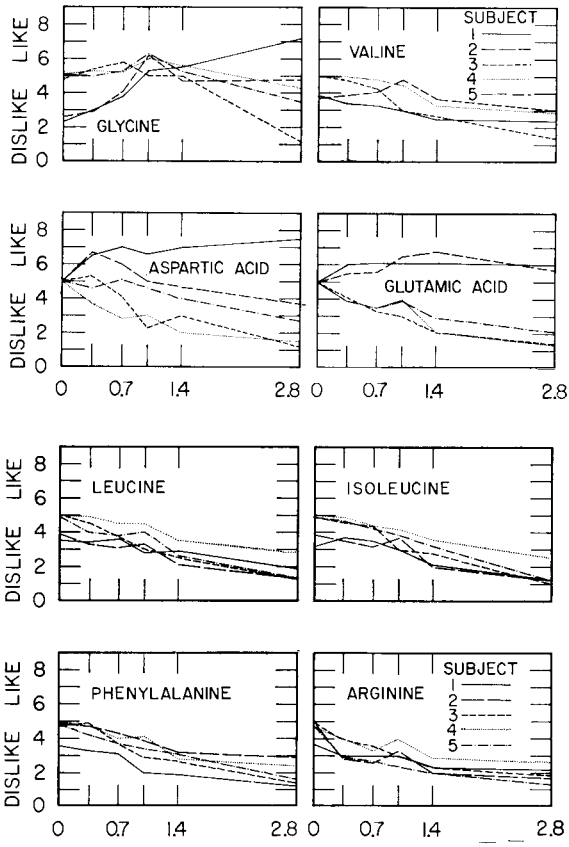
The results of the preference tests are shown in Figs. 2 and 3. Individual responses, plotted for each amino acid, depicted the wide differences in subjective preferences for some amino acids, especially glycine and aspartic and glutamic acids.

With glycine, subjects 1 and 2 indicated a decreasing preference with decreasing concentration; subjects 3, 4, and 5 indicated a maximal preference between 0·050 and 0·025 M, with dislike at the high concentration and no preference at the lower concentration.

For aspartic acid, subject 1 showed the same like–dislike response as for glycine, while subject 2 indicated a peak preference at 0·00625 M. The

remainder of the panel responded with an increase to like (> 5) as the concentration decreased.

Subjective responses to glutamic acid were equally varied: subject 1 indicated no difference in preference at all concentrations; subject 2



FIGS. 2 and 3. Hedonic ratings of the stimuli by the individual subjects. The scale was dislike extremely −1, neither like nor dislike −5, and like extremely −9, with appropriate words at intermediate points.

indicated an increase to like (> 5) for glutamic acid at 0·00625 M; and the rest of the panel indicated an increase to like (> 5) with decreasing concentration.

Probably because of their very similar properties, leucine and isoleucine elicited similar responses from all panelists—extreme dislike at high concentrations with a decrease to "neither like nor dislike" as concentration decreased.

For valine, phenylalanine, and arginine, the panel recorded hedonic behavior similar to that reported for leucine and isoleucine. It was interesting that, for most of the amino acids tested, the panel expressed dislike at all concentrations.

Taste Interaction

The results of the final task, indicating taste impression and degree of contribution to total taste, are shown in Tables 2 to 9. These data provide information on the complex sensory properties of the amino acids. Bitter-

TABLE 2. TASTES PERCEIVED AND CONTRIBUTION TO THE OVER-ALL TASTE OF GLYCINE

Subject	Taste	Concentration — M					
		0	0·125	0·250	0·350	0·500	1·00
1	Sweet	—	4·0	17·0	49·2	49·0	72·0
	Sour	1·0	10·5	21·0	30·0	34·0	26·5
	Salty	—	—	2·0	—	—	—
	Bitter	99·0	85·5	52·0	20·8	17·0	1·5
	Bland	—	—	8·0	—	—	—
2	Sweet	—	9·0	63·9	92·2	84·3	80·5
	Sour	—	4·0	—	7·5	10·2	19·5
	Salty	—	—	—	—	—	—
	Bitter	80·5	87·0	36·1	0·3	5·5	—
	Bland	19·5	—	—	—	—	—
3	Sweet	—	26·0	64·5	78·3	75·0	65·0
	Sour	—	—	—	14·2	—	1·0
	Salty	—	2·5	11·5	0·8	7·0	12·0
	Bitter	2·5	4·5	18·0	6·7	18·0	22·0
	Bland	97·5	67·0	6·0	—	—	—
4	Sweet	—	45·0	48·0	100	72·0	53·0
	Sour	—	3·0	14·0	—	4·0	11·5
	Salty	—	7·0	12·5	—	18·0	3·0
	Bitter	—	35·0	26·5	—	6·0	5·5
	Bland	100	10·0	—	—	—	—
5	Sweet	13·3	73·4	93·3	100	99·9	99·6
	Sour	0·1	0·2	0·1	—	0·1	0·2
	Salty	—	—	—	—	—	0·1
	Bitter	—	—	—	—	—	0·1
	Bland	86·6	26·4	6·6	—	—	—

Values for each stimulus are the mean of 15 replications for each subject. Blandness indicates no taste preceived.

TABLE 3. TASTES PERCEIVED AND CONTRIBUTIONS TO THE OVER-ALL TASTE OF VALINE

Subject	Taste	Concentration — M					
		0	0·0313	0·0625	0·089	0·125	0·250
1	Sweet	0·7	7·7	12·3	17·5	23·7	24·3
	Sour	—	0·3	1·7	10·8	4·3	7·0
	Salty	—	—	—	—	—	—
	Bitter	79·3	92·0	86·0	71·7	72·0	68·7
	Bland	20·0	—	—	—	—	—
2	Sweet	0·1	2·3	7·7	15·8	12·7	14·7
	Sour	—	—	—	14·2	—	—
	Salty	—	—	—	—	—	—
	Bitter	66·6	77·7	92·3	70·0	87·3	85·3
	Bland	33·3	20·0	—	—	—	—
3	Sweet	—	3·3	6·7	11·7	12·7	11·3
	Sour	—	3·0	7·7	10·8	10·0	9·7
	Salty	—	0·3	1·3	1·7	0·3	—
	Bitter	0·3	7·0	45·3	75·8	77·0	79·0
	Bland	99·7	86·4	39·0	—	—	—
4	Sweet	—	2·0	—	5·0	—	—
	Sour	—	—	—	—	—	1·3
	Salty	—	—	—	—	—	—
	Bitter	6·7	84·7	100	95·0	100	98·7
	Bland	93·3	13·3	—	—	—	—

Values for each stimulus are the mean of 15 replications for each subject. Blandness indicates no taste perceived. Subject 5 did not participate in this experiment.

ness appeared to be the predominant characteristic for all the stimuli except aspartic and glutamic acids, explaining the preponderance of use of the dislike portion of the hedonic scale.

Results with glycine (Table 2) give some clues to the complete reversal noted earlier in the hedonic ratings by subjects 1 and 2. Here, both subjects reported bitterness increasing with decreasing concentration, which would account for their hedonic ratings not agreeing with those of the other three subjects.

Data on aspartic and glutamic acids are not clearly delineated but, as might be expected from pH values (2·9–3·9), sourness was the predominant characteristic. Subjects showed greater differences in their sensory impressions to glutamic acid. This is not surprising, as it has an additional CH—OH group and the Na salt possesses sensory properties that elude quantitative description.

TABLE 4. TASTES PERCEIVED AND CONTRIBUTION TO THE OVER-ALL TASTE OF LEUCINE

Subject	Taste	Concentration — M					
		0	0·0156	0·0313	0·044	0·0625	0·125
1	Sweet	—	—	—	—	—	—
	Sour	—	—	—	—	—	—
	Salty	—	—	—	—	—	—
	Bitter	92·0	92·7	92·7	86·7	98·7	100
	Bland	8·0	7·3	7·3	13·3	1·3	—
2	Sweet	0·1	—	—	0·3	—	—
	Sour	—	—	—	—	—	—
	Salty	3·0	3·0	11·9	10·5	12·3	15
	Bitter	30·2	77·0	88·1	89·2	87·7	85
	Bland	66·7	20·0	—	—	—	—
3	Sweet	1·0	3·3	6·7	10·0	7·3	8·7
	Sour	6·3	21·0	40·0	45·0	48·3	45·0
	Salty	1·3	2·3	5·0	8·3	4·7	3·7
	Bitter	6·7	16·0	41·7	36·7	39·7	42·7
	Bland	84·7	57·3	6·7	—	—	—
4	Sweet	2·7	5·7	4·7	4·2	—	—
	Sour	0·7	1·0	2·0	5·8	3·3	3·3
	Salty	5·3	14·7	11·7	4·2	9·4	8·3
	Bitter	18·0	65·3	81·7	85·8	87·3	88·3
	Bland	73·3	13·3	—	—	—	—
5	Sweet	—	0·3	—	—	—	—
	Sour	—	1·7	0·3	—	2·5	2·7
	Salty	—	0·2	0·7	1·7	1·1	1·1
	Bitter	26·7	84·5	92·3	98·3	96·4	96·2
	Bland	73·3	13·3	6·7	—	—	—

Values for each stimulus are the mean of 15 replications for each subject. Blandness indicates no taste perceived.

Responses to leucine and isoleucine were similar, but the latter appeared to impart a stronger impression of bitterness. The response of some of the panel—particularly subjects 1 and 2—to the water (blank) sample was quite interesting: the data suggested taste carryover. In fact, observation of the data for several of the same amino acids revealed that these were not isolated experiences. Since the predominant taste reported for the blank was bitterness, the panel's sensitivity to 6,n-propyl thiouracil is significant. The results (Table 10) showed that all subjects were "tasters", and of essentially equal sensitivity. Thus, the taste carryover that appeared

TABLE 5. TASTES PERCEIVED AND CONTRIBUTION TO THE OVER-ALL TASTE OF ISOLEUCINE

Subject	Taste	Concentration — M					
		0	0·0156	0·0313	0·044	0·0625	0·125
1	Sweet	—	—	—	—	—	—
	Sour	—	—	—	—	—	—
	Salty	—	—	—	—	—	—
	Bitter	87·0	100	87·0	100	100	100
	Bland	13·0	—	13·0	—	—	—
2	Sweet	0·3	—	0·3	1·6	0·1	0·7
	Sour	—	0·3	0·3	0·3	0·3	—
	Salty	2·0	5·4	7·7	10·7	12·8	14·3
	Bitter	31·0	74·3	91·7	87·4	86·8	85·0
	Bland	66·7	20·0	—	—	—	—
3	Sweet	0·7	1·3	3·0	10·0	10·3	8·7
	Sour	4·0	12·7	31·7	40·0	38·0	37·7
	Salty	—	—	—	—	—	—
	Bitter	1·7	16·0	31·0	50·0	49·0	53·6
	Bland	93·6	70·0	34·3	—	2·7	—
4	Sweet	—	—	—	—	—	—
	Sour	—	—	—	—	—	—
	Salty	—	—	1·0	0·3	4·0	7·7
	Bitter	6·7	100	99·0	99·7	96·0	92·3
	Bland	93·3	—	—	—	—	—
5	Sweet	0·1	0·2	0·1	1·7	—	—
	Sour	—	0·7	1·0	1·3	2·0	1·3
	Salty	—	0·3	0·3	—	—	0·7
	Bitter	13·3	58·9	85·3	97·0	98·0	98·0
	Bland	86·6	39·9	13·3	—	—	—

Values for each stimulus are the mean of 15 replications for each subject. Blandness was used to indicate no taste perceived.

to exist in these experiments might have contributed to the variability in the panel's description of the stimuli. However, it cannot be denied that these amino acids do possess rather complex sensory properties.

In the ranking test (Table 11), as might be expected, not all subjects were in agreement on the intensities of the eight amino acids. The mean rank order was: aspartic acid > valine > arginine > glutamic acid > phenylalanine > glycine > isoleucine > leucine. Conceivably, stimuli that were ranked close together were interchangeable; however, it is apparent that phenylalanine, glycine, leucine, and isoleucine possess considerably less intense tastes.

TABLE 6. TASTES PERCEIVED AND CONTRIBUTION TO THE OVER-ALL TASTE OF ASPARTIC ACID

Subject	Taste	Concentration — M					
		0	0·00313	0·00625	0·0088	0·0125	0·0250
1	Sweet	—	—	—	14·3	0·7	5·4
	Sour	6·6	100	100	71·4	86·0	94·6
	Salty	—	—	—	—	—	—
	Bitter	—	—	—	—	6·6	—
	Bland	93·4	—	—	14·3	6·6	—
2	Sweet	—	—	—	—	—	—
	Sour	6·6	100	100	100	100	100
	Salty	—	—	—	—	—	—
	Bitter	—	—	—	—	—	—
	Bland	93·4	—	—	—	—	—
3	Sweet	—	24·3	19·0	15·0	13·6	7·9
	Sour	—	53·7	63·0	70·0	73·0	83·4
	Salty	—	18·7	17·6	15·0	13·3	8·6
	Bitter	—	—	0·3	—	—	—
	Bland	100	3·3	—	—	—	—
4	Sweet	—	—	—	—	—	—
	Sour	—	62·7	66·0	64·3	68·0	68·0
	Salty	—	37·3	34·0	35·7	32·0	32·0
	Bitter	—	—	—	—	—	—
	Bland	100	—	—	—	—	—
5	Sweet	—	—	—	—	—	—
	Sour	—	82·5	77·9	71·4	69·3	68·7
	Salty	—	1·8	2·3	1·1	2·8	2·8
	Bitter	—	15·7	19·7	18·4	28·1	28·5
	Bland	100	—	—	—	—	—

Values for each stimulus are the mean of 15 replications for each subject. Blandness indicates no taste perceived.

DISCUSSION

Gustatory responses to glycine, L-valine, -leucine, -isoleucine, -arginine, phenylalanine, -aspartic and -glutamic acids were studied over a wide range of concentrations by a trained panel. In plotting of intensity ratings, (Fig. 1) the data followed a pattern similar to that encountered in studies with animals: a rapid increase in response at low intensities with a tendency to level off at high intensities.

Intensity ratings of the amino acids revealed an order of effectiveness of: aspartic > valine > arginine > glutamic > phenylalanine > glycine > isoleucine > leucine. Table 11 shows considerable difference between

TABLE 7. TASTES PERCEIVED AND CONTRIBUTION TO THE OVER-ALL TASTE OF GLUTAMIC ACID

Subject	Taste	Concentration — M					
		0	0·00156	0·00313	0·0044	0·00625	0·0125
1	Sweet*	—	1·3	8·7	20·0	20·0	20·0
	Sour	—	96·7	82·7	60·0	60·0	60·0
	Salty	—	1·3	8·7	20·0	20·0	20·0
	Bitter	—	0·7	—	—	—	—
	Bland	100	—	—	—	—	—
2	Sweet*	—	13·0	16·3	22·4	17·5	21·1
	Sour	—	84·3	80·3	73·6	79·7	76·0
	Salty	—	2·0	3·3	4·0	2·8	2·8
	Bitter	—	0·7	—	—	—	—
	Bland	100	—	—	—	—	—
3	Sweet	—	6·7	12·0	8·6	6·7	5·0
	Sour	—	34·0	49·0	63·6	66·0	73·3
	Salty	—	21·0	26·0	22·1	21·3	18·3
	Bitter	—	4·0	7·0	5·7	6·0	3·7
	Bland	100	34·3	6·0	—	—	—
4	Sweet	—	9·0	7·0	10·0	1·7	0·3
	Sour	—	51·7	51·7	51·4	50·0	52·0
	Salty	—	38·0	38·0	38·6	38·7	37·3
	Bitter	—	1·3	3·3	—	9·7	10·2
	Bland	100	—	—	—	—	—
5	Sweet	—	0·1	0·4	0·2	0·7	1·0
	Sour	—	91·6	67·3	56·9	64·7	55·7
	Salty	—	7·0	23·7	35·0	29·3	37·0
	Bitter	—	1·3	8·6	7·7	5·3	6·3
	Bland	100	—	—	—	—	—

Values for each stimulus are the mean of 15 replications for each subject. Blandness indicates no taste perceived.

*Subjects 1 and 2 commented that a "yeasty" quality was evident (possibly a contaminant).

some subjects as to the ranking of some of the amino acids. The greatest differences were found in the rankings of glycine, leucine, and glutamic acid, which were probably influenced by the subject's sensitivity to the particular stimulus. It would have been interesting to rank the samples at other concentrations; however, time did not permit. The evidence suggests that more investigations are needed before definitive conclusions can be reached.

TABLE 8. TASTES PERCEIVED AND CONTRIBUTION TO THE OVER-ALL TASTE OF PHENYLALANINE

Subject	Taste	Concentration — M					
		0	0·0078	0·0157	0·022	0·0313	0·0625
1	Sweet	—	—	—	—	—	—
	Sour	—	—	—	—	—	—
	Salty	—	—	—	—	—	—
	Bitter	100	100	100	100	100	100
	Bland	—	—	—	—	—	—
2	Sweet	—	—	—	—	—	—
	Sour	—	—	—	0·7	0·3	6·3
	Salty	1·0	6·7	10·7	10·7	13·7	13·0
	Bitter	45·7	86·7	89·3	88·6	86·0	80·7
	Bland	53·3	6·6	—	—	—	—
3	Sweet	—	—	0·3	—	1·0	0·3
	Sour	8·3	2·7	26·0	33·6	28·0	29·3
	Salty	—	—	—	—	—	—
	Bitter	11·7	11·7	46·3	66·4	67·7	70·3
	Bland	80·0	85·7	27·3	—	3·3	—
4	Sweet	—	—	—	—	—	
	Sour	—	—	—	—	—	—
	Salty	—	—	—	—	—	—
	Bitter	60·0	86·7	100	100	100	100
	Bland	40·0	13·3	—	—	—	—
5	Sweet	—	—	—	—	—	—
	Sour	—	—	0·7	2·9	3·3	1·3
	Salty	—	—	—	—	—	—
	Bitter	20·0	80·0	99·3	97·1	96·7	98·7
	Bland	80·0	20·0	—	—	—	—

Values for each stimulus are the mean of 15 replications for each subject. Blandness indicates no taste perceived.

Results of the hedonic ratings (Figs. 2 and 3) are not surprising in view of the preponderance of bitterness responses recorded by the panel. Interestingly, the results with glycine show the same type of relationship reported by Halpern *et al.* (1962): greatest acceptance at 0·35 M with rejection (or dislike, in our tests) at higher concentrations. However, subjects 1 and 2 showed a reversal of the dislike trend, endorsing the results reported by Schutz and Pilgrim (1957), and emphasizing the importance of evaluating individual responses before considering pooled information.

TABLE 9. TASTES PERCEIVED AND CONTRIBUTION TO THE OVER-ALL TASTE OF ARGININE

Subject	Taste	Concentration — M					
		0	0·0125	0·0250	0·036	0·050	0·100
1	Sweet	—	—	—	—	—	—
	Sour	9·3	18·7	20·7	30·0	26·0	25·3
	Salty	8·0	13·3	11·3	10·0	14·0	14·0
	Bitter	76·0	68·0	68·0	60·0	60·0	60·7
	Bland	6·7	—	—	—	—	—
2	Sweet	10·7	45·7	44·0	37·1	37·0	34·3
	Sour	—	—	—	—	—	—
	Salty	—	9·3	10·0	24·3	14·0	16·0
	Bitter	2·7	45·0	45·3	37·9	48·7	49·7
	Bland	86·7	—	—	0·3	—	—
3	Sweet	—	40·7	40·7	42·1	37·3	39·7
	Sour	—	20·3	22·0	25·0	27·3	24·7
	Salty	—	21·3	22·3	19·3	18·0	18·0
	Bitter	—	12·3	15·0	13·6	17·3	17·7
	Bland	100	5·3	—	—	—	—
4	Sweet	4·7	6·7	9·3	10·0	10·0	10·0
	Sour	18·7	26·7	37·3	40·0	40·0	40·0
	Salty	14·0	20·0	28·0	30·0	30·0	30·0
	Bitter	9·3	13·3	18·7	20·0	20·0	20·0
	Bland	53·3	33·3	6·7	—	—	—
5	Sweet	6·0	72·9	77·7	64·3	62·7	57·7
	Sour	0·1	—	—	—	1·3	1·3
	Salty	—	—	0·3	—	—	—
	Bitter	7·2	20·2	22·3	35·7	36·0	41·0
	Bland	86·7	6·7	—	—	—	—

Values for each stimulus are the mean of 15 replications for each subject. Blandness indicates no taste perceived.

The data for valine show the same relationship as that found with rats in the Halpern study: rejection or definite dislike at all concentrations. No other comparisons were possible, since the test stimuli were different.

The data describing the taste properties of the amino acids are interesting from several viewpoints. Most of the amino acids possess a primary taste and minor components of other tastes which are not detectable at low concentrations. Although the data are for only a few of the many compounds possessing the $RR'C$ (NH_2) COOH skeleton, they may be applied to the taste and structural criteria described by Kaneko (1939) and Cohn (cited in Moncrieff, 1951). Kaneko indicated that for the amino acids, R

TABLE 10. 6,N-PROPYL THOURACIL DETECTION THRESHOLDS FOR SUBJECTS IN A TRIANGLE DIFFERENCE TEST

Subject	Concentration M $\times 10^{-5}$				Threshold
	8·74	4·37	2·18	1·09	
1	11	12	10	9	1·09–2·18[a]
2	11	9	5	10	2·18–4·37[a]
3	12	10	7	4	2·18–4·37
4	12	10	6	6	2·18–4·37
5	12	12	12	9	1·09

Table values are correct decisions out of a total of 12 tests at each stimulus concentration. All thresholds significant at $P > 0{\cdot}05$.

[a]Thresholds for subjects 1 and 2 were high because of response of bitter to water solutions.

TABLE 11. INDIVIDUAL RANKINGS OF THE AMINO ACIDS

Amino acid	Subject				Average
	2	3	4	5	
Glycine	6 (58)	7(44)	8(44)	3(74)	6(55)
Valine	1 (96)	4(75)	3(71)	2(76)	2(79)
Leucine	8 (50)	6(56)	6(49)	7(46)	8(50)
Isoleucine	4 (71)	8(39)	7(46)	6(49)	7(51)
Phenylalanine	5 (66)	5(59)	4(60)	8(42)	5(57)
Arginine	2·5(90)	3(79)	5(54)	5(64)	3(72)
Aspartic acid	2·5(90)	1(91)	1(87)	1(100)	1(92)
Glutamic acid	7 (51)	2(81)	2(76)	4(72)	4(70)

Subject 1 was not available for this experiment. Values in parentheses are the values assigned on a 100-point scale and are the mean of 8 replications for test days 3–10.

may be any homolog of methyl but R' must be smaller than propyl. Cohn's criteria for α-amino acids indicated only the basic $C(NH_2)COOH$ structure for sweetness but also extended to other types of compounds. Glycine was the only stimulus that met the requirement of R or R' being smaller than a propyl group, and it was predominantly sweet. Threonine was also studied but in less detail. It was reported by three subjects as being primarily sweet, with residual bitterness. The other two subjects said bitterness was the primary taste. These comments are interesting in view of the structural similarities between valine and threonine. The former has an —OH group in place of the valine's $-CH_3$ group, which is a bitter stimulus. A similar condition seemed to hold for glutamic and aspartic acids. Apparently, the presence of the CH—OH moiety between the

COOH groups reduced subjective intensity and sourness and introduced sweetness and saltiness. Increasing chain length also resulted in a change from sweetness to bitterness; however, intensity of bitterness did not increase concurrently. The ranking data indicated valine had a more intense taste than phenylalanine, leucine, or isoleucine. Of course, the molecular weight of the latter three may have been sufficient to reduce their effectiveness as bitter stimuli. However, it must be realized that molecular weight, structure, optical activity, presence of active groups (—OH, —NH_2, —COOH), and other factors may interact to inhibit or enhance the taste intensity and quality of a stimulus.

Taste Theory

Application of some of these data to Beidler's theory of taste stimulation (1954) was considered important to the study. Although the data were obtained in behavioral experiments with man, the basic procedure was the same. If the theory is correct, these data should be adequately described by the taste equation:

$$C/R = C/Rm + 1/KRm,$$

R is response at concentration C,
K is equilibrium constant,
Rm is maximum response.

The theoretical aspects of this theory are described by Beidler (1954, 1961). Their relationship to the data are discussed elsewhere (Stone and Singleton, 1965). Of importance to this discussion is Beidler's plot of the data (C/R vs. C) which yielded a series of equations in accord with his theory of taste stimulation (Fig. 4). The equation of the line for each stimulus was determined by the method of least squares (Goulden, 1952). These and other data (Rm, K, ΔF) are discussed in another report (Stone and Singleton, 1965). That there was some data variability is not surprising, since data represent the pooled behavioral responses of the panel. As a further test of the theory, the fundamental taste equation was rearranged to read

$$R/Rm = \frac{CK}{CK+1},$$

so that a plot should yield a straight line with a slope equal to one ($Y = X$). The fundamental taste equation was used to determine Rm, the maximum

response, and K, the equilibrium constant, for each amino acid. These data were utilized in the revised equation and the resultant values were plotted on logarithmic paper (Fig. 5). Agreement with Beidler's theory of

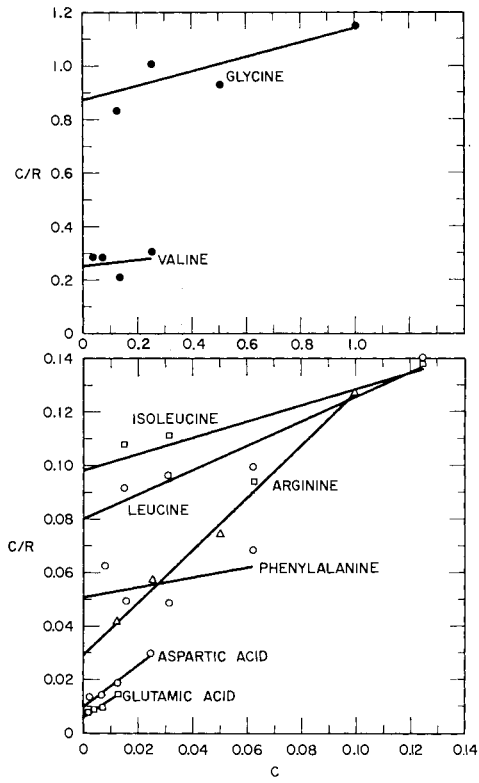


FIG. 4. The ratio of the molar concentration of the stimulus and the magnitude of the subjective response plotted against molar concentration of the stimulus.

taste stimulation is excellent. This is especially significant in view of the species difference of the experimental animal and the different experimental levels at which these data were obtained.

ACKNOWLEDGEMENTS

The author would like to acknowledge the excellent technical assistance of Mrs. Shirley Oliver and the cheerful cooperation of the panel.

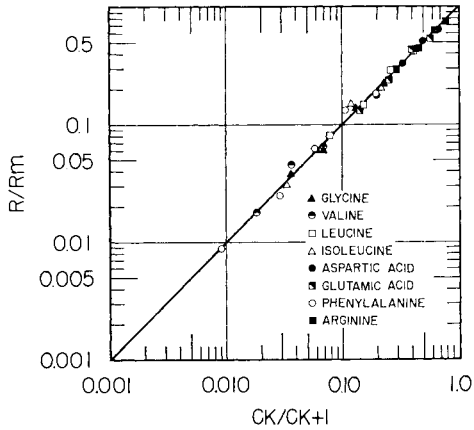


FIG. 5. The ratio of the subjective response and the maximum response plotted against the ratio of the concentration times the equilibrium constant and the concentration times the equilibrium constant plus one. The maximum response and equilibrium constant for each stimulus were determined from the data of Fig. 4.

REFERENCES

BEIDLER, L. M., 1954. A theory of taste stimulation, *J. Gen Physiol.*, **38,** 133.

BEIDLER, L. M., 1961. Taste receptor stimulation, in *Progress in Biophysics and Biophysical Chemistry*, **12,** Pergamon Press, New York.

BERG, C. P., 1953. Physiology of the D-amino acids, *Physiol. Rev.*, **33,** 145.

GOULDEN, C. H., 1952. *Methods of Statistical Analysis*, 2nd edition, New York, J. Wiley and Sons, Inc.

HALPERN, B. P., BERNARD, R. A., and KARE, M. R., 1962. Amino acids as gustatory stimuli in the rat, *J. Gen. Physiol.*, **45,** 681.

JONES, L. V., PERYAM, D. R., and THURSTONE, L. L., 1955. Development of a scale for measuring soldiers' preferences, *Food Research*, **20,** 512.

KANEKO, T., 1938. Taste and constitution of L-amino acids, *J. Chem. Soc. Japan*, **59,** 433 (cited in *Chem. Abst.*, 1938, **32,** 9062).

KANEKO, T., 1939. Taste and constitution of L-amino acids. II Stereochemistry of L-amino acids, *J. Chem. Soc. Japan*, **60,** 531 (cited in *Chem. Abst.*, 1942, **36,** 2874).

LAWRENCE, A. R., and FERGUSON, L. N., 1959. Exploratory physicochemical studies on the sense of taste, *Nature*, **183,** 1469.

PFAFFMANN, C., 1959. The sense of taste, in *Handbook of Physiology*, Section 1. Neurophysiology (J. FIELD, editor), American Physiological Society, Washington, 1, 507.

STONE, H., and SINGLETON, R. C., 1965. On Beidler's theory of taste stimulation, to be published.

SCHUTZ, H. G. and PILGRIM, F. J. Sweatness of various compounds and its measurement. *Food Research*, **22,** 206, 1957.

THE EFFECT OF DENTURE FACTORS ON THE GUSTATORY SENSITIVITY OF DENTURE WEARERS

KRISHAN K. KAPUR,* THOMAS COLLISTER,† and EUGENE FISCHER.†

University of Detroit, School of Dentistry, Detroit, Mich.

RESEARCHERS from many scientific disciplines have long been intrigued by the complexity of the oral cavity. Physiologists, psychologists, food technologists, industrial chemists, physicians, and oral biologists have made many investigations, particularly into the taste phenomenon.

The primary interest of dentists has been to maintain structural and physiological integrity of the oral cavity. Until recently, dental research has been confined primarily to oral diseases. Very little has been done to study the oral sensory mechanisms. Even less is known about how artificial dentures influence these natural processes. Manly, *et al.*[1] reported that the tactile thresholds of teeth in denture wearers were about 10 times as great as in subjects with natural dentition and that the denture wearers exhibited significantly less sensitivity in the acuteness of hardness judgements. Giddon *et al.*[2] found that the denture wearers failed to perceive subtle differences in the sweet taste of certain solid foods when compared with subjects having natural dentition.

Since the work done by these investigators, virtually no research has been reported in the area of stomato-sensory phenomena of denture wearers. Gustatory studies in denture wearers are not only of practical significance but also offer a promising means by which the masticatory-salivary reflex may be studied.

The purpose of this investigation was to determine the influence of

This investigation was aided by Public Health Service Research Grant DE 02117 from the National Institute of Dental Research.

* Professor and Chairman, Department of Oral Biology, Director of Research and Graduate Studies.

† Research Associate, Department of Oral Biology.

dentures and their palatal surface topography and material on the gustatory sensitivity of denture wearers. Taste studies have been made to determine taste thresholds.[3, 4, 5] These studies used subjective methods of tasting solutions. Quantitative measurements of gustatory sensitivity have been obtained by determining the parotid gland secretion rate.[6, 7, 8] The latter technic was employed in this study.

METHOD OF PROCEDURE

The first investigation was conducted to study the effect of masticatory and gustatory stimuli on parotid and whole saliva secretion rates in edentulous subjects wearing complete dentures. Thirty-one denture wearers, ranging in age from 24 to 80 years with a median age of 48 years, were selected for this experiment. Subjects on medication or having a history of medical complications were rejected during the screening examination. Determinations of secretion rates of parotid fluid and whole saliva were recorded for each subject on 2 consecutive days. One day the subject wore artificial dentures, the other day he wore none. The order of testing these 2 conditions was alternated among subjects.

TYPICAL TEST PROCEDURE

The subject was first familiarized with the procedure so that his apprehension would be minimized. He was seated in an upright position in a dental chair. His head was supported by the headrest for the parotid secretion collection and was slightly tipped forward for the whole saliva collection. The subject was asked to rinse his mouth thoroughly so as to flush out all food debris. Secretion rate tests were run. The sequence of the whole and parotid secretion rate determinations was alternated among the subjects.

PAROTID SECRETION RATES

A vacuum cup was used to obtain the parotid saliva.[9] Samples were collected in tubes graduated to 0·1 ml. The flow rate was calculated by measuring the volume of the sample secreted in a standard time of 10 min. After the initial placement of the vacuum cup, it took about 15 min for the collecting ring and tube to be filled with parotid fluid and to be free of air bubbles. An additional 5 min was given as an acquaintance interval and the "clearance sample" so obtained was discarded. This was followed by a 10 min collection of the "experimental sample" for the rest

flow rate. During the collection period the subject was instructed not to engage in any oral activity other than swallowing. Subjects were not permitted to go to sleep.

The effect of the gustatory and masticatory stimuli was evaluated by the method described by Chauncey and Shannon.[10] For the 4 modalities of taste, solutions of 30 per cent sucrose, 2 per cent citric acid, 0·15 per cent quinine and 15 per cent sodium chloride were used as the test solutions. The method of application of a test solution involved swabbing the entire dorsum of the tongue including the lateral margins with wetted cotton swab applicator sticks, every 15 sec. A 5 min clearance sample was followed by the collection of a 10 min experimental sample for each of the 4 test solutions. The testing sequence of the 4 gustatory stimulants was selectively rotated among the subjects. Before taking the clearance sample for each test solution, the subjects received 20 cc of water to swallow through a pipette.

For the masticatory stimulus, 3 rubber bands of 16 gauge, knotted in the form of a bow tie, were employed. Each subject was told to chew the rubber bands on the side opposite to which the vacuum cup was placed. Again, a 10 min experimental sample following a 5 min clearance sample was obtained. The same side was used for the collection of parotid fluid in a given subject for all tests.

WHOLE SALIVA SECRETION RATE

Whole saliva was collected by instructing a subject to accumulate the saliva in his mouth and spit every 30 sec into a tube graduated to 0·5 ml. Subjects were often reminded to refrain from swallowing the saliva. After discarding a 5 min clearance sample, the 10 min experimental sample for rest flow was taken. During this collection period the subject was told not to engage in any oral activity. The effect of chewing upon flow rate was determined by having the subject chew the 3 knotted rubber bands. A 10 min experimental sample followed a 5 min clearance sample.

Effect of Palatal Surface Topography and Material on Secretion Rates

Twelve denture wearers who needed new dentures participated in this experiment. Three maxillary dentures, differing only in their palatal surface topography and material and fitting to one mandibular denture, were constructed for each subject. Specifically, the 3 maxillary dentures differed in that Denture 1 had a vitallium metal palate with the simulated rugae

pattern of the given subject, Denture II was an all acrylic denture with the simulated rugae pattern of the given subject, and Denture III was an all acrylic denture with a smooth palatal surface. The flasking and processing technique reported by Marcroft[11] was used to ensure the duplication of these maxillary dentures in all other respects. All 3 maxillary dentures were adjusted to the mandibular denture for the same occlusal relationship. In this way the same maxillo–mandibular relationship and the same denture forms, with the exception of the palatal surfaces, were maintained for all 3 combinations.

PROTOCOL OF TESTING

Whole and parotid secretion rate determinations were made in each subject for each of the 3 denture combinations and without any dentures. These 4 conditions were tested 4 days in a row, one day for each condition, prior to the delivery of dentures for home use. The testing of these conditions was selectively rotated among the 12 subjects (Table 1). The same typical test procedure as described above was followed at each test sitting.

After having tested the 4 conditions, each subject was given one set of

TABLE 1. TESTING PROTOCOL FOR 12 SUBJECTS

Prior to any denture delivery					Before and after using dentures for 3 weeks					Additional test
Sitting no.:	1	2	3	4	5	6	7	8	9	10
Subject no.:					After	Before	After	Before	After	
1	0	*M	A_1	A_2	M	A_1	A_1	A_2	A_2	M
2	A_2	0	M	*A_1	A_1	A_2	A_2	M	M	A_1
3	A_1	A_2	0	*M	M	A_2	A_2	A_1	A_1	M
4	*A_1	M	A_2	0	A_1	M	M	A_2	A_2	A_1
5	A_2	A_1	*M	0	M	A_2	A_2	A_1	A_1	M
6	M	*A_2	0	A_1	A_2	A_1	A_1	M	M	A_2
7	0	*A_1	M	A_2	A_1	M	M	A_2	A_2	A_1
8	A_1	0	*A_2	M	A_2	A_1	A_1	M	M	A_2
9	A_2	0	*A_1	M	A_1	M	M	A_2	A_2	A_1
10	M	*A_1	A_2	0	A_1	A_2	A_2	M	M	A_1
11	*A_2	M	0	A_1	A_2	A_1	A_1	M	M	A_2
12	0	*M	A_2	A_1	M	A_2	A_2	A_1	A_1	M

M—Metal palate with rugae.
A_1—Acrylic palate with rugae.
A_2 — Acrylic palate without rugae.
* — Denture delivered for home use after 4th test sitting.

preassigned dentures for 3 weeks. At the end of this period, the subject was scheduled for 2 consecutive days. On the first day, the dentures which the subject had worn for 3 weeks were tested. A new maxillary denture was substituted and tested on the second day. At this time, the subject was asked to use this new combination of dentures for a period of 3 weeks. The same procedure was repeated for the second and third combinations of dentures. This protocol permitted us to allow every subject to become used to each combination of maxillary and mandibular dentures for 3 weeks. The sequence in which the 3 dentures were assigned is shown in Table 1.

RESULTS AND DISCUSSION

The means for the parotid and whole saliva secretion rates of the same 31 denture wearers were separately calculated for the presence and absence of dentures. Comparisons of the parotid response for these two conditions with different stimulants are presented in Table 2. As was expected, in both instances the stimulated outputs were higher than the resting flow and the secretion rates varied considerably with different stimulants. In each case, with or without dentures, the variations between mean parotid responses for different stimuli were statistically significant ($P < 0{\cdot}01$). The order of the effectiveness of the 5 stimulants in the two situations also remained unchanged. Citric acid acted as the most effective parotid stimulant with mean flow rates of 0·248 and 0·232 (ml/min/gland) in the presence and absence of dentures respectively. These mean values are quite lower than

TABLE 2. EFFECT OF MASTICATORY AND GUSTATORY STIMULI ON THE PAROTID SECRETION RATES IN 31 DENTURE WEARERS WITH AND WITHOUT DENTURES

Stimulus	Without dentures ml/min/gland			With dentures ml/min/gland			Comparison	
	mean	S.D.	range	mean	S.D.	range	%rise*	*t*-ratio
Resting	0·037	0·006	0·00–0·20	0·044	0·008	0·00—0·15	20	0·70
Chewing rubber band	0·071	0·009	0·01–0·25	0·090	0·011	0·01—0·19	26	1·28
Sucrose	0·116	0·013	0·01–0·35	0·135	0·018	0·02—0·28	16	0·80
Citric acid	0·232	0·028	0·06–0·67	0·248	0·027	0·01–0·80	7	0·41
Sodium chloride	0·166	0·020	0·01–0·42	0·192	0·022	0·02–0·51	16	0·84
Quinine	0·093	0·012	0·00–0·30	0·117	0·016	0·01–0·03	26	1·18

* Per cent rise in secretion rate with dentures over the secretion rate without dentures.

those reported by Chauncey and Shannon.[10] The fact that the population in their study was younger and had natural dentition may account for higher secretion rates.

Other investigators[6, 8] have reported increases of 7–10 times the resting flow of the parotid gland secretion with ipsilateral chewing of wax or rubber bands. This led to the belief that periodontal receptors play a direct role in the masticatory-salivary reflex. However, contralateral chewing of rubber bands in this study produced much lower increases, 1·9 times the resting flow rate without dentures and 2 times with dentures. Contralateral chewing was preferred over ipsilateral by the investigators because of the problem of maintaining the parotid cup in place for long periods of time. Further, it was considered that the denture would act as a solid object and transmit chewing pressure to the underlying tissues on both sides. Bilateral occlusal contact of dentures during mastication has been substantiated by previous reports.[12, 13] Therefore, contralateral chewing in denture wearers was expected to produce substantially the same results as ipsilateral.

The extremely low parotid response to contralateral chewing in this study suggested two possible explanations: either, our original assumption was wrong and contralateral chewing in denture wearers does not produce substantially the same results as does ipsilateral chewing; or, edentulous subjects did not respond as well to masticatory stimulus as do persons with natural dentition because of the loss of periodontal receptors.

Therefore, an experiment was conducted on 6 subjects wearing complete dentures to compare the effects of ipsilateral and contralateral chewing on parotid secretion rate. The results contradicted our original expectation that contralateral chewing would be as effective as ipsilateral chewing in provoking parotid secretion. In all 6 subjects the parotid output was considerably higher with ipsilateral chewing than with contralateral. The mean parotid outputs were 0·05 ml/min/gland for resting flow, 0·10 ml/min/gland for contralateral chewing, and 0·25 ml/min/gland for ipsilateral chewing. The marked effect of ipsilateral chewing on the parotid output in denture wearers was quite similar to that found by Kerr[8] and Lashley[6] in subjects with natural dentition. It demonstrates that the masticatory salivary reflex was not affected by the loss of teeth. This finding either refutes the concept of the direct role of periodontal receptors in the masticatory-salivary reflex and or substantiates the possibility that this role has been transfered to receptors in edentulous mucosa.

Comparison of the parotid outputs with and without dentures showed slight increases with dentures for both the resting and stimulated secre-

tions. The percent rise in secretion rates with the presence of dentures is also shown in Table 2. These increases, however, were found to be statistically insignificant. This demonstrates that the gustatory sensitivity was not affected by the presence of dentures in edentulous persons. This finding supports our previous results* that the taste thresholds of denture wearers were not markedly altered by the presence of dentures. Slight increases of secretion rates with dentures were probably caused by the tactile stimulation of oral mucosa by dentures. The long experience this group of subjects had with dentures may have kept the rise in secretion rates at a minimum.

TABLE 3. COMPARISON OF MEAN VOLUMES OF WHOLE SALIVA OBTAINED IN 31 DENTURE WEARERS WITH AND WITHOUT DENTURES

Secretion[3]	Without dentures (ml/min)			With dentures (ml/min)			Comparison	
	mean	S.D.	range	mean	S.D.	range	%rise*	*t*-ratio
Resting	0·50	0·05	0·07–1·18	0·51	0·05	0·08–1·30	2	0·13
Chewing rubber bands	0·84	0·07	0·11–1·85	1·02	0·10	0·25–2·40	22	1·52
Resting vs. Chewing *t*-ratio	4·41‡			2·38†				

* Per cent increase in secretion output with dentures over the secretion output without dentures.

† Significant at 5% level.

‡ Significant at 1% level.

Similar comparisons between the presence and absence of dentures were made for the total salivary secretions and are presented in Table 3. Stimulated secretions were significantly greater than the resting flow. The total salivary responses to chewing rubber bands were slightly higher with dentures than without; however, these variations were not statistically significant. It was expected that chewing in edentulous subjects would provide greater stimulation and would involve a larger receptor field in the underlying mucosa with dentures than without dentures. The failure of bilateral chewing with dentures to produce much more secretion than without dentures suggests that neither the periodontal receptors nor the receptors of edentulous mucosa are directly involved in the masticatory-

* Unpublished data.

salivary reflex. The reduced tactile sensitivity of teeth in denture wearers reported by Manly *et al.*[1] also supports the fact that there is no adequate reorganization of the receptors in edentulous mucosa after the loss of teeth.

It can be hypothesized that receptors other than the periodontal receptors control the reflex under consideration. A most likely place for these receptors seems to be in the masticatory muscles, especially the masseter. Taking this hypothesis one step further, such receptors would be increasingly stimulated by the rapidly changing contraction of muscles that takes place during chewing of masticatores or during swallowing. A certain amount of resistance between teeth or ridges is necessary to actively stimulate these receptors. Such a resistance requirement, which is not met during a simple opening and closing of the mandible, could explain the limited amount of parotid response.

EFFECT OF PALATAL SURFACE TOPOGRAPHY AND MATERIAL ON THE SECRETION RATES

The observations from the tests performed on four consecutive days, prior to delivery of the dentures, were used for comparing the 3 types of dentures to the control (no dentures).

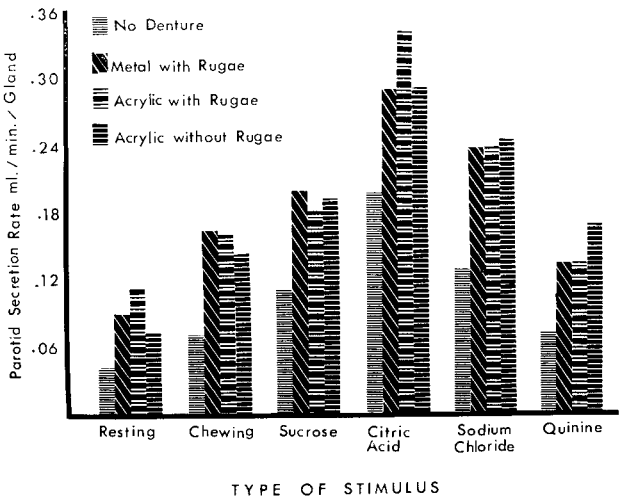


FIG. 1. Comparison of mean outputs of the parotid secretion collected with three types of maxillary dentures and without dentures in 12 denture wearers under different gustatory and masticatory stimulation.

TABLE 4. MEAN DIFFERENCES IN PAROTID OUTPUTS BETWEEN CONTROL (NO DENTURES) AND THREE TYPES OF DENTURES FOR EACH STIMULUS WITH PER CENT CHANGE AND *t*-RATIOS

Stimulus	Metal denture with rugae ml/min/gland			Acrylic denture with rugae ml/min/gland			Acrylic denture without rugae ml/min/gland			*F*-ratio among dentures
	Md	% rise‡	*t*-ratio	Md	% rise‡	*t*-ratio	*Md*	% rise‡	*t*-ratio	
Resting	0·05	110	1·49	0·08	179	1·91*	0·03	77	1·32	<1
Chewing rubber bands	0·08	87	2·13*	0·08	85	1·39	0·06	64	1·35	<1
Sucrose	0·09	85	2·13*	0·10	91	1·93*	0·09	79	2·01*	1·65
Citric acid	0·09	43	1·47	0·14	66	2·26*	0·09	42	1·57	1·36
Sodium chloride	0·08	57	1·76	0·08	58	2·99†	0·10	68	2·39*	<1
Quinine	0·07	89	1·60	0·07	83	2·23*	0·09	116	2·25*	<1

* Significant at 5% level.
† Significant at 1% level.
‡ Per cent increase in secretion output with dentures over the secretion output without dentures.

The mean parotid secretion rates of 12 subjects were calculated separately for the resting flow and for each of the 5 stimuli. These means are shown in Fig. 1. In all instances the output of parotid secretion was higher with dentures than without dentures.

For each stimulus and denture differences between the dentures and control observations were determined. The *t* test against zero mean difference hypothesis was run. In addition, for each stimulus an analysis of variance was calculated to test for mean differences among the 3 dentures. Results are summarized in Table 4, which shows the mean difference, the percent mean change from the control, and the value of *t*. All mean differences are positive and 10 of the 18 *t* values reach the 5 per cent of significance or better using a one-sided *t*. Substantial increases in the resting flow rates were noted with the presence of dentures. These increases were considerably greater than were those seen in the initial group. All *F* ratios showed that variations among secretion rates with the 3 dentures were insignificant. Thus, for each stimulus it is not possible to distinguish statistically among the 3 dentures with respect to change in salivary output from the control.

TABLE 5. COMPARISON OF MEAN VOLUMES OF WHOLE SALIVA COLLECTED IN 12 DENTURE WEARERS WITHOUT DENTURES AND WITH 3 TYPES OF DENTURES

Condition	Resting		Chewint rubber bands	
	(ml/min) Mean S.D.	Comparison with control *t*-ratio	(ml/min) mean S.D.	Comparison with control *t*-ratio
No denture (Control)	0·47 0·14	—	0·74 0·14	—
Metal denture with rugae	0·68 0·16	2·89†	1·05 0·15	3·26†
Acrylic denture with rugae	0·69 0·14	2·51†	0·93 0·17	1·48
Acrylic denture without rugae	0·59 0·13	1·92*	1·07 0·17	3·40†

* Significant at 5% level.
† Significant at 1% level.

The results of the output of whole saliva with the 3 dentures and the control are presented in Table 5. Both the resting and stimulated flow rates were higher with dentures than with no dentures. These increases in flow rates with dentures were statistically significant at the 5 per cent level or better for all dentures except for the stimulated flow rate with the acrylic dentures with rugae. Secretion rate differences among the dentures were insignificant.

COMPARISON OF FLOW RATES BEFORE AND AFTER USE OF DENTURES FOR THREE WEEKS

The effect of adaptation was studied by examining the mean differences in the flow rates before and after the use of each denture for 3 weeks. These mean differences in parotid secretion rates for the 3 types of dentures, and *F* ratios for differences among dentures are given in Table 6. Negative differences indicate reductions in the flow rates after 3 weeks of denture use. None of the differences was significant although there was a suggestion of a pattern in the mean differences. Acrylic dentures with smooth palatal surfaces showed an increase in the parotid output, whereas, dentures with metal palates and simulated rugae showed a decrease in the parotid output, and acrylic dentures with simulated rugae fluctuated around zero.

Similar analysis was made on whole saliva flow rates. Mean differences obtained from observations made before and after the 3 week use of each

TABLE 6. MEAN DIFFERENCES IN PAROTID OUTPUTS BETWEEN OBSERVATIONS MADE BEFORE AND AFTER USING DENTURES FOR 3 WEEKS

Stimulus	Metal denture with rugae ml/min/gland	Acrylic denture with rugae ml/min/gland	Acrylic denture without rugae ml/min/gland	*F*-ratio among dentures
F. R.	−0·051	−0·011	0·033	1·89
Chewing rubber bands	0·054	0·012	0·030	< 1
Sucrose	−0·043	−0·031	0·029	1·23
Citric acid	−0·049	0·016	0·016	1·57
Sodium chloride	−0·008	−0·017	0·033	< 1
Quinine	−0·035	0·001	0·017	< 1

Negative value indicates reduction in parotid output after using the denture for 3 weeks.

denture are given in Table 7. These mean differences were tested against the hypothesis of zero difference and were found to be insignificant.

TABLE 7. MEAN DIFFERENCES IN VOLUMES OF WHOLE SALIVA COLLECTED BEFORE AND AFTER USING DENTURES FOR 3 WEEKS

	Metal denture with rugae ml/min		Acrylic denture with rugae ml/min		Acrylic denture without rugae ml/min	
	Md	*t*-ratio	*Md*	*t*-ratio	*Md*	*t*-ratio
Resting	−0·120	< 1	0·032	< 1	0·023	< 1
Chewing rubber bands	−0·150	1·57	0·149	< 1	−0·006	< 1

Negative value indicates reduction in total salivary output after using the denture for 3 weeks.

COMPARISON OF WHOLE SALIVA FLOW RATES FOR THREE SITTINGS

Each denture was tested 3 times for salivary flow rate in this study. Mean values and the analysis of variance are given in Table 8. The differences between the stimulated and resting flow rates were found to be highly significant, while denture differences were insignificant. Subject differences and subject inter-actions were highly significant, showing that inter- and intra-subject variations of salivary responses were large. Variations in the whole saliva flow rates over the 3 sittings were found to be insignificant.

TABLE 8. MEAN WHOLE SALIVA VOLUMES BY SITTING, DENTURE AND STIMULUS WITH ANALYSIS OF VARIANCE

Secretion	Resting ml/min			Chewing rubber bands ml/min		
Sitting	1	2	3	1	2	3
Metal denture with rugae	0·68	0·52	0·66	1·05	1·12	1·10
Acrylic denture with rugae	0·69	0·56	0·61	0·97	1·14	1·01
Acrylic denture without rugae	0·59	0·58	0·64	1·07	1·03	1·07

Source	d.f.	Mean square	*F*-ratio
Dentures	2	0·0988	<1
Stimuli	1	31·3368	48·90†
Denture × Stimuli	2	0·0920	1·10
Subjects	11	13·4397	20·97†
Subjects × Dentures	22	0·2265	2·70*
Subjects × Stimuli	11	0·6408	7·65*
Residual	22	0·0838	

* Significant at 1% level.
† Significant at 5% level.

Of interest is the possibility of systematic changes in flow rates over the 3 sittings. This aspect of design was studied by using orthogonal polynominals to fit linear and quadratic functions. No significant patterns were found, although there was a suggestion from the means that the flow rates tended to rise slightly over the 3 sittings under stimulation, with no such pattern evident in the resting flow rates. This effect, however, was not significant and must be taken only as a slight indication.

SUMMARY AND CONCLUSIONS

A study of the parotid and total salivary responses in denture wearers revealed that the gustatory sensitivity of edentulous subjects was not affected by the presence of dentures. The mean responses to 4 gustatory stimulants, representing the 4 modalities of taste, were considerably lower than those reported by other investigators for younger groups with natural dentition.

The chewing of rubber bands by edentulous subjects produced significant increases in parotid and total salivary secretions. The parotid output was substantially greater with ipsilateral chewing than with contralateral. These findings discount the possibility that periodontal receptors play the prime role in the masticatory-salivary reflex.

The insertion of new dentures, even in subjects who had had previous denture experience, increased the resting flow rates. Three weeks of adaptation to their new dentures failed to cause any significant changes in flow rates. Palatal surface topography and material did not affect salivary responses.

ACKNOWLEDGEMENTS

We wish to thank Dr. Edward Kozlow, Dental Director, and Mr. William Bannan, Superintendent of the Detroit House of Correction, and their infirmary staff for their kind co-operation which enabled us to complete this study.

REFERENCES

1. MANLY, R. S., PFAFFMAN, C., LATHROP, D. D., and KEYSER, J. Oral Sensory Thresholds of Persons with Natural and Artifical Dentitions, *J. Den. Res.* **31**, 305, 1952.
2. GIDDON, D. B., DREISBACK, M. E., PFAFFMAN, C., and MANLY, R. S. Relative Abilities of Natural and Artificial Dentition Patients for Judging the Sweetness of Solid Foods, *J. Pros. Den.* **4**, 263, 1954.
3. RICHTER, C. P., and CAMPBEL, I. K. Sucrose Tase Thresholds of Rats and Humans, *Am. J. Physiol.* **128**, 291, 1940.
4. COOPER, P. M., BILASH, J., and ZUBEK, J. P. The Effect of Age on Taste Sensitivity, *J. Geront.* **14**, 56, 1959.
5. COHEN, T., and GITMAN, L. Oral Complaints and Taste Perception in the Aged, *J. Geront.* **12**, 294, 1959.
6. LASHLEY, K. S. Reflex Secretion of the Human Parotid Gland, *J. Exp. Psychol.* **1**, 461, 1916.
7. ALLEN, F. The Secretory Activity of the Parotid Gland, *Quart. J. Exp. Physiol.* **19**, 337, 1929.
8. KERR, A. C. *The Physiological Regulations of Salivary Secretions in Man.* International Series of Monographs on Oral Biology No. 1, New York, 1961. Pergamon Press, pp. 48–73.

9. SHANNON, I. L., PRIGMORE, J. R. and CHAUNCEY, H. H. Modified Carlson-Crittenden Device for the Collection of Parotid Fluid, *J. Den. Res.* **41**, 778, 1962.
10. CHAUNCEY, H. H., and SHANNON, I. L. Parotid Gland Secretion Rate as Method for Measuring Response to Gustatory Stimuli in Humans, *Proc. Soc. Exp. Biol. and Med.*, **103**, 459, 1960.
11. MARCROFT, K. R. Fabrication of Identical Duplicate Dentures. *J.A.D.A.* **64**, 476, 1962.
12. YURKSTAS, A. A., and EMERSON, W. H. A Study of Tooth Contact During Mastication with Artificial Dentures, *J. Pros. Den.* **4**, 168, 1954.
13. BREWER, A. A., and HUDSON, D. C. Application of Miniaturized Electronic Deviceto the Study of Tooth Contact in Complete Dentures, *J. Pros. Den.* **11**, 62, 1961.

ON THE MECHANISM OF THE TASTE DEFECT IN FAMILIAL DYSAUTONOMIA

ROBERT I. HENKIN

Clinical Endocrinology Branch
National Heart Institute
National Institutes of Health
Bethesda, Maryland

DURING our continued study of taste we became interested in patients with familial dysautonomia because of their decreased taste sensitivity.[1] Familial dysautonomia is a congenital disorder characterized by many findings, including orthostatic hypotension, absence of lacrimation, hyporeflexia and relative insensitivity to temperature and pain. Since methacholine has been shown to initiate lacrimation in these patients,[2] we administered this drug to see what effect it would have on their ability to taste. Subsequently, we have used this disease as model system to elucidate some of the mechanisms underlying taste perception in the normal individual.

Thirteen solutions each of sodium chloride (NaCl), sucrose, urea and hydrochloric acid (HCl) ranging in concentration from 3×10^{-1} to 1×10^{-3} M/l. and saturated solutions of NaCl, sucrose, urea and phenylthiocarbamide were used to measure detection and forced choice recognition thresholds in six patients. Thresholds for the smell of pyridine and thiophene in water and thiophene and nitrobenzene in mineral oil were also studied repeatedly. Details of these techniques have been previously reported.[1,3] Methacholine (0·05 to 0·10 mg/Kg) and/or acetylcholine (0·70 to 1·0 mg/Kg) were administered on the tongue, subcutaneously and/or intravenously to three patients to observe the effects on taste thresholds. In addition, 3×10 mm biopsies of the anterior and posterior portions of the tongue were carried out in two of these patients. Methacholine was given to five control patients, aged 6–33, in doses similar to those used in the patients with familial dysautonomia. Patients and controls were tested with isotonic sodium chloride, given subcutaneously; in addition, a double blind procedure was used for drug administration and taste testing in the patients.

The median detection thresholds (MDT) for sodium chloride as salt, for sucrose as sweet, for urea as bitter and for HCl as sour were determined in normal subjects and in patients with familial dysautonomia (Fig. 1). For the normal subjects (the enclosed boxes without dots) the range of median detection threshold for the taste of sodium chloride and of sucrose are the same, with a range of 6–60 mM/l. and a median of 12 mM/l. In normal subjects the range for urea is 90–150 mM/l., and the median, 120 mM/l.; for HCl the range is 0·8–6 mM/l., and the median 3 mM/l. These

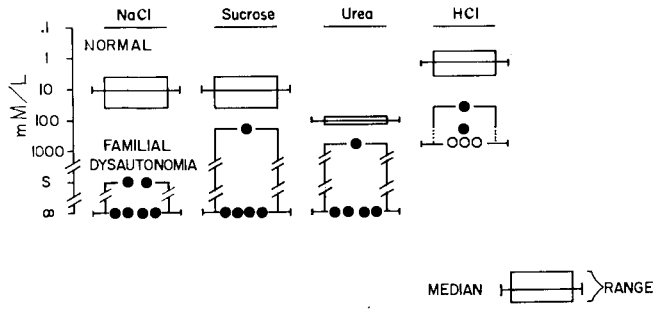


FIG. 1. Median Detection Thresholds (MDT) for taste in normal subjects and in patients with familial dysautonomia. The black and white dots indicate the individual detection thresholds for the patients with familial dysautonomia for each modality tested. The lines enclosing the dots indicate the range of response of the patients. The enclosed boxes without dots indicate the upper and lower limits of the range of response for normal subjects for each of the modalities tested. The lines through the enclosures represent the MDT for all subjects. The scale on the left is in mM/l. The letter *S* refers to a saturated aqueous solution. A black dot at this level indicates that the patient detected this concentration as different from water. A dot level with the infinity sign (∞) indicates that the patient could not detect a difference between a saturated solution of the substance and two comparison stimuli of water. The three white dots and the dotted lines in the column under HCl indicate that these patients could not detect 0·3 N HCl as different from water.

thresholds correspond closely with those reported by other investigators.[4, 5, 6] In the patients with familial dysautonomia, (enclosures with dots) median detection thresholds for the taste of NaCl, sucrose, urea and HCl are incalculably higher than those in the normal subjects. For sodium chloride, the range of response was from *S* (two patients could detect a saturated solution of NaCl as different from H_2O) to ∞ (the remaining four patients could not even detect a saturated solution of NaCl as different from H_2O). Similarly, detection thresholds for sucrose, urea, and HCl were significantly higher than those in normal subjects. Four of the five patients tested could not detect a saturated solution of sucrose or

urea as different from two comparison solutions of H_2O. For HCl (open circles), three of the five patients could not detect any difference between a 0·3 N solution and water.

Notice that there is no overlap in response between the normal subjects and the dysautonomic patients. Every patient with familial dysautonomia exhibits a detection threshold significantly higher than that of the most insensitive of the normal subjects. Whereas there is a pattern of response to various taste modalities in normal subjects, this is not the case with

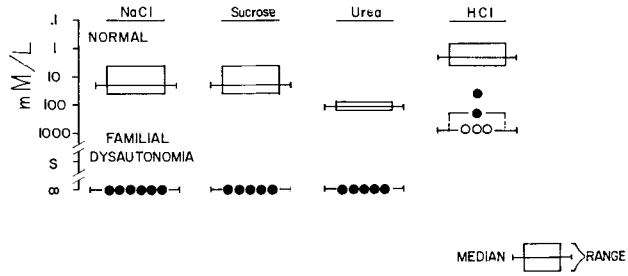


FIG. 2. Median recognition thresholds (MRT) for taste in normal subjects and in patients with familial dysautonomia. Data indications are similar to those in Fig.1.

patients with familial dysautonomia. In the normals the median detection thresholds for sodium chloride and sucrose are about the same, that for urea is approximately four times higher, and that for HCl approximately one tenth as high. Median detection thresholds in the patients with familial dysautonomia are uniformly at the highest concentrations presented to them. The mechanism of this defect is probably not related to cell membrane permeability, since most membranes are permeable to urea, yet urea itself cannot initiate a taste response. Ionization is not a factor, since sucrose does not ionize while sodium chloride does, yet neither substance produces a taste in these patients.

Median recognition thresholds (MRT) for these tastes were also determined in normal subjects and in patients with familial dysautonomia (Fig. 2). As with the MDT, recognition thresholds for patients with familial dysautonomia are significantly higher than those in normal subjects by an incalculable amount for each of the taste modalities tested. Not only were the patients with familial dysautonomia unable to detect the test substances as different from water, but they were all unable to recognize a saturated solution of sodium chloride as salty, a saturated solution of sucrose as sweet, or a saturated solution of urea as bitter.

Metacholine has been administered previously to patients with familial dysautonomia, and shown to initiate lacrimation.[2] With this experiment in mind, we gave varying doses of methacholine subcutaneously and/or intravenously to two of our patients (Fig. 3). Prior to drug administration, thresholds for NaCl in patient S.L. were unobtainable; in patient E.S. thresholds were obtainable only at a saturated solution of sodium chloride.

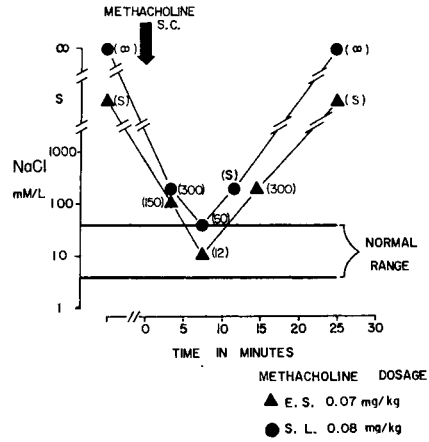


FIG. 3. The effect of methacholine administration upon taste detection and recognition of NaCl in two patients with familial dysautonomia. Concentration of NaCl in mM/l. is on the ordinate, time in minutes on the abscissa. The detection threshold for each of the two patients is represented by the dot and the triangle, respectively, the recognition threshold by the number in parenthesis beside each symbol. Threshold values for NaCl ranging from 6 to 60 mM/l. are within the normal range.

At time 0, methacholine was injected subcutaneously. Shortly, the patients noted flushing, warming of the skin, eructations and flatulence, hyperhydrosis and moderate dyspnea. Beginning at time 0, detection and recognition thresholds for the taste sodium chloride were determined at three- to six-minute intervals. Gradually, over the next few minutes, the patients became able to detect sodium chloride as different from water. Coupled with this phenomenon, but lagging behind it in time, these patients became able to recognize sodium chloride as salty, an ability which they had never before exhibited. At the point of maximal systemic drug effect, coincident with the appearance of tears, the detection thresholds in the patients were 12 mM/l. in one case, 60 mM/l. in the other. These are within normal limits. At this time the patients recognized the taste of these

same concentrations as salty. As the effect of the drug wore off, recognition thresholds increased and then became unobtainable. Finally, the detection thresholds returned to pre-treatment, insensitive levels, with the final wearing off of the drug effect. This return of detection and recognition thresholds to normal occurred with methacholine given either subcutaneously or intravenously. However, if methacholine or acetylcholine was applied directly to the surface of the tongue, even in concentrations ten times those administered subcutaneously or intravenously, the patients exhibited no alteration in taste sensitivity, although the systemic effects of flushing, dyspnea, coughing, and so forth did occur. This apparent inconsistency may serve to emphasize the central nervous system defect in these patients. The symptoms produced by oral administration of small amounts of acetylcholine are in the super-sensitive response of peripheral receptors. Taste thresholds are not altered under these conditions. It is impossible to specify the actual dose of acetylcholine delivered to its effective site of action and the effects of different routes of administration cannot be directly compared. However, if peripheral receptors were affected by oral acetylcholine while taste thresholds were affected only by parenteral administration then it would be tempting to suggest that the changes in taste threshold occurred through the action of acetylcholine at a central nervous system site which is unaffected by oral acetylcholine.

To evaluate these phenomena further, we carefully observed the tongues of these patients. We noted that their tongues were quite different from any tongues we had ever seen. Comparison of the tongues of the mothers of two of these patients with the tongues of their afflicted children (Fig. 4) shows that filiform, fungiform and circumvallate papillae are present on the mothers tongue, but that the tongues of their children are smooth, with only filiform papillae present. These observations have now been confirmed by other workers.[7]

In an effort to evaluate these clinical observations further we have obtained biopsies from the anterior and posterior portions of the tongue in two of the patients with familial dysautonomia, in the areas where we would normally expect to find fungiform and circumvallate papillae, respectively. Methylene blue was injected into the subepithelial areas of the tongue 10–20 minutes before the excision of the sample. The biopsy material was appropriately imbedded, stained and evaluated by light microscopy and by electron microscopy.[8] In confirmation of our clinical impression, there were no fungiform papillae, circumvallate papillae, or taste buds by either microscopic technique in any of the biopsy specimens. These histological observations are shown in Fig. 5.

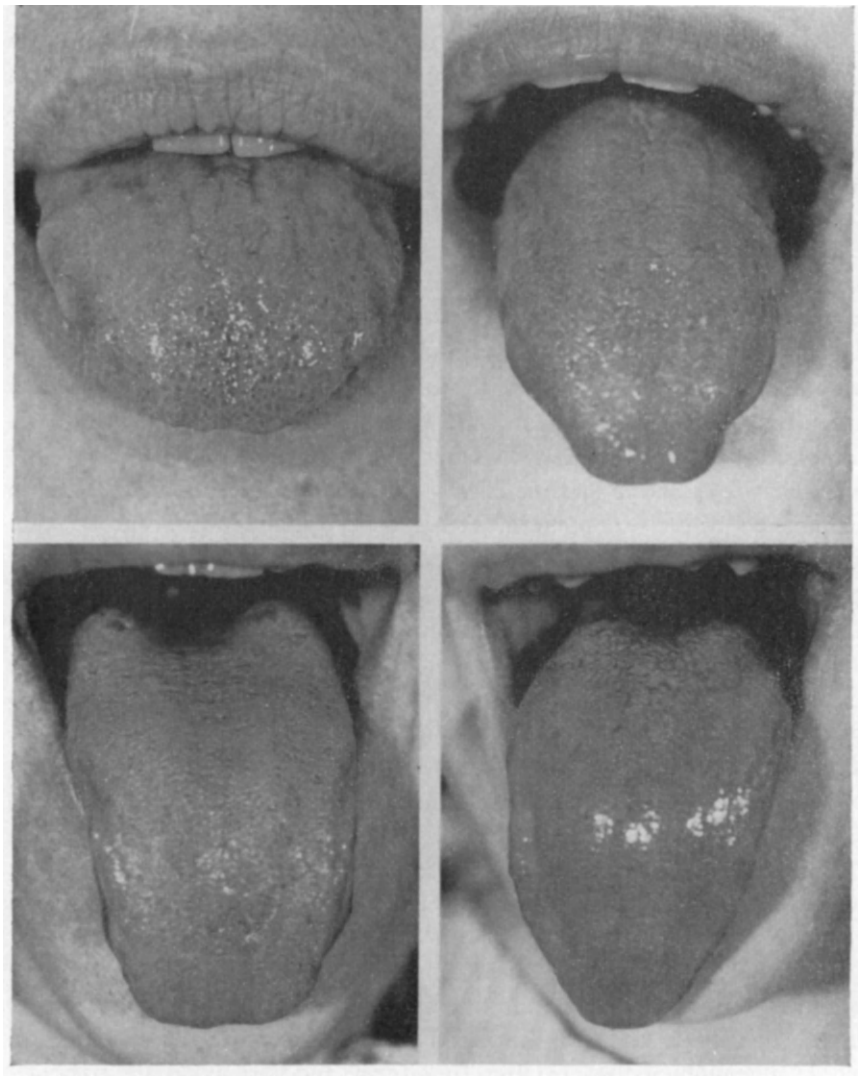


FIG. 4. Comparison of photographs of the tongues of two patients with familial dysautonomia with those of their unaffected mothers. On the right are photographs of the tongues of two patients with familial dysautonomia. Notice that the tongues are smooth, without the normal anatomical architecture and without fungiform or circumvallate papillae. On the left are photographs of the tongues of the unaffected mothers of these patients. Notice that all three types of papillae are present.

On the left (Fig. 5) is an artist's illustration of a normal tongue with normal filiform, fungiform and circumvallate papillae. The left lower insert shows an idealized composite histological section of a fungiform papilla drawn from our observations. Taste buds with their nerve fibers are easily seen as well as a collection of subepithelial nerve fibers which are not in association with taste buds. These are most probably free nerve fibers. These fibers were especially well demonstrated by supravital staining with methylene blue. The upper left insert shows an idealized composite histological picture of a normal circumvallate papilla taken from our data. Taste buds are more numerous here than in fungiform papillae. The neural innervation of these taste buds is similar to that observed in fungiform papillae. A dense network of subepithelial nerve fibers which are not connected with the taste buds is apparent. These fibers, which appear to terminate as free nerve endings, are more numerous in circumvallate than in fungiform papillae.

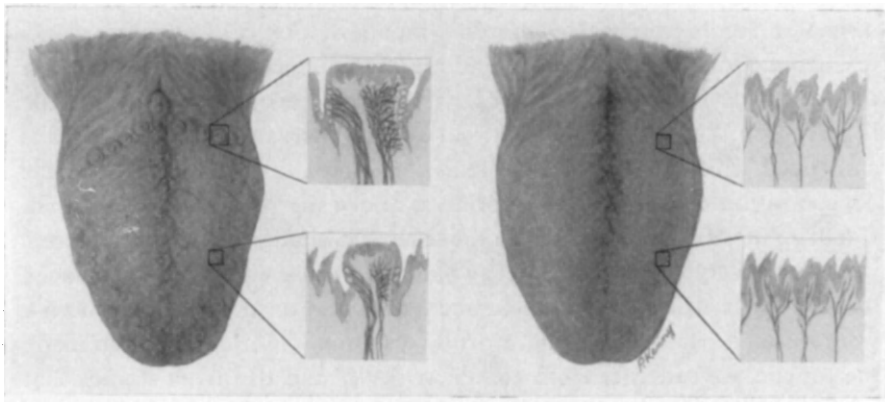


FIG. 5. Comparison of gross and histological structure in the tongues of normal subjects and of patients with familial dysautonomia. On the left is an artist's representation of a normal tongue, showing filiform, fungiform and circumvallate papillae. The lower and upper inserts show an idealized composite histological section through a fungiform and circumvallate papilla, respectively. On the right is an artist's representation of a tongue from a patient with familial dysautonomia. The lower and upper inserts show an idealized composite section through anterior and posterior areas of the tongue which correspond to those areas where fungiform and circumvallate papillae would appear in the normal subject. See text for full explanation.

On the right (Fig. 5) is an artist's illustration of a tongue from a patient with familial dysautonomia. The surface of this tongue appears uniform, without specialized anatomical detail, covered only with filiform papillae. The upper and lower inserts illustrate the histological appearance derived

from biopsy material. Whereas the biopsies from the anterior and posterior portions of the normal tongues showed fungiform and circumvallate papillae, respectively, the anterior and posterior portions of the patient's tongue were similar histologically: both showed an epithelial surface without the specialized structures of taste buds. The only neural elements present were nerve fibers similar in all respects to the ones which we have interpreted as free nerve fibers and which terminate as free nerve endings. There are significantly fewer neural elements in the tongues of dysautonomic patients than in normal tongues. This pattern holds for both the anterior and posterior portions of the tongue in our patients. What role this anatomical abnormality plays in this syndrome cannot be fully evaluated.

With these physiological and histological data in mind, it is possible to formulate some hypotheses about the mechanism of taste in these patients and about the role of acetylcholine-like drugs in returning taste sensitivity to normal (Fig. 6). On the left is illustrated a normal papilla with an idealized taste bud. Below it is an idealized reproduction of the electrical discharge recorded from the chorda tympani, as observed by many workers, from recordings in animals and recently in man.[9,10,11] Initially, we observe the level of spontaneous activity. When a drop of sodium chloride is placed on the papilla, the electrical activity increases markedly. This is associated with the subject's appreciation of the taste as salty when the concentration of the sodium chloride is above the recognition threshold. If a drop of water were placed on the tongue, a similar electrical discharge would be observed, and the subject would report that water had been placed on his tongue. If an acetylcholine-like compound or an anticholinesterase were injected parenterally and a drop of sodium chloride then placed on the tongue, we can infer from animal work[9,10] and also from studies that we have undertaken[1] that there would be a marked increase in electrical activity, recorded from the chorda tympani. The subject would feel slightly flushed and diaphoretic. He would detect the drop of sodium chloride as salty. Our data in five control patients indicate that there is no change in either taste detection or recognition thresholds under these conditions.[1]

On the right in Fig. 6 is illustrated the histological appearance of a comparable anatomical area from the tongue of a patient with familial dysautonomia. The nerve fibers which extend up to the epithelium, and which are similar to free nerve endings, are the only neural elements present. Below the histological section is a reproduction of an idealized recording from the chorda tympani of these patients. We have not carried out these recordings, and the only purpose of this picture is to illustrate our hypothesis better. We postulate that there would be the same sponta-

neous activity of the chorda tympani as in normal subjects. When a drop of sodium chloride was placed on the tongue there would be a discharge of electrical activity. At this time, the patients have stated that they felt a drop of liquid on their tongue but they could not identify this drop correctly as salty, bitter, sweet or sour. Thus, the same type of electrical discharge must take place in patients with familial dysautonomia. Evidence for this hypothesis comes from our own recording of electrical activity in the ulnar nerve of two of these patients in response to electrical, sensory and

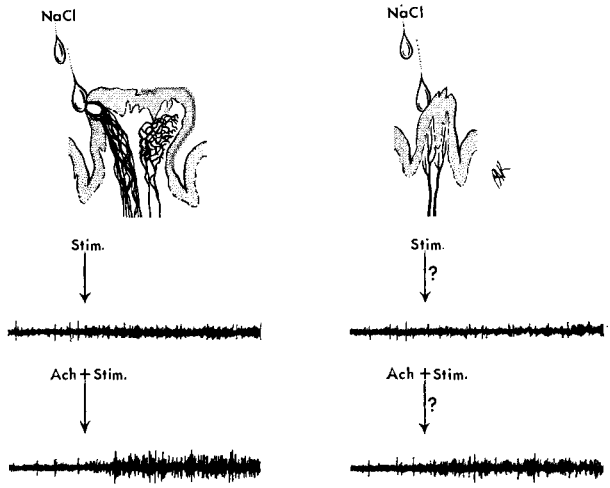


FIG. 6. Comparison of the electrical response to a drop of NaCl placed on a fungiform papilla of a normal subject and on a comparable tongue area of a patient with familial dysautonomia.

In the left upper panel is an artist's representation of a drop of NaCl being placed on an idealized fungiform papilla on the tongue of a normal subject. The left middle panel shows a representation of the electrical recording of the event at the chorda tympani. Initially background activity is recorded. At the arrow the NaCl drop is applied to the papilla and shortly after the electrical discharge is recorded. The left lower panel shows a representation of the same type of recording but after the preparation had been treated with either acetylcholine or one of its homologues. Initially background activity is observed. At the arrow NaCl is applied to the tongue and shortly after the electrical discharge is observed. This is significantly greater than that observed without pretreatment. In the right upper panel is an artist's representation of a drop of NaCl being placed on an area from the anterior two-thirds of the tongue of a patient with familial dysautonomia. This area is where a fungiform papilla would be observed in a normal subject. The right middle panel shows a hypothetical recording of the event at the chorda tympani. By our hypothesis this recording should not differ from that made in the normal subject. The right lower panel shows the same type of recording after the patient had been treated with methacholine. Again, by our hypothesis, the recording should not differ from that made in the normal subject under the same conditions. See text for full explanation.

motor stimulation. Normal ulnar nerve conduction velocity was recorded in the patients. Response to touch or to electrical stimulation was associated with a normal appearing electrical discharge. The electrical response did not fatigue or alter its form with continued supramaximal stimulation. It is reasonable to assume that what occurred in the periphery, in the ulnar nerve, in response to sensory and electrical stimulation, would also occur in the nerves from the tongue.

If an acetylcholine-like drug were administered parenterally to these patients and a drop of sodium chloride were placed on the tongue as shown at the bottom of the figure, we would hypothesize that there would be an increase in electrical activity in the chorda tympani just as there would be in normal individuals. At this time, however, the patients with familial dysautonomia state that they can detect the drop as different from water and that it tastes salty, as they could not do previously. This result allows us to formulate an hypothesis for the mechanism of taste in these patients and quite possibly for that in normal subjects. The patients lack papillae and taste buds, but they do have what appear to be free nerve endings as the only sensory innervation in their tongues. Under baseline conditions they cannot taste, but after receiving methacholine they can taste normally. Thus, it must be possible for them to taste normally through the action of nerve fibers which closely resemble free nerve fibers terminating in free nerve endings without the presence or action of taste buds. It is well known that taste buds are present in areas of the mouth and pharynx other than on the tongue.[12] It appears logical to assume that these areas should subserve the appreciation of taste in these patients. However, this is not the case. Careful questioning of these patients shows that without acetylcholine, taste does not occur on the tongue, on the palate, on the pharynx or on the larynx. With methacholine, however, the patients indicate that taste takes place on the anterior two-thirds of the tongue. The only exception to these observations is that of one patient who was unable to detect or recognize saturated solutions of sodium chloride, sucrose, urea or 0·3 N HCl over the anterior two-thirds of the tongue, but who could recognize saturated solutions of sucrose, urea and 0·3 N HCl over the posterior third. Despite this exception, we present these data as evidence that these patients can taste without the presence of taste buds through the use of nerve fibers which resemble free nerve fibers and through a mechanism which is potentiated by the action of acetylcholine. Support for this hypothesis that free nerve endings can mediate perception of sensory stimuli comes from a number of investigators. Weddell has shown that free nerve endings in the cornea can mediate perception of pain,

touch and temperature.[13] Bardach and Fujigo have observed that the sea robin, a fish that possesses free nerve endings but no taste buds is capable of responding to some gustatory stimuli in a manner similar to fish who possess taste buds.[14] Thus, the absence of taste buds does not preclude the ability to taste.

The data presented here further suggest that the effect of acetylcholine in allowing these patients to perceive taste sensation correctly is a central one, and that the integration which resulted in the correct taste responses in these patients occurred centrally rather than peripherally. Support for this hypothesis is given by findings from an ancillary experiment dealing with the effects of training on taste recognition in these patients. Attempts were made to teach four of our patients to respond correctly to the taste of saturated solutions of NaCl, sucrose, and urea and a 0·3 N solution of HCl. This was done by carefully placing a few drops of each solution on the anterior two-thirds of the tongue and telling the patients that the drops were salty, sweet, bitter or sour, respectively. The procedure was repeated 2 to 3 times for each patient. After training, the patients were retested. Two of the four patients were able to recognize the taste of sucrose, urea and HCl for a few minutes; in five to ten minutes they could not consistently identify any of the solutions. This transient improvement in recognition after training occurred on each of 3 separate days. This improvement of taste after training suggests that there is some characteristic of the solutions presented which allowed the patients to integrate this information about the taste and respond to it properly.

The mechanism by which this training experiment was successful may lie not in the taste modality itself but through the sense of smell. Only those 2 dysautonomic patients whose smell thresholds were normal were capable of being trained. Most probably olfactory information, picked up from the solutions presented to the patients to taste, was integrated and then, after a short length of time, confused or forgotten. We have previously observed this phenomenon of obtaining information about solutions to be tasted through olfactory cues in patients with adrenocortical abnormalities, although mainly with respect to increases in perceptual ability.[15]

These results suggest a number of hypotheses. Since some dysautonomic patients exhibit normal smell thresholds and can be trained transiently to taste, while others exhibit abnormal smell thresholds and cannot be so trained, it appears that patients within the syndrome of familial dysautonomia require further classification. In general, those children who exhibited abnormal smell thresholds were more severely handicapped by their disease than those in whom smell thresholds were normal. In those

children who exhibited normal smell thresholds, we have evidence for a complete sensory system, from receptor to central integration, that is working effectively in spite of the gross abnormalities in the taste system.

If the essential disorder in these patients were in the central nervous system, e.g. in the reticular formation, their multifold neurological abnormalities might be more understandable. It is important to point out that taste abnormalities are not the only oral sensory deficiencies in these patients. Tests for oral stereognosis were performed in those patients who were involved in the training experiments, and were found grossly abnormal, whether or not the patients could be trained to taste. In these studies small plastic forms in various geometric shapes (Fig. 7) were placed in the

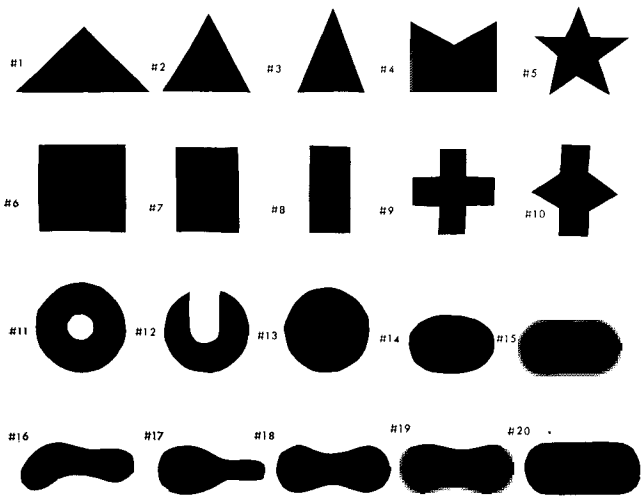


FIG. 7. Photograph of geometric forms used to test oral stereognosis, approximately actual size. See text for explanation. (Courtesy, Dr. J. Bosma, National Institute of Dental Research, Bethesda, Md.)

mouths of each of the patients. They were required to mouth the form and then to identify it, while still in their mouth, from a group of similar plastic forms spread out on a table in front of them. These dysautonomic patients, aware that some object was in their mouth, could identify only the simplest of the forms. They made these identifications inconsistently, with a great deal of effort and only after considerable prodding by the experimenter. The patients took much longer periods of time to make these discriminations than do normal subjects. Thus, not only is their sensory perception of taste impaired but their oral perception in general is markedly abnormal. How this relates to the anatomical findings of lack

of taste buds and other specialized receptors on the tongue, and to the paucity of neural innervation in this area, is not known. However, it is the integrative aspects of these sensory perceptions that are lacking, and not the appreciation of the stimuli themselves. Oral stereognosis is a complex crossed-system task, involving the senses of touch and vision. It is the ability to integrate the sensory components of this task that is lacking in these patients.

Furthermore, the mechanism underlying this disease and the biochemical mechanism through which their taste can be returned to normal offers a unique opportunity to evaluate taste perception. Figure 8 illustrates the biosynthesis of acetylcholine. Acetate, through the action of coenzyme A,

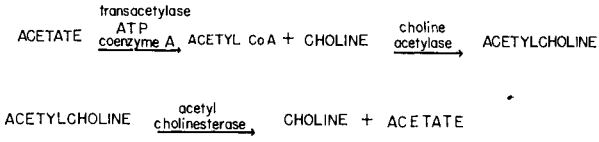


FIG. 8. The biosynthesis and metabolism of acetylcholine. See text.

ATP and transacetylase, forms acetyl CoA. Acetyl CoA plus choline, through the action of cholineacetylase forms acetylcholine. Acetylcholine is metabolized by the enzyme acetylcholinesterase to choline and acetate. Our limited studies suggest that acetylcholinesterase is present in normal concentrations in the red blood cells of these patients, and that pseudocholinesterase is present in normal concentration in their serum.[1] Whether acetylcholinesterase is present in normal amounts in other tissues is not known. However, this observation suggests that the metabolism of acetylcholine to choline and acetate is normal. Similar evidence is indicated from the failure to produce systemic effects of alterations in taste thresholds in the one patient to whom we administered Tensilon. There is little evidence to suggest a specific deficiency in acetylcholine as transmitter substance, for a myasthenia-like response pattern could not be demonstrated after 10 minutes of supramaximal tetanizing stimulation of the ulnar nerve. This observation would tend to rule out any failure of the enzymes transacetylase or cholineacetylase or failure of any of the cofactors taking part in the synthesis of acetylcholine as important in the mechanism underlying this syndrome.

If the metabolism of acetylcholine were normal, one possible mechanism for this abnormality could be the presence of a "false" neurotransmitter. If this were the case then some substance (e.g. a hydrocarbon group-

choline derivative) might substitute, albeit with poor efficiency, for acetylcholine, the "true" neurotransmitter. This false neurotransmitter might allow function at the sensory receptors, although not with normal efficiency. If an acetylcholine-like drug were then administered to the patients, the receptor would function normally or even supramaximally. Some such reaction may well take place for the supersensitivity to acetylcholine we have observed in these patients is quite striking. Some investigators have shown supersensitivity of the pupillary response to local application of $2\frac{1}{2}$ % per cent methacholine[16]. Our experience indicates that acetylcholine administered orally even in 1/100th the quantity known to affect normal subjects can produce cough and dyspnea in these patients.[1].

Although we cannot specify the biochemical defect underlying this syndrome or the taste abnormality, all the data strongly suggest that the disorder involves the cholinergic system. When we more fully understand the physiology and biochemistry of this system, we will know more about this syndrome in particular and about the mechanism of taste in general.

REFERENCES

1. HENKIN, R. I. and KOPIN, I. J. Abnormalities of taste and smell thresholds in familial dysautonomia. Improvement with methacholine. *Life Sciences*, **3**, 1319, 1964.
2. KROOP, I. G. Production of tears in familial dysautonomia. *J. Pediat.* **48**, 328, 1956.
3. HENKIN, R. I., GILL, J. R., Jr. and BARTTER, F. C. Studies on taste thresholds in normal man and in patients with adrenal cortical insufficiency; the role of adrenal cortical steroids and of serum sodium concentration. *J. Clin. Invest.* **42**, 727, 1963.
4. PFAFFMAN, C. "The sense of taste", in *Handbook of Physiology*, Vol. 1, Neurophysiology. *Am. Physiol. Assoc.*, Washington, D. C., 1959.
5. SCHUTZ, H. G. and PILGRIM, F.J. Sweetness of various compounds and its measurement. *Food Research*, **22**, 206, 1957.
6. CRAGG, J. H. The sour taste: threshold values and accuracy, the effects of saltiness and sweetness. *Trans. Roy. Soc. Can.* **31**, (3): 131, 1937.
7. SMITH, A. A. and DANCIS, J. Absence of taste-bud papillae in familial dysautonomia. *Science*, **147**. 1040, 1965.
8. The author wishes to thank Dr. Robert Hoye for his assistance in performing the biopsies in these patients and Dr. Bruce Wetzel for his preparation of the light microscope and electron microscope sections.
9. ZOTTERMAN, Y. The response of the frog's taste fibres to the application of pure water. *Acta Physiol. Scand.* **18**, 181, 1949.
10. LANDGREN, S., LILJESTRAND, G. and ZOTTERMAN, Y. Chemical transmission in taste fibre endings. *Acta Physiol. Scand.* **30**, 105, 1954.
11. ZOTTERMAN, Y. A comparison of neural and psychophysical response to taste. *2nd Int. Symp. on Olfaction and Taste*, Tokyo, Japan, 1965. In press.
12. LALONDE, E. R. and EGLITIS, J. A. Number and distribution of taste buds on the epiglottis, pharynx, larynx, soft palate and uvula in a human newborn. *Anat. Rec.* **140**, 91, 1961.

13. WEDDELL, G. Somesthesis and the chemical senses. *Ann. Rev. Psychol.* **6,** 119, 1955.
14. BARDACH, J. and FUJIGO, M. Investigations of external chemoreceptors of fishes. *2nd Int. Symp. on Olfaction and Taste*, Tokyo, Japan, 1965. In press.
15. HENKIN, R. I., GILL, J. R., Jr. and BARTTER, F. C. Effect of adrenal insufficiency and of corticosteroids on smell threshold. *Clin. Res.* **10,** 400, 1962.
16. SMITH, A. A. and DANCIS, J. Physiologic studies in familial dysautonomia. *J. Pediat.* **63,** 838, 1963.

STUDY ON THE PAROTID SALIVARY SECRETION DUE TO TASTE STIMULI IN RABBITS

ROKURO SUHARA, HIROO TAKASHITA and SHINOBU YAZAKI

Department of Physiology, Nihon University
School of Dentistry, Tokyo, Japan

INTRODUCTION

Here we report our findings on the measurement of the parotid salivary secretion in rabbits which was induced by a few solutions like tartaric acid, sodium chloride, sucrose and hydrochloride quinine in different concentrations as taste stimuli.

METHODS

By way of experimental animals, use was made of more than 60 healthy rabbits, each about 2·0 kg in weight, without distinction of sexes. In order to make the insertion of a polyethylene tube easy into the parotid gland duct on the right side of rabbits, a small surgical operation was performed at their mouth corner. After the operation, rabbits were fed in an animal cage for about 10 days under careful treatment.

For our purposes, we inserted a narrow polyethylene tube, 3/4 mm in outside diameter, into the parotid gland duct and the tube was then connected to a pipette-like manometer filled with water for the measurement of minute volume. As the coloring reagent inside the manometer changed according to the secretion of parotid saliva, changes were read in terms of scales and then plotted.

RESULTS AND DISCUSSIONS

When the secretion of parotid saliva was studied, it was made clear that a certain volume of parotid saliva constantly secreted without any external stimulus. We called this salivary secretion "resting saliva"[1, 2, 3] and with

this "resting saliva" as an indicator, we tried to compare reflex saliva secretions brought about by various taste stimuli above mentioned.

Figure 1 showed the volume curves of parotid "resting saliva" in rabbits. These (*A* and *B*) were measured for 60 minutes, no anaesthesia. Ordi-

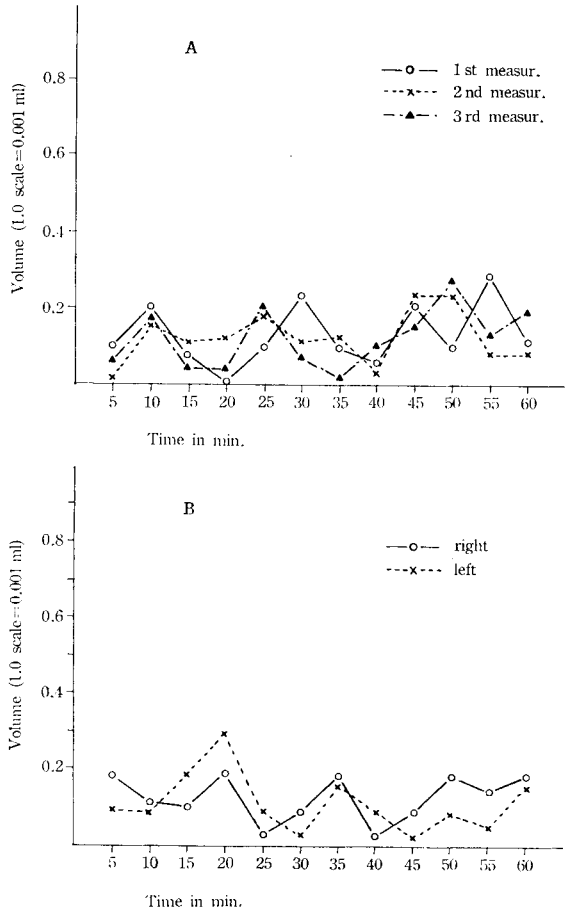


FIG. 1. The volume curves of parotid resting saliva in rabbits. *A*—Degrees of difference by days. *B*—Differences at right and left sides.

nate showed the volume of parotid "resting saliva" for each 5 min 1 scale stood for 0·001 ml. Abscissa showed the secreting time in minutes. *A* showed the volume curves of a similar tendency on different days. The 1st measurement was June 1st, 2nd measurement was June 3rd, and 3rd measurement was June 5th. It showed that there was not so much difference

at each day. And *B* showed the volume curves of the differences of parotid "resting saliva" on right and left sides. These showed almost the same tendency of the curves as the "resting saliva" on right and left sides of the parotid gland in a rabbit.

Thus, we knew that "resting saliva" can be measured in every rabbit regardless of right and left sides if tested at the same time at a different day.

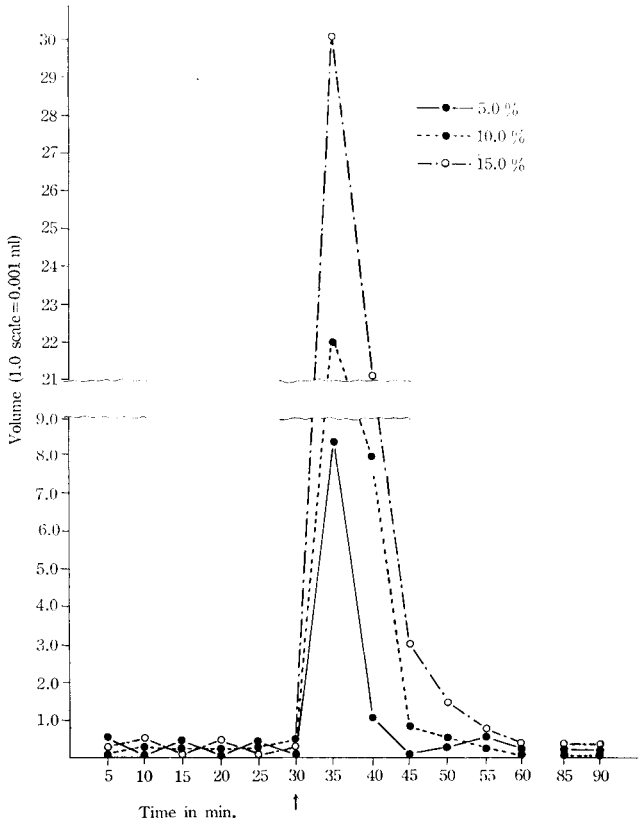


FIG. 2. The saliva curves when the solution of tartaric acid is poured into the mouth. ↑ = Infusion of tartaric acid. (2·0 ml.)

Figure 2 showed three curves of parotid saliva, when the tartaric acid solution of different concentrations was poured into the mouth of a rabbit. ↑ = the infusion of tartaric acid into the mouth. At first, the volume of "resting saliva" was measured for 30 minutes. Afterward, a 5 per cent (0·33 mol) tartaric acid solution of 1·0–2·0 ml was administered to a rabbit. After the passage of a certain interval, the same animal was subjected

to a 10 per cent (0·67 mol) tartaric acid solution. Then, in the same way, a 15 per cent (1·0 mol) tartaric acid solution was administered to the rabbit.

Compared with "resting saliva", "reflex saliva" showed to be noticeably more. While the volume of "resting saliva" was 0·5–1·0, the administration of tartaric acid brought about the secretion of saliva 8·5 at a 5 per cent level, 21·5 at 10 per cent and 30·5 at 15 per cent respectively. Thus the volume of salivary secretion was in parallel to the concentration of an acid used. The secretion continued for 5–10 minutes at a 5 per cent level, 15–20 minutes at a 10 per cent level and 25–30 minutes at a 15 per cent level.

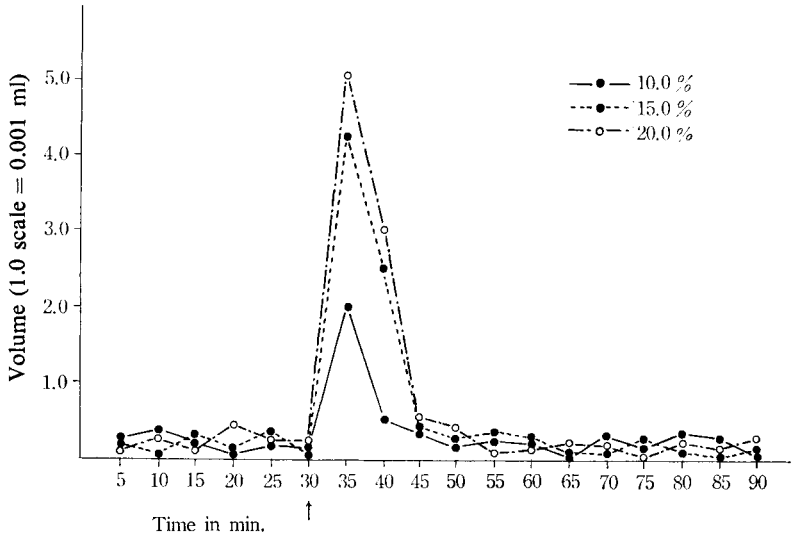


FIG. 3. The saliva curves when the solution of sodium chloride is poured into the mouth. † = Infusion of sodium chloride. (2·0 ml.)

As given in Fig. 3, with the use of sodium chloride the maximum secretion at 10 per cent (1·7 mol) was 2·0, 4·5 at a 15 per cent (2·6 mol) level, and 5·0 at a 20 per cent (3·4 mol) level. The tendency was in parallel to that of the previous amount of tartaric acid. In terms of the duration of secretion, it continued 5–10 minutes at a 10 per cent level and 15–20 minutes at a 20 per cent level, the secretory duration of a 15 per cent level occurring in the middle.

With three different concentrations of sucrose, 20 per cent (0·6 mol), 30 per cent (0·9 mol), and 40 per cent (1·2 mol); the maximum secretions were respectively 3·5 at a 20 per cent level, 9·5 at a 30 per cent and 10·0

at a 40 per cent level (Fig. 4). Here again, we noticed the same tendency as the previous two tests. As a result, we found that cats are non-responsive to a sweet taste,[4,5] but rabbits respond to a sweet taste. The secretory durations at these percentage levels were respectively 5–10 minutes, 10–15 minutes and 15–20 minutes.

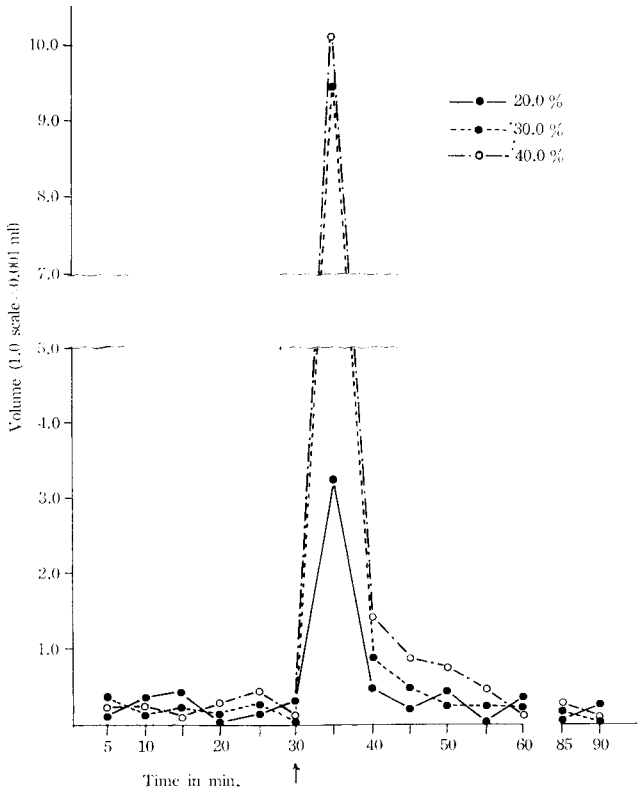


FIG. 4. The saliva curves when the solution of sucrose is poured into the mouth. ↑ = Infusion of sucrose. (2·0 ml.)

Lastly, we used three different concentrations of hydrochloride quinine (Fig. 5). The reflex secretions were respectively 0·8 at a 1·0 per cent (0·003 mol) level, 2·0 at a 3·0 per cent (0·008 mol) level and 3·5 at a 5·0 per cent (0·013 mol) level. The secretory duration was about 10 minutes in the case of a 1·0 per cent level, while it was 15 minutes in the range of 3·0 to 5·0 per cent. Incidentally we noticed that in case of quinine in the range of a 3·0 to 5·0 per cent concentration the resting saliva of rabbits was quite reduced for 25 minutes after reflex secretion.

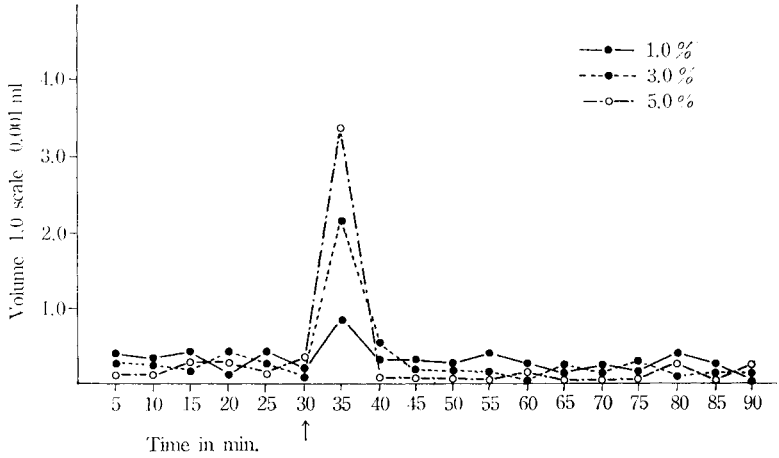


FIG. 5. The saliva curves when the solution of hydrochloride quinine is poured into the mouth. ↑ = Infusion of quinine. (2·0 ml.)

SUMMARY

Based on these findings, we reached the conclusion that as in the same case with dogs and humans parotid saliva by a suction cup method had a distinct resting secretion without any external stimulus. As for the reflex saliva, rabbits showed to have receptors for tartaric acid, sodium chloride and sucrose. And the volume of reflex saliva of course was in parallel to the concentration of above each agent used.

REFERENCES

1. SUHARA, R. and ASAKAWA, H. On the Composition of Human Parotid Resting Saliva and Reflex Saliva, *Dent. J. Nihon Univ.*, **1**, 153, 1958.
2. HAYASHI, T., SUHARA, R., TAKASHITA, H. and OHKAME, K. Mechanism of Secretion of Resting Saliva in Men, *Keio J. of Med.*, **14**, 19, 1965.
3. LASHLEY, K. S. Reflex Secretion of the Human Parotid Gland, *J. Exper. Psychol.*, **1**, 461, 1916.
4. BEIDLER, L. M., FISHMAN, I. Y. and HARDIMAN, C. W. Species Differences in Taste Responses, *Am. J. Physiol.*, **181**, 235, 1955.
5. PFAFFMANN, C. Gustatory Nerve Impulses in Rat, Cat and Rabbit, *J. Neurophysiol.*, **18**, 429, 1955.

THE ELECTROLYTIC CONCENTRATION OF SALIVARY CONDITIONAL AND UNCONDITIONAL REFLEXES

CLARA H. WILLIAMS, ANDREW LIVINGSTON, JR., and W. HORSLEY GANTT

Food and Drug Administration, Washington, D.C., U.S.A.

THERE are conflicting views as to whether conditional reflexes (CR) and unconditional reflexes (UR) in response to stimuli are compensatory or duplicating. Kimble[1] maintains that the CR's and UR's are never strictly the same; that the CR is not a duplicate of the UR; and that imprecise measurements or incomplete recording of the details of a reaction have produced cases where the CR does appear to be an exact replica of the UR. Pavlov[2], on the other hand, concluded from his analyses of salivary secretion in dogs that the responses to a conditional stimulus (CS) were the same as to the unconditional stimulus (US). The work of Gantt[3] also points out the similarities, both qualitative and quantitative, between the CR and UR.

The present study attempts to resolve this question, at least for the salivary response, by comparing the electrolytic concentration of saliva collected from dogs during presentation of several different conditional and unconditional stimuli.

EXPERIMENTAL

Saliva Collection

The arrangement for collecting saliva during the conditioning experiments in a soundproof camera is shown in Fig. 1. The trap of an i.v. infusion tubing set was cut on a slant to form a cup which could be attached to the dog's cheek over the externalized parotid fistula by means of methyl 2-cyanoacrylate. The saliva flowed down the transparent tubing into a series of beakers in the saliva collector shown in Fig. 1. The beakers were

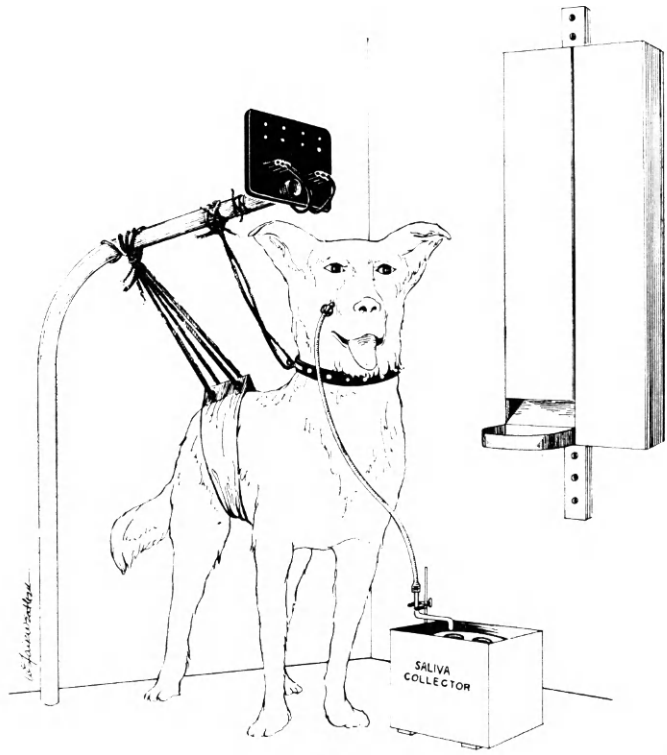


FIG. 1.

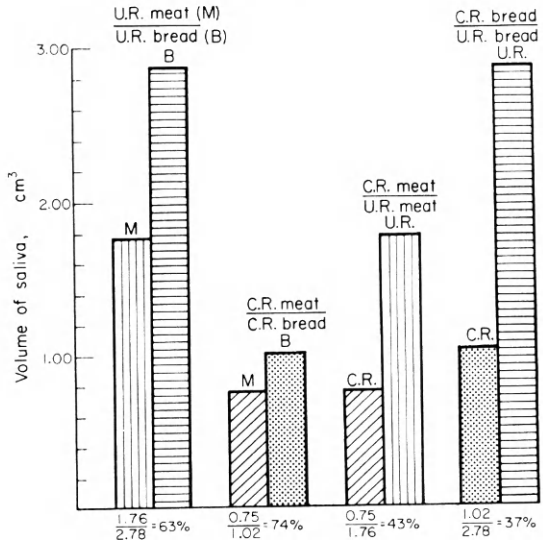


FIG. 2. Salivary secretions under various conditions.

rotated electrically from outside the camera, so that saliva from the different stimuli could be separately collected. The dog was held in position by a sling around the hind quarters and by a rope attached from the collar to the side wall, thus providing the dog with enough movement to reach the feeder, but not enough to pull the cup loose from its cheek. (Heart rate and respiratory recordings made during the experiment will be reported elsewhere.) Food was withheld from the dogs for 48 hours before the experiments, to increase the CR. Saliva was collected (1) under normal eating conditions; (2) after mecholyl injection (1 – 3 mg subcut.) and (3) after histamine (0·3 mg histamine – PO_4 subcut.). These latter injections were administered just before the start of the experiment.

Conditional Reflex Experiment

Two tones, 500 and 1000 cycles per second, were used alternatively as conditional stimuli throughout, the former reinforced with meat (Pard canned meat preparation in 15 gram balls) and the latter with different types of food (depending upon what the dog would eat). The tone was sounded for 30 sec (in one series of experiments for 60 sec) and the food discharged from the automatic feeder into a tray during the final 2 sec of the tone. The interval between the tones was 3 min (for one experiment this was shortened to 1 min). The saliva collected from each day's experiment represented the total volume collected during the presentation of ten conditional stimuli and ten unconditional stimuli for meat, and ten conditional stimuli and ten unconditional stimuli for a different food.

Chemical Analyses

Analyses were made on fresh saliva shortly after each experiment. Preliminary studies showed that centrifuging did not alter the concentration of the electrolytes. The concentration of sodium and potassium was determined using a Coleman Model 21 Flame Photometer, chlorides by Sendroy's method,[4] CO_2 by the Peters and Van Slyke[5] manometric method, protein by the Greenberg[6] method for cerebrospinal fluid protein. Total solids were measured on one-drop samples using a Goldberg Refractometer. pH was measured on a Beckman pH meter using a one-drop glass electrode.

RESULTS

All of the saliva samples collected from the four dogs were alkaline, the pH averaging 8·8, the range being 8·2–9·3. There was no correlation between pH and the various experimental conditions under which saliva was collected.

The average concentrations of Na, K, and Cl, the CO_2 combining capacity and the volume of saliva secreted are shown in Tables 1 and 2 for the *four dogs*. The data on "Teddy" is incomplete because he became so agitated in the camera after fourteen sessions that it was impossible to keep the plastic cup attached to the cheek and no further experiments could be run.

The data shows:

(1) That the volume of saliva secreted to the conditional stimuli was much less than that secreted to the unconditional stimuli, and the amount from the CR for meat was less than the CR for Ritz crackers, Zweibach and lollipups. Abuladze,[7] working with dogs with salivary fistulae and exteriorized tongue segments, also found less secretion to a conditional stimulus than to a unconditional stimulus (Fig. 1).

(2) The volume of saliva secreted to meat as unconditional stimulus was usually less than that to Zweibach, cheese crackers or lollipups, thus paralleling the volumes from the CR's. It is a well-known fact that meat produces less parotid salivary secretion than does dry food, and the saliva collected here was exclusively parotid saliva.

(3) The Na concentration in saliva was less than in the dog's sera, ranging from 96–134 mEq/l. for the different dogs. It was constant for each set of experimental conditions, there being no significant difference in the concentration in saliva secreted to the CS or to the US.

(4) The K concentration of the unstimulated saliva ranged from 29 to 56 mEq/l. or seven times the dogs' serum concentration. The concentration was constant for each of the different experimental conditions.

(5) The Na/K ratio was constant for each given experiment upon each dog.

(6) The chloride content in saliva was about the same as in serum chloride, and was rather constant under a given set of experimental conditions. The Na/Cl ratio was constant.

(7) The CO_2 combining capacity was about 20 per cent higher in the saliva than in the serum. Again there was no difference in the combining capacity in saliva from the CR and UR collections, in the cases where enough saliva was available for determinations.

TABLE 1. SALIVARY ELECTROLYTES—4 DOGS

(Average values under various conditions)

No. of Dogs	No. of Days	Conditions of Experiment	Na mEq/l.	K mEq/l.	Na/K	Cl mEq/l.	Na/Cl	CO_2 cap. Vol%	Volume secreted cc
A.									
4	42	T500	116	47	2·4	105	1·1	q.n.s.	0·38
		30″ tone							
a, b,	(*a*=15)	Meat	96	41	2·3	93	1·0	64	1·27
*c, d**	(*b*=10)	3′ interval							
	(*c*= 5)	T1000	104	44	2·4	100	1·0	q.n.s.	0·58
	(*d*=12)								
		RC, Z, or L†	96	41	2·3	92	1·0	58	2·47
B. 3	12	T500	130	19	6·8	70	1·9	114	2·35
		30″ tone							
a, b,	(*a*=4)	Meat	117	16	7·3	64	1·8	105	5·97
*c**	(*b*=3)	3′ interval							
		T1000	128	17	7·5	65	2·0	116	2·82
	(*c*=5)	(Mecholyl)							
		RC, Z, or L†	120	17	7·0	64	1·9	107	5·62
C.									
3	8	T500	111	44	2·5	104	1·1	q.n.s.	0·82
		30″ tone							
a, b,	(*a*=4)	Meat	96	35	2·7	87	1·1	q.n.s.	1·66
*d**	(*b*=3)	3′ interval							
	(*d*=1)	T1000	100	36	2·8	90	1·1	q.n.s.	1·17
		(Histamine)							
		RC or Z†	96	36	2·7	88	1·1	54	3·08
D.									
2	10	T500	122	35	3·5	96	1·3	q.n.s.	1·24
		30″ tone							
*a, b**	(*a*=5)	Cracker							
	(*b*=5)	3′ interval & Meat	103	29	3·6	78	1·3	68	5·74
		(Different							
		food) T1000	122	32	3·8	92	1·3	q.n.s.	1·08
		Candy	108	30	3·6	86	1·3	74	5·43
E.									
3	14	T500	104	32	3·2	83	1·2	q.n.s.	0·68
		30″ tone							
a, b,	(*a*=6)	Meat	101	30	3·4	77	1·3	q.n.s.	1·85
*c**	(*b*=5)	1′ interval							
	(*c*=3)	T1000	107	32	3·3	81	1·3	q.n.s.	0·96
		RC, Z or L†	103	30	3·4	75	1·4	q.n.s.	2·48

No. of Dogs	No. of Days	Conditions of Experiment	Na mEq/l.	K mEq/l.	Na/K	Cl mEq/l.	Na/Cl	CO_2 cap. Vol%	Volume secreted cc
F.									
3	15	T500 60″ tone	134	56	2·4	122	1·1	q.n.s.	0·50
*a, b, c**	(*a*=1) (*b*=7) (*c*=7)	Meat 3′ interval	119	50	2·4	105	1·1	q.n.s.	0·98
		T1000	129	55	2·3	118	1·1	q.n.s.	0·50
		RC, Z or L†	113	46	2·5	101	1·1	q.n.s.	1·69
G.									
4 *a, b, c, d**	4 (*ea.*=1)	Blood Serum	156	5·8	26·9	102	2·2	46	

* *a*, Bernice; *b*, Sam; *c*, Penny; *d*, Teddy
† RC, Ritz Cracker; Z, Zweibach; L, Lollipups
q.n.s., quantity not sufficient

TABLE 2.

(Median values under various conditions of data in Table 1)

4 dogs, 105 exp-days of 10 ea for meat and for bread, i.e. 1050 samples of ea.

	UR Meat	UR Bread	CR Meat	CR Bread
Na(mEq/l.)	102	106	126	114
K(mEq/l.)	32	33	40	34
Cl(mEq/l.)	82	87	100	91
CO_2(Vol %)	68	66	114	116
Vol (cc)	1·76	2·78	0·75	1·02

Ratio $\frac{\text{saliva}}{\text{serum}}$ Na = 104/156 K = 33/6 Cl = 85/102 CO_2 = 67/46

(8) When the salivary secretion was increased by mecholyl injections there was a marked but constant drop in the K concentration in all four dogs, no real change in the Na concentration, but a three-fold increase in

the Na/K ratio of unstimulated saliva. The pH was unchanged, the CO_2 capacity was increased, and the Cl concentration fell. Here again, the concentrations of the inorganic ions in the saliva secreted to the CR was about the same as to the UR.

(9) The dosage of histamine used here produced no marked increases in the salivary secretion. We discovered later that Stavraky[8] found that 4 mg of histamine-PO_4 i.v. was needed to increase the flow and the duration was only $3^1/_2$ min.

(10) When the inter-trial interval was shortened to 1 min there was an increase in the rate of flow in the 3 dogs (Sam, Bernice and Penny) with a slight drop in K concentration, (E) in Table 1.

(11) The electrolytic concentration seems to be independent of the rate of flow. While the K concentration was lowered after mecholyl stimulation and when the inter-trial interval was shortened, it was still independent of the rate of flow for that particular experiment. Some other factor must be responsible for the altered K concentration.

(12) The total solids were measured in all samples and the protein content in the last few experiments, but there was no correlation between protein concentration, electrolytic concentration or the volume. The average protein content for "Bernice" ranged from 508 to 561 mg%, for "Penny" from 315 to 359 mg%, and for "Sam" from 466 to 565 mg%.

DISCUSSION

The electrolytic concentrations of the saliva secreted to the conditional stimulus and to the unconditional stimulus under the experimental conditions in four dogs described here are practically identical, but the volumes vary, more being secreted in each case to the US than to the CS. Thus one can conclude that the physical responses to the CR and to the UR differ, but not the inorganic chemical reponses. This agrees with Pavlov's [2] findings that the responses of an animal to conditional and unconditional stimuli are similar.

There are conflicting reports in the literature as to the relationship between the electrolytic composition of saliva and the rate of secretion. Pavlov[2] found that the percent of inorganic salts was not influenced by the rate of secretion of saliva in dogs. Werther[9] reported that an increase in the rate of secretion caused a higher concentration of salts in submaxillary and parotid saliva from rabbits and dogs. Bailey and Balch[10] found in studies of saliva from a parotid fistula of a small steer that Na increased as the rate of secretion increased, but the K and Cl decreased. Shannon[11]

in studies with human saliva found that at low rates of secretion the correlations between volume and inorganic ions become obscure and that "stimulus intensity modifies response predictability". Baxter[12] in fistulated dogs found 20 mEq/l. of K in 33 cc of parotid saliva secreted to 5 min of bread and meat consumption, and 20 mEq/l. in 5·1 cc of saliva secreted to 5 min of milk drinking. The chloride concentrations were 45·7 and 53 mEq/l. respectively.

The concentration of Na, K, and Cl ions is apparently associated with some factor other than the rate of flow, because for a given set of conditions the concentrations remain the same regardless of the amount secreted. For example, the shortening of the inter-trial interval to 1 min caused "Bernice" and "Sam" to become more alert and more anxious and the saliva flow increased as well as the Na/K ratio. The dog "Penny" was unaffected by the shortening and the rate of secretion and the Na/K ratio remained the same. Also mecholyl altered the electrolyte concentration, but independently of the rate of flow.

Babkin[13] states that a salivary gland does not secrete as a unit, but that the different sets of epithelia in the gland contribute different components to the secretion and local productivity depends upon the intensity of excitation received from the salivary center.

Baxter[12] discusses the relationship between stimulus, rate of flow, and inorganic salt concentration.

CONCLUSION

Using meat and bread as unconditional stimuli for forming parotid salivary conditional reflexes, we measured the sodium, potassium, chlorides, carbon dioxide constituents and the volume of 1050 samples collected from 4 dogs for 105 experimental days. We found:

1. There were no significant differences of the above 4 constituents in the unconditional reflex salivary secretion to meat and to bread.

2. The volume of saliva secreted as a UR to bread (16 g lollipups, 6 g Zweibach or 3·5 g cheese crackers) was nearly twice that secreted as a UR to meat (15 g).

3. The CR electrolytes (mEq/l.) secretion for both meat and bread was greater than the UR secretions.

4. There was little or no difference in the electrolytic (mEq/l.) secretion between the CR to meat and the CR to bread.

5. The volume of saliva secreted as a CR to bread was about one-third more than the volume of the CR to meat.

6. Thus in comparing the salivary secretion to meat and to bread, UR and CR, there is no marked differences in the composition of the saliva under the conditions of these experiments.

7. The volume of saliva secreted in a given time was greater to the bread than to the meat, and the CR volume was roughly parallel to the UR volume to meat and bread respectively.

All the determinations were made on the parotid secretion.

ACKNOWLEDGEMENT

We are indebted to Mr. James Reus of the Instrument Laboratory for the fabrication of the "Saliva Collector".

REFERENCES

1. KIMBLE, G. A. In: *Hilgard and Marquis Conditioning and Learning*. Appleton-Century-Crofts, Inc., 1961, pp. 52–53.
2. PAVLOV, I. P. In: *The Work of the Digestive Glands*, Charles Griffin and Co., Ltd., Philadelphia, 1910, pp. 84–86.
3. GANTT, W. H. The nervous secretion of saliva: the relation of the conditioned reflex to the intensity of the unconditioned stimulus. *Amer. J. Physiol.*, **123**, 74, 1938.
4. SENDROY, J. Photoelectric microdetermination of chloride in biological fluids and of iodate and iodine in protein-free solutions. *J. Biol. Chem.*, **130**, 605, 1939.
5. PETERS, J. P., and VAN SLYKE, D. D. In: *Quantitative Clinical Chemistry*, Baltimore, Williams and Wilkins, 1939, Vol. 11, pp. 296–298.
6. GREENBERG, D. M. The colorimetric determination of the serum proteins. *J. Biol. Chem.*, **82**, 545, 1929.
7. ABULADZE, K. S. In: *The Functioning of Paired Organs*, Pergamon, N. Y. MacMillan. Translated by Dr. R. Crawford, p. 92, 1963.
8. STAVRAKY, G. W. The response of the submaxillary and parotid glands of the dog to histamine. *J. Pharmacol.*, **43**, 265, 1931.
9. WERTHER, M. Einige Beobachtungen über die Absonderung der. *Arch. Ges. Physiol.*, **38**, 293, 1886.
10. BAILEY, C. B. and C. C. BALCH. Saliva secretion and its relation to feeding in cattle. 1. The composition and rate of secretion of parotid saliva in a small steer. *Brit. J. Nutr.*, **15**, 371, 1961.
11. SHANNON, I. L. and J. R. PRIGMORE. Whole saliva volume, sodium, potassium and chloride: day-to-day comparisons under identical and diverse forms of stimulation. *J. Dent.* Res., **38**, 843, 1959.
12. BAXTER, H. Variations in the inorganic constituents of mixed and parotid gland saliva activated by reflex stimulation in the dog. *J. Biol. Chem.*, **102**, 203, 1933.
13. BABKIN, B. P. In: *Secretory Mechanism of the Digestive Glands*. Paul B. Hoeber, Inc., 1950, p. 697.

BEHAVIORAL TECHNIQUE TO ANALYZE A DOG'S ABILITY TO DISCRIMINATE FLAVORS IN COMMERCIAL FOOD PRODUCTS*

VERA P. ROGERS,† GLENN T. HARTKE and RALPH L. KITCHELL‡

PRODUCERS of commercial dog foods often list a designated ingredient as a flavor on the label attached to their products without possessing the knowledge that the dog can actually distinguish the presence of the ingredient so labeled. Preference trials yield useful information if a distinct preference or avoidance can be demonstrated. Naturally, if an animal shows a preference or avoidance to a product with, or without a specific ingredient he must be able to perceive that ingredient. It is in the acceptance category (non-discrimination) where it is not possible to determine whether the animal does not discriminate because he cannot perceive the ingredient or whether he can perceive the ingredient but does not discriminate because he is not motivated to discriminate.

The purpose of this study was to develop a conditioning procedure which would motivate the animal to give a conditioned response if he perceived the selected ingredient in a commercially canned dog food.

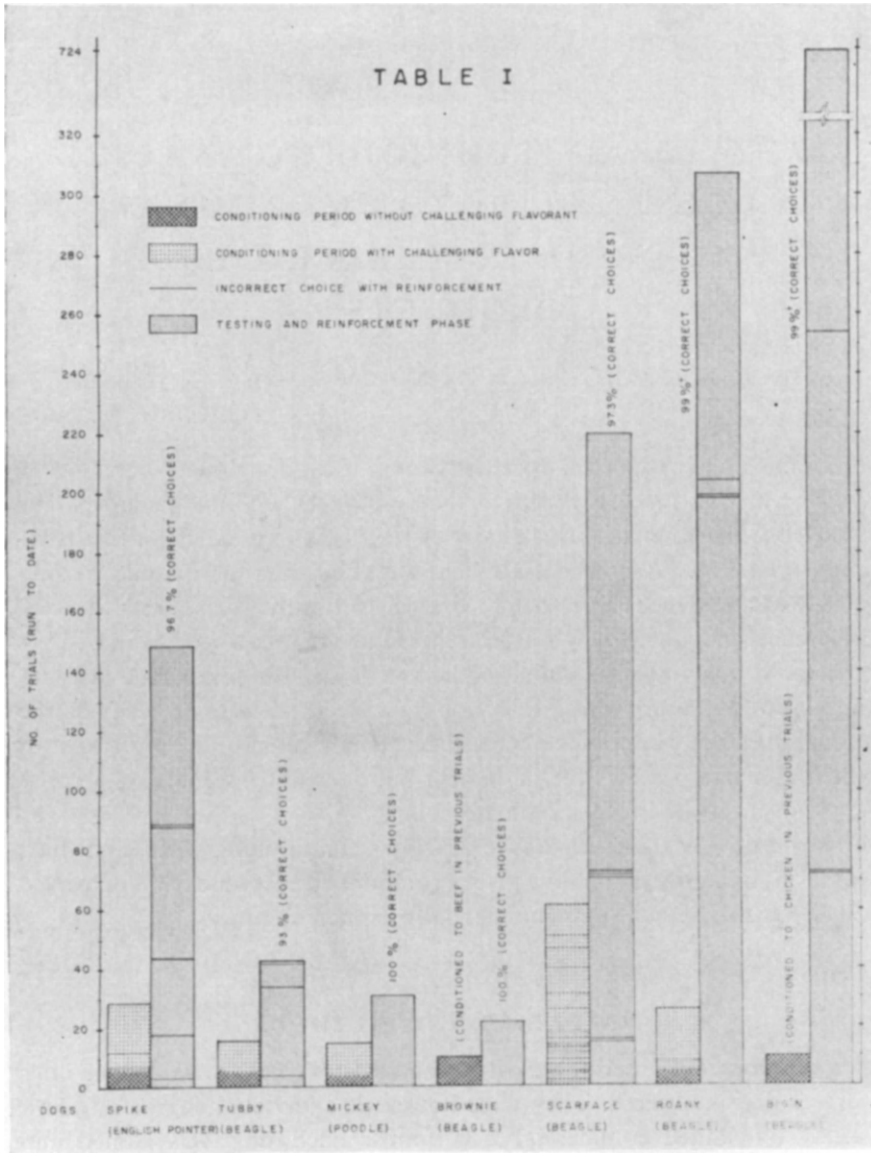
MATERIALS AND METHODS

Seven dogs were used for most of the trials (Tables 1 and 2). The dogs were of various breeds. They were housed in individual cages and fed a variety of canned commercial dog foods. Each dog was named and handled individually.

* Supported, in part, by a grant from the Pet Food Institute, Chicago, Illinois.

† Instructor, Department of Anatomy, College of Veterinary Medicine, Kansas State University, Manhattan, Kansas.

‡ Dean, College of Veterinary Medicine, Kansas State University, Manhattan, Kansas.



The food products used were commercially canned dog foods purchased from local supermarkets in the area. Each brand was given a Roman numeral code number which was placed on the can after removal of the label (Fig. 1). If the label indicated a specific ingredient (flavor) as being present, this was identified by printing on the can in capital letters, L for liver, B for beef, F for fish, C for chicken and O for no labeled flavor.

TABLE II

DOG	CONDITIONING FLAVOR	CHALLENGING FLAVORS IN PREPARED DOG FOODS				
		BASE	BEEF	CHICKEN	FISH	LIVER
SPIKE	FISH	X	X	X		X
TUBBY	BEEF	X		X	X	X
MICKEY	BEEF	X		X	X	X
BROWNIE	BEEF	X		X	X	X
SCARFACE	LIVER	X	X	X	X	
ROANY	CHICKEN	X	X		X	X
BIG'N	CHICKEN	X	X		X	X

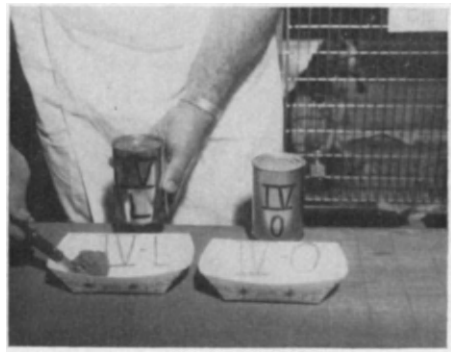


FIG. 1. Can of dog food is coded in Roman numerals. If a flavorant is added this is indicated by a capital letter (L-liver or *O* if none is added). A small portion of the food is placed in a plastic-coated meat tray which is used only once. The spatula is cleaned after being used once.

The experimental procedure was divided into 3 phases: (1) the habituation-training (conditioning period without challenging flavorant) phase;

(2) the conditioning (conditioning with challenging flavorant) phase; and (3) the testing and reinforcement phase.

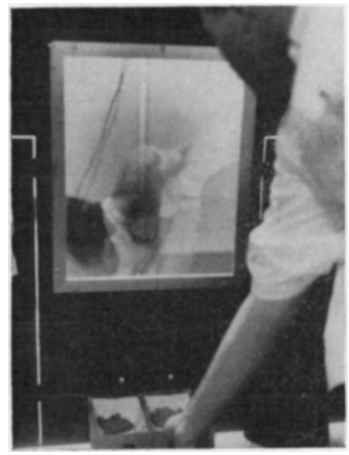


FIG. 2. Front of the testing area. The dog may be seen through the two-way mirror. The plastic-coated trays are presented to the dog by placing them randomly in the drawer and pushing the drawer into the enclosure. In later trials 6 trays were presented simultaneously.

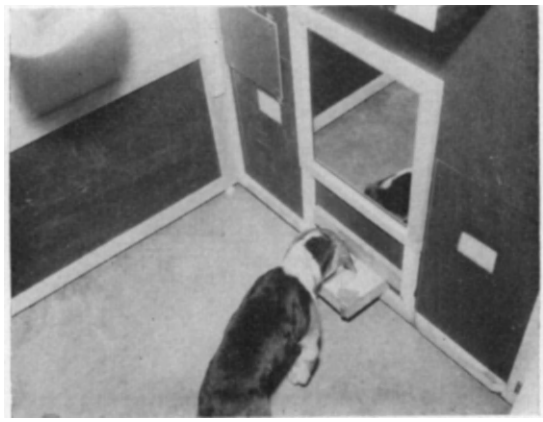


FIG. 3. Interior of the testing area The dog is making a selection from the two trays. The dog cannot see the operator through the two-way mirror.

Each dog was placed in a testing area where he could be observed through a two-way mirror (Fig. 2). Flood lights were directed towards the mirror so the dog could not see the observer through the mirror (Figs. 2 and 3).

A drawer which was located below the mirror, was used to deliver two or six different canned dog foods to the animal. The food was placed in plastic-coated trays (Figs. 1, 2 and 3) which were presented to the dog by pushing the drawer into the testing area. Each tray was used only once.

In the habituation-training phase the dog was placed in the testing area and, using the drawer and the plastic-coated trays, was fed the food (flavor) he was to be conditioned to recognize (conditioning flavor, CF). All dogs but one were presented with a natural product using either chicken (CFC), beef (CFB), or liver (CFL). The dog conditioned to fish (Table 2) was conditioned to a fish-flavored commercial dog food (CFF) because no fresh fish product was available. The CF in each instance was presented to the dog in small amounts using a new tray for each presentation. As soon as the dog readily approached the drawer and ate the CF, phase two was begun.

The conditioning phase consisted of the presentation of the natural product (CF) and randomly selected commercial dog food products containing no labeled flavor (dog foodbase product or dog food, no flavor DFN) or a commercial product containing a labeled flavor (dog food, beef flavored, DFB; dog food, liver flavored, DFL; dog food, chicken flavored, DFC; or dog food, fish flavored, DFF). In many instances one company would use the same base formula but just increase the concentration of the labeled ingredient. If the dog selected the natural or that specific flavor to which he was to be conditioned, he was permitted to eat the food from that tray. If he selected the commercial products, (DF's) the drawer was quickly pulled out of the training area. Each conditioning or test session consisted of ten to twelve presentations. The dogs conditioned rapidly and performed at an efficiency level of 100 per cent after as few as two conditioning sessions (Table 1). A dog was considered conditioned when one session (10 trials) was completed without error.

The testing and reinforcement phase consisted of the presentation of either the pure CF (such as beef, CFB or the same designated flavor, using a variety of different brands of commercial dog foods (DFB), in one tray and a series of different challenging flavors (DFC, DFL, DFF) or a base product with no labeled flavor (DFN) in the other tray or trays. The trays were randomly placed in the drawer so no pattern could be followed in the selection of the food. Each conditioned flavor was tried against the complete list of challenge-flavored products. The foods were presented as rapidly as possible depending upon how quickly the animals responded. An experimental session was controlled by the appetite of each individual dog.

RESULTS AND DISCUSSION

The dog conditioned rapidly to the conditioning flavor by simply rewarding him by permitting him to eat the conditioning flavor if he made the correct choice. If he did not, the drawer was pulled away from him. This negative reinforcement was apparently quite effective as many of the dogs approached the drawer cautiously after having made an incorrect choice.

Dogs conditioned to a natural product, such as beef (CFB), would select commercial dog foods, beef flavored (DFB) when these products were paired with commercial dog foods with no flavor (DFN) or other foods with different labeled flavors (DFC, DFF, DFL). They would also select the correct flavored product from 5 other products. The performance level of all the dogs was above 93 per cent. The presentation of two products prepared by the same company, for example Brand IL (liver flavored, DFL) and Brand IO (no flavor, DFN) to a dog conditioned to liver, resulted in the dog selecting the dog food with liver in it. The same dog correctly selected the liver flavored product of a different brand (Brand IIL) over Brand IO (no liver). The dog conditioned to a natural product like liver selected a variety of commercial products containing liver; this indicates that the dog distinguished that liver, or a product similar to liver, was in each of the commercial products.

No attempts have been made to date to determine thresholds based upon concentration of the ingredient. It is of interest to note that dogs conditioned to the natural product (such as CFB), selected the commercial product with this specific labeled flavor (DFB) over the same brand base product not labeled with the specific flavor (DFN) even though many of the base products contained the natural product (beef or beef by-products); the dogs did this in the multiple presentations where one product labeled beef (DFB) was randomly placed among several other products containing beef but not labeled beef (DFN). In these instances it appears that the dogs must have been distinguishing on the basis of the higher concentration of the beef in labeled product.

The technique described in this study does not provide any direct information as to whether or not the dogs preferred one product over another product. One of the dogs (Scarface, Tables 1 and 2) when presented with a flavored product and a non-flavored product in his home cage would eat only the non-flavored product. This dog was conditioned, and responded 97 per cent, to the liver flavored product.

In this study it appeared that the dogs were using their olfactory systems primarily although taste certainly would seem to be involved in

reinforcement of the conditioning procedure. The dogs would make their selection, in most instances, without touching the food. Some of the dogs, particularly when presented with six choices, would approach each of the six trays and then return to the correct tray. Discrimination by vision was made more difficult in selected trials by adding dyes to the various products. This did not seem to interfere with the dog's ability to discriminate among the various products.

SUMMARY AND CONCLUSIONS

A technique has been developed whereby it can be determined whether a dog will distinguish that a specific ingredient is present in a variety of commercially prepared dog foods. This technique is not limited by the dog's preference (or avoidance) of the ingredient. The technique is based upon motivating (or forcing) the dog to direct his attention to the input of receptors activated by the ingredient. A positive response does not indicate the dog's preference (or avoidance) of the ingredient. A positive response does suggest that the dog can be conditioned to respond to the presence of an ingredient. The presence of neural activity from receptors in response to the application of an ingredient to peripheral receptors does not indicate that the animal will respond to this input. This technique should permit closer correlation between neurophysiological studies and behavior. This study also indicates, from the applied aspects, that dogs can distinguish that beef, liver, fish and chicken have been added to the base products of a number of commercially available products in the municipality where this study was conducted.

THE SENSORY AND BEHAVIORAL FACTORS IN TASTE PREFERENCES*

CARL PFAFFMANN,† G. L. FISHER,‡ and M. K. FRANK

Brown University

THE study of preference behavior toward nutritive and nonnutritive substances in solution requires analysis at several levels of the factors which (a) initiate, (b) maintain and (c) terminate the behavior. One level includes the afferent inputs, largely from the olfactory, gustatory and somatosensory head receptors. Another includes those effects contingent upon ingestion, the immediately postingestive interoceptive (sensory), and/or metabolic consequences which are followed by the longer lasting metabolic effects that change the nutritional state of the organism. Both the immediate and long-term effects play back upon or interact with the afferent input in such a manner that preference and ingestive behavior may be modified. The nature and mechanism of this feedback is as yet only partially understood.

In addition to the more physiologically defined variables one can specify certain environmental or experiential determinants although in the last analysis all determinants of behavior will have a physiological substrate. Past experience may modify through learning, the response to a sensory stimulus. Or under other circumstances the discrimination of stimuli may be obscured by the arrangement of the test situation itself. Or different motor responses, learned or unlearned, which serve as the behavioral index, might yield different results to the same stimulus.

In the first part of this paper I shall present some additional evidence pertaining to the total gustatory afferent field by describing new observations on the IXth nerve. In the second part we shall discuss the relationship among different behavioral preference methods as they reflect the gustatory control of behavior of the laboratory rat.

* Supported in part by a grant from the National Science Foundation.

† Present address – Rockefeller University.

‡ Present address – University of Maryland.

I. TASTE SENSITIVITY OF RAT CIRCUMVALLATE PAPILLA

The sensory fibers for mammalian taste travel in three mixed nerve trunks: the lingual, the glossopharyngeal, and the vagus; no one of these subserving taste alone. The lingual nerve, more specifically, its branch, the chorda tympani innervates taste buds of fungiform papillae of the anterior portion of the tongue; the lingual branch of the glossopharyngeal innervates taste buds in the foliate and circumvallate papillae of the posterior third of the tongue; and the pharyngeal branch of the vagus, the extra-lingual taste buds in the mouth cavity (Andrew and Oliver, 1951).

The whole nerve as well as single fiber responses of the chorda tympani has been studied intensively in several mammals in response to many chemicals (e.g. Beidler, 1953; Erickson, 1963; Fishman, 1957; Gordon *et al.*, 1959; Pfaffmann, 1941, 1955; Zotterman 1935, 1961). On the other hand, neither the glossopharyngeal nor the vagus has been studied in anything like the same detail.

Zotterman (1935) reported that stroking the caudal tongue with a brush dipped in salty, bitter, and sour substances produced mechanical (large spike) and chemical (small spike) responses in the whole glossopharyngeal nerve of the cat; Pfaffman (1941), recording from the same nerve in the same species, found acid and quinine sensitivity for the posterior tongue. Appelberg (1958) described differences in the response of the monkey, cat, dog, and rat glossopharyngeal nerve; all responded to ipsilateral mechanical and thermal stimulation, as well as to salty, bitter and sour substances; all but the cat nerve responded to sweet substances, and only the monkey nerve responded to water. Also, touching circumvallate papillae produced a discharge of small taste spikes when a solution was already on the tongue. Baldwin, *et al.* (1959) reported responses to "acid, salt, and less to quinine" in the glossopharyngeal of the sheep, goat, and calf; there were also responses to sugar, if the circumvallate was moved at the same time. Kitchell (1963) reported responses of the lingual branch of the glossopharyngeal nerve of the goat, pig, and dog; stimulus flow over the posterior tongue often did not yield a nerve response; but stroking of the vallate papilla with a wooden probe when the stimulus was on the tongue produced a phasic response to the deformation and a secondary discharge to the chemical stimulus; the nerve of each of these mammals responded to sour, sweet, bitter, and salty stimuli. Finally, Bernard (1964) reported only low magnitude responses in the glossopharyngeal of the calf, even to high concentrations of the stimuli, and a decrease in response magnitude with

repeated stimulation; only sodium chloride and acetic acid reliably elicited a response, hydrochloric acid, sugars and quinine did not; although mechanical responses were easily obtained. Since large amplitude responses in the chorda tympani were obtainable to chemical stimulation of the posterior tongue, Bernard concluded that the glossopharyngeal is probably not important for taste in the calf; the chorda tympani seemed to be responsible for anterior and posterior fields of taste.

Most recently Yamada (1965) has used the integrator method to quantify the IXth nerve responses in three species, the rat, rabbit and cat. He enclosed the tongue in a flow box such that solutions flowed over the circumvallate and foliate papillae. In all species he observed that quinine elicited greater responses than did the comparison salts. In the rat circumvallate papillae, the ratio of responses of 0·02 M quinine to 0·5 M NaCl was 1·22, for rabbit foliate papillae, 1·17 and for cat circumvallate, the 0·02 M quinine to 1·0 M KCl ratio was 1·63. He notes that the response magnitude to salt increased linearly with log concentration but does not give the concentration functions. He also reported differences in the responses to other stimuli.

Certain facts call for systematic investigation of the response characteristics of the glossopharyngeal to taste stimuli in the rat: (1) taste preference–aversion and taste discrimination thresholds have been found at lower concentrations of certain substances (quinine, hydrochloric acid) than the threshold response of the chorda tympani; (2) the magnitude of the response of the chorda tympani to sugars, considering their behavioral effect, has been disappointing (Hagstrom and Pfaffmann, 1959); and (3) denervation of the chorda tympani alone has little effect on taste preference–aversion behavior, whereas, denervation of both the chorda tympani and the glossopharyngeal has a discernible effect (Pfaffmann, 1952). In short, the contribution of the IXth nerve to the total sensory field must be taken into account before further attempts to relate neurophysiology of taste to behavior are made.

Stimulation of the taste buds deep in the furrows of the circumvallate and foliate papillae probably requires repeated opening and closing of the papillary furrows. This is normally accomplished by tongue movement and the enhancing effect of touching or rubbing the vallate noted by several prior experimenters is probably due to the increased circulation of the stimulus solutions into the papillae.

More adequate stimulation of the glossopharyngeal taste buds might be effected by a system providing an actual flow of stimuli into the papillae. In the present experiment, taste solutions were infused directly in to the

trench of the single rat circumvallate papilla. The resulting responses in the lingual branch of the glossopharyngeal nerve were recorded electrophysiologically.

METHOD

Each of seven albino male COBS rats (mean weight, 494 g) was prepared in the following manner. The animal was anesthetized by interperitoneal injection of pentobarbital sodium (Diabutal, 60 mg/ml); initial dosage was 1·0 cc per 100 g of weight and additional doses were of 0·1–0·2 cc given at hourly intervals or wherever necessary. An incision was made in the ventral lower neck and the trachea incannulated; the upper jaw was stabilized with a head clamp and the head positioned to facilitate deep dissection of the left side.

In order to expose the glossopharyngeal nerve, the submaxillary and major sublingual glands, the posterior half of the digastric muscle, the posterior horn of the hyoid bone, and the ascending branch of the hypoglossal nerve were removed in that order under binocular magnification. The glossopharyngeal nerve was dissected free and its pharyngeal branch cut; the nerve trunk was cut near its exit from the posterior lacerated foramen and was drawn under the carotid artery with a fine thread tied to its cut end. The ventral neck incision was then closed.

In order to expose the posterior third of the tongue, a second incision was made on the left side of the face from the corner of the mouth to above the angle of the jaw; the masseter muscle was removed and the lower mandible exposed, its upper processes chipped off to the level of the teeth; branches of the posterior facial vein were tied off and cut in order to reach the lining of the mouth, which was cut to expose the entire tongue; and finally, the jaw was freed from the pterygoid muscles. The single circumvallate papilla of the rat was then exposed by tension on the lower incisor teeth.

The ventral neck incision was then reopened and the animal's head positioned so that the nerve and the papilla could be visualized with a minimal adjustment of the binocular microscope. The nerve was placed over a three wire (nichrome) electrode, positioned within the cavity of the dissection and the incision was covered with Saran wrap to prevent drying of the nerve. The recording circuit included a Grass preamplifier, an integrating circuit and Esterline Angus Graphic Ammeter. A Dumont Cathode-ray Oscillograph and an audio amplifier and speaker provided visual and auditory monitoring of the nerve response.

The tip of a glass pipette (inner diameter of 0·1–0·2 mm) was inserted in the trench of the circumvallate papilla with a micromanipulator. The tip could be observed as it slid into the ipsilateral (to the nerve) crevice at the same time that the recorder showed a small neural response to the mechanical disturbance of the papilla. Stimulus solutions were delivered through the pipette into the papilla, flowed out over the contralateral (to stimulation) side of the tongue and mouth into a drain via a wick; thus, the simultaneous stimulation of the foliate papillae (located on the ipsilateral edge of the tongue) was avoided.

Since the receptors of the glossopharyngeal respond to temperature change (increase in activity to cold and decrease to warm) as well as mechanical deformation of the tongue, the temperature of the stimulus solutions was maintained at a temperature of 34 degrees and the rate of flow was controlled at 0·68 cc per minute by a Harvard infusion pump. Solutions were changed by changing the syringe containing the taste stimulus. Since the taste solution remained in the polyethylene tubing from the B–D stopcocks to the pipette each stimulus was in effect given twice, first for the 1 $\frac{1}{2}$ min of regular stimulation following a prior water rinse, then a second time for half a minute while the taste stimulus was being flushed out of the tube. This was followed by air and then rinse water. The second brief flow of stimulus often produced a second response but its magnitude was below that of the first stimulation. This can be seen in figures showing the IXth nerve response. Only the first response of the two was used in plotting the quantitative results.

Water was flowed through the furrow of the papilla before each stimulus so that the regularly repeated baseline water response could be used to monitor the viability of the preparation. All chemical responses are given as percentages of the magnitude of the response to water flow.

RESULTS

Figures 1 and 2 are tracings of the recorded nerve response to 0·003 M quinine hydrochloride and 0·01 M hydrochloric acid, to 1·0 M sodium chloride and 1·3 M sucrose. The higher concentrations of sodium chloride and hydrochloric acid show an obvious transient and steady state response during solution flow into the papilla; at lower concentrations the steady state was not easily distinguishable from that to water. The flow of quinine hydrochloride and sucrose, on the other hand, produced slow transient responses with no steady state greater than that to water flow. Thus all stimuli produced their major effects during flow, the nerve response to the

acid and salts falling to baseline immediately after flow terminated, but the response to quinine and the sugars continued at a level somewhat above baseline. Renewal of flow of an acid or salt stimulus already in the papilla produced an immediate and large transient (somewhat less in amplitude than the initial response), whereas recurrence of sugar or quinine flow pro-

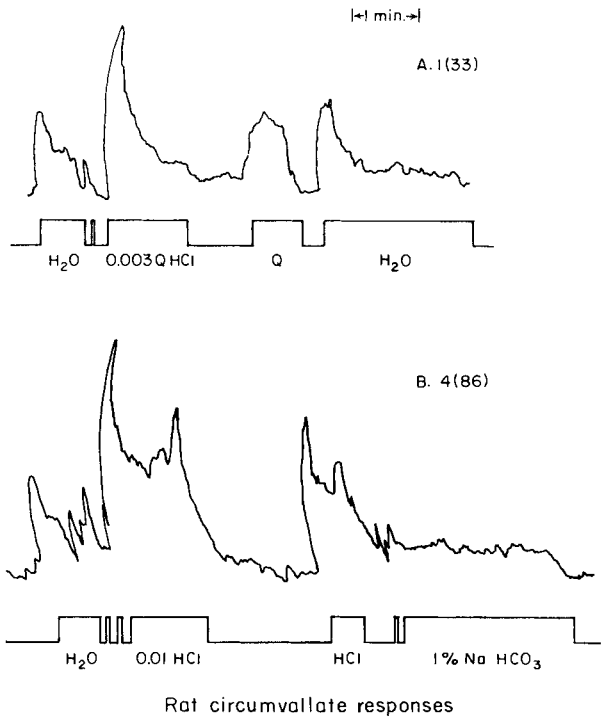


FIG. 1. Tracings of integrated responses to quinine and hydrochloric acids infused into the rat circumvallate papilla. Signal marker indicates duration of flow of indicated solution.

duced a response much less than that to the initial flow. It appears then that stimulation by salts and acids is more dependent upon flow than is the case for sugars and quinine.

The mean of the response to water after water was 28·5 and its standard deviation was 5·1. Since, if these responses are normally distributed, 90 per cent of them would fall between 21 and 36, a mean response of 21 or less and 36 or more is arbitrarily considered different from that of water and "threshold" will be below or above the concentration which produced this response.

The quinine hydrochloride response is a rising function of concentration, the response to 0·01 M being more than twice that to 0·003 M and the response to 0·001 M only 1 $\frac{1}{3}$ times the response to 0·0003 M with threshold between 0·000003 M and 0·00001 M. The hydrochloric acid response, also a rising function of concentration, has a threshold between 0·0001 M and

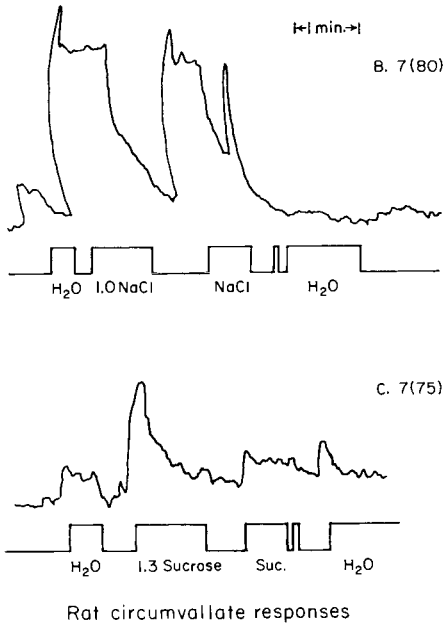


FIG. 2. Same as Fig. 1 for NaCl and sucrose.

0·0003 M. Responses to both quinine and hydrochloric acid are positively accelerated functions of log concentration. The response to sucrose is a rising function of concentration above the threshold stimulus between 0·03 M and 0·1 M.

The response to sodium chloride, on the other hand, is a nonmonotonic function of concentration. Between 0·01 M and 0·1 M the nerve response was less than that to water, to lower concentrations responses were similar to water. Concentrations above 0·1 M gave responses much greater than water (response in this range is a steep positive function of log concentration). There is therefore a "suppression" of the water response by weak concentrations of sodium chloride. The threshold for "suppression" lies between 0·003 M and 0·01 M. The threshold for a response greater than

water lies between 0·1 M and 0·2 M. Figure 3A presents the IXth nerve response as a function of concentration of each of the basic taste stimuli. Figure 3B shows the comparable responses for the chorda tympani.

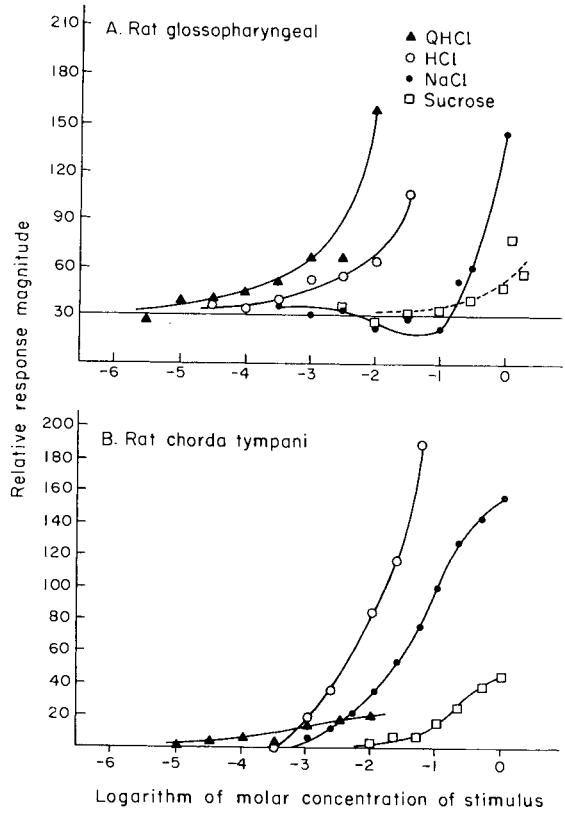


FIG. 3. Comparison of relative response magnitudes for rat glossopharyngeal and chorda tympani nerves. Responses equated at 1·0 M NaCl.

DISCUSSION

Previous investigators had noted the enhancing effect of moving the papilla. We used a more effective stimulation of receptors in the depths of the circumvallate papilla with the pipette flow system. With this method of stimulation, the glossopharyngeal response was found not only to be a systematic function of concentration of all chemicals used but the nerve response appeared to be directly dependent upon stimulus flow. In the normal awake animal the orifices of all tongue papillae presumably are

opened and closed by the action of the tongue muscles allowing chemicals to flow in and out of the papillary fissure. The pipette method seems an effective method of stimulation in the experimental situation.

Since the glossopharyngeal nerve responds strongly to mechanical stimulation of the posterior tongue, especially near the papillae, the response to flow of water (or any stimulus) in the papilla probably is partly mechanical. Evidence that the response to water flow is not entirely mechanical derives from the response "suppression": 0·1 M and 0·01 M NaCl produce smaller responses than does water. Of interest is the fact that the response to 0·01 M potassium chloride is also consistently smaller than the "water" response. Thus both NaCl and KCl solutions give evidence of suppression. These concentrations of sodium and potassium chloride are approximately those found in mammalian extra-cellular fluid (Ringer's solution: 0·15 M sodium chloride, 0·04 M potassium chloride).

The biphasic nature of the sodium chloride function might be described in the following way: stimuli below 0·003 M and 0·01 M produce the "water" response; stimuli above this "suppress" the water response; and stimuli above 0·1 M–0·2 M produce a "salt" response (see Figure 3A). Appelberg (1958) found no "water" response in the rat glossopharyngeal, but this may have been due to his method of stimulation.

The response functions of the glossopharyngeal (Fig. 3A) and chorda tympani (Fig. 3B) of the rat to several chemicals show the following differences. The quinine threshold is about one logarithmic unit lower in the glossopharyngeal and the relative magnitude of response is much greater; in fact, quinine is the most effective stimulus (in terms of magnitudes) for the glossopharyngeal, and the least effective for the chorda tympani (Beidler, 1953; Beidler, *et al.*, 1955; Pfaffmann, 1955). The response of the rat chorda tympani to 0·01 M quinine was only 15 per cent of the response to 1·0 M sodium chloride; whereas, the response of the glossopharyngeal to 0·01 M quinine was 115 per cent of that to 1·0 M NaCl. These values are in general agreement with those reported by Yamada (1965).

The hydrochloric acid threshold is also approximately one log unit lower in the glossopharyngeal, but the relative response magnitude to acid is about the same in the two nerves. The sucrose threshold is almost the same in the two taste nerves and, except for one preparation, sugar responses are small in the glossopharyngeal as they are in the chorda tympani (Pfaffmann, 1955).

The response functions to sodium chloride for the two nerves are considerably different; the chorda tympani shows no "water" response, the direction and magnitude of the change in level of firing of the nerve is de-

pendent upon the concentration of the solution bathing the tongue (Pfaffmann and Powers, 1964), and the threshold, for an increase in activity to the sodium chloride stimulus on a water rinsed tongue lies between 0·001 M and 0·003 M (Pfaffmann, 1955). The glossopharyngeal, on the other hand, responds to water with an increase in activity, responds less to 0·01 M and 0·1 M, and responds more to 0·1–0·2 M. Yamada (1965) did not report a water response or evidence of suppression. This may have reflected our different methods of stimulation. Our curves show that threshold for increase in response to NaCl is much higher in the IXth that in the chorda tympani nerve. This is consistent with the continued increase in discharge with concentrations as high as 4 M reported by Yamada.

Therefore, there appear to be two different sensory "mechanisms" in one species by which sodium chloride on the tongue can be distinguished from water: one (via the chorda tympani) signals minute differences in concentration of the bathing solution by appropriate increases or decreases in the level of response, the other (via the glossopharyngeal) operates by opposition of responses to "water" and "salt". This latter "mechanism" is similar to that found in the cat chorda tympani: threshold for an increase in nerve activity is high (approximately 0·1 M), and a "water" response is present (Zotterman, 1961). Behaviorly, the cat shows a slight preference for sodium chloride which begins at about 0·1 M (Carpenter, 1956), while the rat can discriminate much lower concentrations (0·001 M) from water.

Taste thresholds for preference and aversion as well as absolute behavioral thresholds have been determined; it would be expected that these threshold responses would not occur to concentrations below those which first produce neural activity in any of the nerves mediating taste. A fairly good correspondence between the behavioral measures and the response of the chorda tympani alone has been noted (Pfaffmann, 1960); since some information is now available about both of the major taste nerves of the rat, the correspondence seems even better.

Sucrose preference thresholds (Richter and Campbell, 1940) and absolute thresholds (Koh and Teitelbaum, 1961) agree with the neural response threshold to this substance in the chorda tympani (Hagstrom and Pfaffmann, 1959); the glossopharyngeal threshold is a half log unit higher. Sodium chloride preference thresholds in adrenalectomized rats (Richter, 1939) and absolute thresholds in normal rats (Koh and Teitelbaum, 1961) coincide with the chorda tympani response threshold (0·001 M); the glossopharyngeal's first response to sodium chloride is to 0·1 M.

Hydrochloric acid aversion begins at a considerably higher concentration (0·002 M) than the chorda tympani response threshold (0·001 M) (Ben-

jamin, 1953), but this threshold is higher than the absolute taste threshold determined by Koh and Teitelbaum (1961), which was 0·0005 M; the glossopharyngeal nerve responds to concentrations as low as 0·0003 M. The concentration of quinine which is first avoided 0·00001 M (Benjamin and Pfaffmann, 1955) and its absolute behavioral threshold are identical but this value is considerably below the threshold for the chorda tympani which is 0·0001 M; however, the first obtained response of the glossopharyngeal is to 0·00001 M. That is, the glossopharyngeal responds first to concentrations of quinine and hydrochloric acid which are equal to or lower than behavioral threshold concentrations of these substances, whereas the chorda tympani does not respond to these concentrations.

Thus, behavioral thresholds can be accounted for by considering both major taste nerves; it appears that the fine discrimination of sodium chloride in the rat is mediated mainly through the chorda tympani, that sugar is detected at slightly lower concentrations by the chorda tympani; that hydrochloric acid aversion may be mediated by either nerve, but its absolute detection is mediated through the glossopharyngeal, and that quinine aversion and absolute threshold is mediated through the glossopharyngeal. The extreme sensitivity of the glossopharyngeal to quinine localizes most "bitter" taste in the rat at the back of the tongue, where it is classically located in humans; and the sensitivity of the chorda tympani to sodium chloride localizes the "salty" taste at the front of the tongue, although strong salt solutions excite the posterior receptors too. Finally, it should be noted that the chemical sensitivities of the foliate papillae have not yet been determined; information regarding the response of the glossopharyngeal to foliate stimulation is necessary to complete the determination of the major sensory field of taste in the rat. Such determinations are currently under way.

II. ENVIRONMENTAL DETERMINANTS OF BEHAVIOR

Such results indicate a good correspondence between sensory mechanisms of taste and behavior. But the correspondence refers primarily to threshold values and the concentration ranges over which increasing aversion to certain stimuli may be found. In the case of the positively attractive stimuli, there is a typically nonmonotonic preference aversion function in which the just supra threshold solution elicits preferential ingestion which increases up to a peak concentration point beyond which preference de-

clines give way to an aversion. Osmotic effects (McLeary, 1953; Shuford, 1959) plus caloric effects in the case of sugars (Jacobs, 1964) it has been suggested, act to brake or to turn off the ingestion of attractive stimuli such as sucrose.

The role of the purely gustatory factors in the case of NaCl has been examined by a number of methods. Thus the esophageal fistulated animal with no input to the digestive tract shows a preference–aversion function for NaCl. Sugar solutions show largely positive responses to sugar (Stellar *et al.*, 1954; Mook, 1963). By-passing all taste and smell by the Epstein gastric cannula shows complete elimination of salt and sugar preferences (Borer and Epstein, 1965). Oral denervation, and ablation of the thalamic taste nuclei clearly attenuate preferential selection (Richter, 1939; Pfaffmann, 1952; Ables and Benjamin, 1960; Oakley and Pfaffmann, 1962).

Multiple stimulus tests which permit tasting of all concentrations simultaneously show the NaCl peak preference, although at a lower concentration than in the successive test. In the multiple test, sugar shows only a monotonic function of increasing intake with increased concentrations as do short term relative intake tests or rates of response in operant bar pressing situations, especially where volume of intake is minimized (Carpenter, 1958; Collier and Myers, 1961).

I shall restrict the present discussion to the stimulus values for NaCl on the rising part of the preference–aversion function to show the complexity for interpretations from one behavioral test to another.

Anyone who has ever used the two bottle preference test has been plagued by the ever present position habits that develop or are expressed. One animal may develop such a strong preference for one side that alternation of tubes from one side to the other has little effect except in the case of the very strong stimuli. In these certain instances, the animal ignores the taste stimulus and measures of both preference and aversion are attenuated.

We thought that one simple solution would be to eliminate position entirely by using only a simple aperture behind which each of two solutions would be automatically alternated, at regular one minute intervals (Fisher, 1964). As a control the same device was used with two apertures at which salt and water were presented alternately at 1 minute intervals, each at its own aperture. The positions of saline and salt containers were reversed at successive 12 hour periods and each animal lived continuously in the automated living cage. Food was available throughout the test, drinking records were obtained by means of an electronic drinkometer circuit.

In general, each animal produced 6000–8000 licks within each 12 hour period (1 ml of liquid per 200 licks). Figure 4 shows graphically the devel-

opment of licking behavior by all animals in the two- and single-aperture situations. A stable preference appeared during the fifth period in the two-aperture situation following some vacillation. In the single-aperture modification the preference is far less stable. There is an obvious discrepancy between the licking patterns shown in the two modifications of the apparatus. The dialysis was performed as a control to determine whether some construction feature in the apparatus prevented any preference behavior. Figure 4 suggests a trend for enhanced salt preference soon after the dialysis.

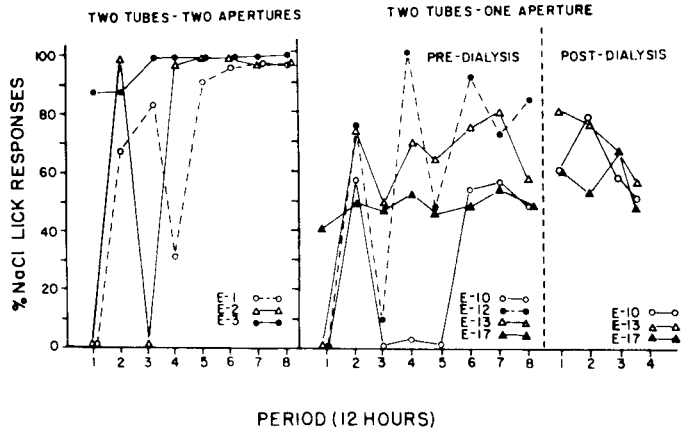


FIG. 4. Per cent of licks at tube containing saline solution for all animals. Percentages are based on licks-at-saline-tube divided by the total number of licks × 100 for each 12-hour period. (Reproduced from *Psychon. Sci.* with permission.)

We were clearly surprised to find that by removing position we had reduced or almost removed the taste preference. In the single-aperture situation the animal must lick the individual tubes to determine the contents. In the two-bottle situation, once a given tube is licked, a distinctive body stance, position in cage or specific visual cues may become associated with the slightly reinforcing saline tube. Once the animal has made a preferential choice, he may remain near the preferred solution. Even if the animal prefers a given solution only very slightly over another, inertia may keep him licking at that solution to a greater extent than is warranted by the degree of the taste preference *per se*. This is in contrast to the need for recurrent sampling of the drinking tubes to determine contents when the solutions are presented at the same aperture. Here, once the thirsty animal licks a tube, response momentum keeps him licking; the saline may taste better but distilled water quenches thirst. The resulting data yields no consistent taste preference. This suggests that the mildly preferred taste solutions may

not be capable of maintaining preferential responding without the aid of supplementary cues. Conversely, the very *clear* preference for 0·1 M NaCl demonstrated by rats in the usual two-bottle preference test may be abetted by the wealth of cues available in that procedure.

Overall, the data suggests a slight preference in albino rats for 0·1 M NaCl when alternated with distilled water. In the two-aperture procedure, the weak saline preference is perhaps supported by positional and visual cues to give a clearer saline preference.

The fact that animals rely upon position, as in the case of salt preference, however, can be taken as an evidence that taste stimuli can and do reinforce approach responses to a particular place in the cage where the reinforcing stimulus was located, that is, they are reinforced to perform a specific motor response in a particular location by the gustatory stimulus.

Subsequent work by Fisher (1965) indicated that other distinctive cues associated with the taste stimulus, such as an auditory stimulus, can enhance the intake to strengthen a demonstrated preference. In the two-bottle test we believe that a place or position was associated with the particular taste stimulus.

This is germane to the question of whether animals can learn a locomotor response as a result of reinforcement by saline solutions. At first glance it might be expected that preference can be taken as an index of relative reinforcing value, the more reinforcing a stimulus, the more it would be preferred to a less reinforcing one when both are presented together. Deutsch and Jones (1960) were the first to show the disparity between the *T* maze situation in which water and salt solutions are each located in opposite arms of the *T* maze as compared with the preferential intake of salt in the usual two-bottle test. They found that the mildly thirsty rat would learn to run preferentially to the water rather than the salt in the *T* maze. The same animal in a two-bottle preference test showed the usual strong NaCl preference.

Chiang and Wilson (1963) and others have confirmed this effect. One difficulty with the maze test, however, is that in order to make the animal run in the maze, it must be thirsty, or otherwise motivated. When thirst motivated the animal is reinforced more by water than saline, that is it prefers water.

Stearns (1965) recently repeated the *T* maze test between water and saline under somewhat different conditions as follows: In the home cage half the animals received water and half received NaCl on day 1 as drinking fluid. On days 2 and thereafter the solution which each animal received was determined in an irregular order. On days 21 to 25 each subject was

given one unrewarded exploration trial in the *T* maze daily. The subject was placed in the start box and removed about 30 sec after entering one of the goal boxes. On the 26th day the water trials in the *T* maze began and preference tests discontinued. Each subject was given one trial per day in the *T* maze, NaCl being located in the goal box chosen less often during the exploration trials for each subject. The subject was left in the goal box for 15 min after the first lick. On the next day each subject was forced to the solution not chosen on the previous day and for the following 47 days free and forced choices were alternated, fluid consumption, running times and choices being recorded. Thus in this procedure the animals were given one trial a day and allowed to remain in the goal box for 15 min.

Under these circumstances the blocks of free trials showed an increasing choice of the arm of the *T* maze which contained saline solutions. The group data showed an increasing number of runs to NaCl throughout the experiment. For the last two blocks of free choice trials the increase in the choice of sodium chloride side was statistically significant at better than the 0·01 level.

On the other hand, the group data does not completely give the whole picture, that is although all the *S*'s showed NaCl preferences in the home cages, only 7 of the 10 learned to run to sodium chloride, that is ran at least 7 times in the last 10 free choice trials to sodium chloride. Two other animals seem to be choosing at two sides with about the same frequency as before training and one subject continued to run consistently to water. Nevertheless, these results, contrary to the findings of Deutsch and Jones, and Chiang and Wilson indicate that a preferential runing to the *T* maze arm containing saline could be demonstrated under different experimental conditions. These data suggest that the length of drinking time as well as the quality of the taste stimulation are important in determining preference. Perhaps the longer drinking time and one trial a day permitted the satiation of thirst which might be one factor interfering with the earlier use of the *T* maze to demonstrate salt preference.

Brookshire (personal communication) has also found evidence for another factor in the *T* maze, namely prior experience. Two groups of animals were reared, one only on water, the other on hypotonic saline solution. In subsequent *T* maze tests those reared on water ran to the water arm, those reared on saline ran to the saline arm. Both groups, however, showed the usual two-bottle preference for saline in the home cage *ad lib.* test. Thus an interaction between thirst and deprivation and prior experience with the stimulus seems to be significant in determining the ultimate results of the *T* maze as a behavioral test.

Further indication that saline has a reinforcing effect is given in the results of a new contingent lick procedure (Fisher, 1965), in which licking itself is treated as an instrumental response somewhat after the manner of Hulse *et al.* (1960). In this situation there are two tubes, one fixed, the other retractable so that after the animal licks a specified number of times at the fixed tube, the second will be presented for a predetermined period. It is then withdrawn and in order to reintroduce the second tube the animal must again make the prescribed number of licks at the fixed tube. The presentation of the second tube, therefore, is contingent upon behavior toward the first. The amount of licking at the two tubes was monitored by a drinkometer circuit, appropriate counters and recorders.

The animals were first trained to lick at the retractable tube with no fixed tube in place. The retractable tube was elevated and the fixed tube containing distilled water introduced. Initially one lick at the fixed tube would lower the retractable tube for 15 sec. After ten presentations, the schedule was changed to a ratio of ten licks at the fixed tube to bring down the retractable tube. The fixed tube was always present so that the animal could continue on it or shift to the second tube when it was presented. Thus the presence of both tubes provided a choice but the requirement of a fixed number of licks at the first tube insured that the animal always sampled tube No. 1.

Eight animals were run in a series of three 15 min sessions at each level of water deprivation typically of 23 hr, 6 hr, 3 hr and 1 hr in which the fixed tube contained water and the second tube saline ($W \rightarrow S$ series). Then this order was reversed and the fixed tube contained saline, the retractable tube water ($S \rightarrow W$). The third session repeated the $W \rightarrow S$ sequence followed by a control and final session with water in both tubes ($W \rightarrow W$).

Lick rates were determined by dividing the total number of licks at a tube by the time that tube was available. Thus, the number of licks on the fixed tube was divided by the duration of the session. The number of licks on the retractable tube was divided by the total duration of the tubes' availability at each session. The median lick rates of all animals on both tubes for the last session under each level of deprivation are presented graphically in Fig. 5. Each indicates the mean lick rates on the two tubes, left-hand bar the fixed tube, right-hand bar the retractable one. The unfilled bars indicate water licking, the filled ones salt licking. It can be seen that in the early periods of the test sequence the animal licked primarily at the first tube during high water deprivation, and began to drink less water with lower deprivation and continued training. In the second stage, the $S \rightarrow W$ phase, rates of licking at the fixed tube (containing saline) were higher at

all levels of deprivation. In the second $W \rightarrow S$ phase there was a general decline in licking at the fixed tube (containing water) when compared with the first $W \rightarrow S$.

It is quite apparent that after training, the animal stays on the saline tube and does not shift to water which becomes available. When the water tube brings saline he immediately shifts over to the saline. It would be expected

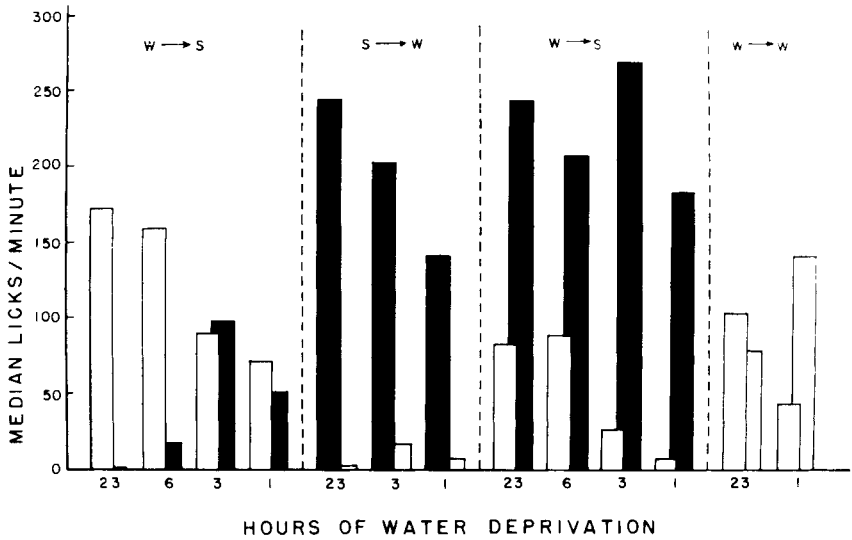


FIG. 5. Median rates of licking for all animals on the third day of each level of water deprivation. The filled bars indicate saline licking; the unfilled bars indicate water licking. $W \rightarrow S$ indicates that licking at the water tube resulted in presentation of saline tube, $S \rightarrow W$ indicates that licking at saline tube presents the water tube, $W \rightarrow W$ indicates that licking at the water tube resulted in presentation of another water tube.

that if the animal indeed preferred water, he would shift to it or stay with it depending upon the arrangements of the fixed and retractable tubes. There is clear indication that the animal prefers saline even in the presence of water and at different deprivation levels.

The same effect is reflected in the pattern of drinking behaviour following training. When the animal is required to make ten licks to bring down the retractable tube with salt there is a clear stepwise progression in the cumulative record of licking on the fixed tube (H_2O), that is, the animal makes only the necessary number of licks to bring the more preferred saline in the second tube (see Fig. 6, $W \rightarrow S$).

On the other hand, in the $S \rightarrow W$ sequence the animal shows a continuously steady lick rate on the fixed saline tube and does not shift to water,

except for a few sampling trials. Thus the animal is exposed to both stimuli but clearly demonstrates a saline preference here also. We conclude along with other investigators that the *T* maze is relatively insensitive for preference testing. Behavior displayed in it is complicated by the interaction of thirst, past experience and possibly other factors which have not yet been entirely analyzed.

Actually, it now seems clear, from the new electrophysiological data that correlation between the properties of the chorda tympani nerve alone and

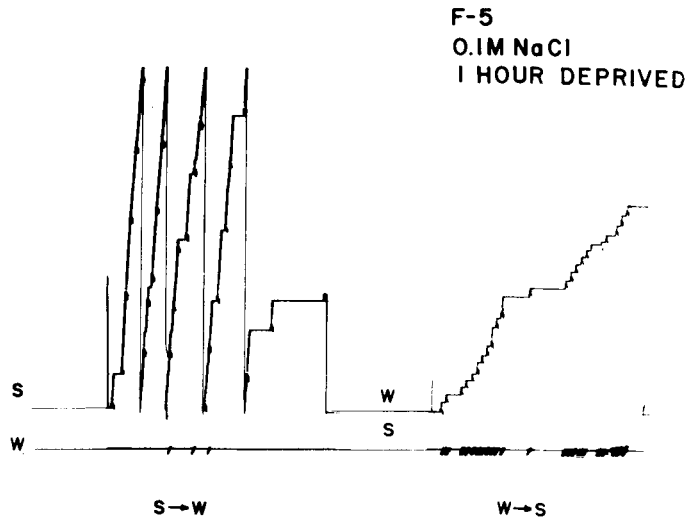


FIG. 6. Two cumulative drinking records from the contingent lick method. *S* indicates record for salt tube, *W* indicates record for water tube. *S*→*W* and *W*→*S* same as in Fig. 5.

behavior must be made with some caution. The present writer has sinned in this way in the past. The most unusual observations on the IXth nerve receptive fields of the rat is that the back of the tongue possesses a functionally different salt receptive system from that of the chorda tympani. There are clear indications of a water receptive system and its inhibition by saline solutions. Until a single unit analysis has been carried out, however, we cannot be certain that the IXth nerve contains a dual fiber system for saline and water like that described for the cat (Zotterman, 1956).

We can ask to what extent is the behavior of an animal under the control of one afferent input as opposed to the other. Presumably the saline response in the chorda tympani occurs earlier, i.e. has a shorter latency, be-

cause normally the solution touches the tip of the tongue before the IXth nerve receptors are reached as the solution travels from the front to the back of the mouth. In addition, there is a delay resulting from flow of solution into the trenches surrounding the papillae. Since earlier experiments on tongue denervation indicated a more significant effect on behavior in the combined chorda–IXth nerve denervations than in the chorda denervation alone (Pfaffmann, 1952) it would seem that correlations of behavior with the chorda tympani alone are incomplete.

SUMMARY

In this paper I have reviewed some new data relevant to preferences or taste-motivated behavior. The first section gave a further electrophysiological inventory of the sensory input from the IXth nerve receptor field of the rat. As expected, a greater sensitivity of the posterior tongue to quinine compared to the anterior tongue was observed, thus providing a closer correspondence between taste electrophysiology and behavior. We have not found an especially good sugar response although there is a definite and clear reaction. Unexpectedly we did find a significant functional difference in the sodium chloride response of the circumvallate IXth nerve area and the chorda tympani nerves. "The rat is like a cat" in the back of its mouth, for we saw some indications of a water response and its inhibition by hypotonic saline solutions. This observation calls for further electrophysiological study.

The difference between the chorda tympani and IXth nerve response to sodium chloride brings into question hypotheses about the control of behavior derived purely from the properties of the chorda tympani. Both nerves presumably are activated in the behaving animal. How does the initial stimulation of the chorda tympani field followed by the later stimulation of the IXth nerve with its different functional properties interact in the control of behavior. Prior studies involving denervation of the IXth and chorda tympani nerves independently and in combination give little hint except to show that the overall behaviour is more influenced by removal of both IXth and chorda tympani nerves than by either alone.

Environmental or situational factors interact with the more purely sensory determinants so that apparently similar behavioral methods may inadvertently focus upon different processes and thereby give apparently contradictory results. Stearns (1965) under different experimental conditions found the *T* maze to give signs of a saline preference. The contingent lick method, which utilized licking both as an instrumental as well as con-

summatory response, provided stronger evidence for a clear saline preference over water. Rats would lick on a water tube only just long enough to bring down the retractable tube containing saline. When the saline tube was presented first they remained at it and, except for a few samplings, rarely shifted to water.

These results indicate that apparently similar behavioral tests must be scrutinized carefully to determine differences amongst them. Physiological and behavioral analyses when so carried out complement each other.

REFERENCES

ABLES, M. F. and BENJAMIN, R. M. Thalamic relay nucleus for taste in albino rat. *J. Neurophysiol.*, 1960, **23**, 376–382.

ANDREW, B. L. and OLIVER J. The epiglottal taste buds of the rat. *J. Physiol.*, 1951, **114**, 48P.

APPELBERG, B. Species differences in the taste qualities mediated through the glossopharyngeal nerve. *Acta Physiol. Scand.*, 1958, **44**, 129–137.

BALDWIN, B. A., BELL, F. R. and KITCHELL, R. L. Gustatory nerve impulses in ruminant ungulates. *J. Physiol.*, 1959, **146**, 14P.

BEIDLER, L. M. Properties of chemoreceptors of tongue of rat. *J. Neurophysiol.*, 1953, **16**, 595–607.

BEIDLER, L. M., FISHMAN, I. Y. and HARDIMAN, C. W. Species differences in taste responses. *Amer. J. Physiol.*, 1955, **181**, 235–239.

BENJAMIN, R. M. *Cerebral mechanisms in gustatory discrimination.* Doctoral dissertation, Brown University, 1953.

BENJAMIN, R. M. and PFAFFMANN, C. Cortical localization of taste in the rat. *J. Neurophysiol.*, 1955, **18**, 56–64.

BERNARD, R. A. An electrophysiological study of taste reception in the peripheral nerves of the calf. *Amer. J. Physiol.*, 1964, **206**, 827–835.

BORER, K. T. and EPSTEIN, A. N. Disappearance of salt and sweet preferences in rats drinking without taste and smell. *The Physiologist*, 1965, **8**, 118, and this symposium.

CARPENTER, J. A. Species differences in taste preferences. *J. Comp. Physiol. Psychol.*, 1956, **49**, 139–144.

CARPENTER, J. A. A comparison of stimulus-presentation procedures in taste-preference experiments. *J. Comp. Physiol. Psychol.*, 1958, **51**, 561–564.

CHIANG, H. and WILSON, W. A. Jr. Some tests of the diluted-water hypothesis of saline consumption in rats. *J. Comp. Physiol. Psychol.*, 1963, **56**, 660–665.

COLLIER, G. and MYERS, L. The loci of reinforcement. *J. Exp. Psychol.*, 1961, **61**, 57–66.

DEUTSCH, J. A., and JONES, A. D. Diluted water: An explanation of the rat's preference for saline. *J. Comp. Physiol. Psychol.* 1960, **53**, 122–127.

ERICKSON, R. P. Sensory neural patterns and gustation. In Y. ZOTTERMAN (Ed.), *Olfaction and Taste.* Oxford: Pergamon Press, 1963, 205–213.

FISHER, G. L. Measured saline preference in the albino rat: The role of nongustatory factors. *Psychon. Sci.*, 1964, **1**, 45–46.

FISHER, G. L. Measured sucrose preference in the albino rat: The influence of an auditory cue. *Psychon. Sci.*, 1965, **2**, 9.

FISHER, G. L. Saline preference in rats determined by contingent licking. *J. Exp. Beh.* in press. 1965.

FISHMAN, I. Y. Single fiber gustatory impulses in rat and hamster. *J. Cell. Comp. Physiol.*, 1957, **49**, 319–334.

GORDON, G. R., KITCHELL, R. L., STRÖM, L. and ZOTTERMAN, Y. The response pattern of taste fibers in the chorda tympani of the monkey. *Acta Physiol. Scand.*, 1959, **46**, 119–132.

HAGSTROM, E. C. and PFAFFMANN, C. The relative taste effectiveness of different sugars for the rat. *J. Comp. Physiol. Psychol.*, 1959, **52**, 259–262.

HULSE, S. H., SNYDER, H. L. and BACON, W. E. Instrumental licking behavior as a function of schedule, volume, and concentration of a saccharine reinforcer. *J. Exp. Psychol.*, 1960, **60**, 359–364.

JACOBS, H. L. Evaluation of the osmotic effects of glucose loads in food satiation. *J. Comp. Physiol. Psychol.*, 1964, **57**, 309–310.

KITCHELL, R. L. Comparative, anatomical and physiological studies of gustatory mechanisms. In Y. ZOTTERMAN (Ed.), *Olfaction and Taste*, Oxford: Pergamon Press, 1963, 335–355.

KOH, S. D. and TEITELBAUM, P. Absolute behavioral taste thresholds in the rat. *J. Comp. Physiol. Psychol.*, 1961, **54**, 223–229.

MCLEARY, R. A. Taste and post-ingestion factors in specific hunger behavior. *J. Comp. Physiol. Psychol.*, 1953, **46**, 411–421.

MOOK, D. G. Oral and postingestional determinants of the intake of various solutions in rats with esophageal fistulas. *J. Comp. Physiol. Psychol.*, 1963, **56**, 645–659.

OAKLEY, B. and PFAFFMANN, C. Electrophysiologically monitored lesions in the gustatory thalamic relay of the albino rat. *J. Comp. Physiol. Psychol.*, 1962, **55**, 155–160.

PFAFFMANN, C. Gustatory afferent impulses. *J. Cell. Comp. Physiol.*, 1941, **17**, 243–258.

PFAFFMANN, C. Taste preferences and aversions following lingual denervation. *J. Comp. Physiol. Psychol.*, 1952, **45**, 393–400.

PFAFFMANN, C. Gustatory nerve impulses in rat, cat and rabbit. *J. Neurophysiol.*, 1955, **18**, 429–440.

PFAFFMANN, C. The pleasures of sensation. *Psychol. Rev.*, 1960, **67**, 253–268.

PFAFFMANN, C. and POWERS, J. B. Partial adaptation of taste. *Psychon. Sci.*, 1964, **1**, 41–42.

RICHTER, C. P. Salt taste threshold of normal and adrenalectomized rats. *Endocrinology*, 1939, **24**, 367–371.

RICHTER, C. P. Transmission of taste sensation in animals. *Trans. Amer. Neurol. Ass.*, 1939, **65**, 49–50.

RICHTER, C. P. and CAMPBELL, K. H. Sucrose taste thresholds of rats and humans. *Amer. J. Physiol.*, 1940, **128**, 291–297.

SHUFORD, E. H. Palatability and osmotic pressure of glucose and sucrose solutions as determinants of intake. *J. Comp. Psychol.*, 1959, **52**, 150–153.

STEARNS, E. B. Reward value of saline and water for the rat. *Psychon. Sci.*, 1965, **2**, 193–194.

STELLAR, E., HYMAN, R. and SAMET, S. Gastric factors controlling water- and salt-solution-drinking. *J. Comp. Physiol. Psychol.*, 1954, **47**, 220–226.

YAMADA, K. The glossopharyngeal nerve response to taste and thermal stimuli in the rat, rabbit and cat. *Kumamoto Medical J.* 1965, **18**, 106–108.

ZOTTERMAN, Y. Action potentials in the glossopharyngeal nerve and in the chorda tympani. *Skand. Arch. Physiol.*, 1935, **72**, 73–77.

ZOTTERMAN, Y. Species differences in the water taste. *Acta Physiol. Scand.*, 1956, **37**, 60–70.

ZOTTERMAN, Y. Studies in the neural mechanism of taste. In W. A. ROSENBLITH (Ed.), *Sensory Communication*. New York: Wiley, 1961, p. 205–216, Ch. **12**.

SUGAR RECEPTOR AND α-AMINO ACID IN THE RAT

HIDEKI TATEDA

Department of Biology, Faculty of Science
Kyushu University

INTRODUCTION

As pointed out by Moncrieff (1946), α-amino acids are known as a group of sweetish organic substances. There have been, however, few electrophysiological studies on the effect of α-amino acids on the sugar receptors. Halpern *et al.* (1962) studied the taste responses of the rat to glycine and DL-alanine using electrophysiological and behavioral methods. They showed that the receptor of α-amino acid was different from that of sodium chloride but the relation between sugar receptor and α-amino acid was not studied.

On the primary process of gustation Beidler (1954) assumed that taste stimulus reacts with specialized receptor sites by adsorption. According to him, the adsorption was described by a monomolecular reaction and of the form of the Langmuir's adsorption isotherm in the response to salt in the rat.

On the sucrose receptor, Beidler (1955) reported that the hamster was sensitive to sucrose. From his figure of the curve of the integrated response recorded from chorda tympani nerve in response to various concentrations of sucrose solution, it seems that his taste equation may also be as applicable to the response of the sugar receptor as the salt receptor. Diamant *et al.* (1963) reported the response to sucrose in human chorda tympani. In their report the relation between peak height of response to sucrose and the stimulus concentration also seems to fit Beidler's taste equation. However, this equation does not seem to offer an adequate description of the integrated responses of the rat to various concentrations of α-amino acid reported by Halpern *et al.* (1962).

It, therefore, seems important to study whether or not these substances stimulate the same receptor site, and if so, do they stimulate the same re-

ceptor site in different ways? This was done in the present paper and the results show that sugars and α-amino acids stimulate the same receptor site.

MATERIAL AND METHODS

Albino rats (Wister-King A) from the Animal Supply of Kyushu University Medical School, were used for experiments. Methods of making preparation, stimulating the tongue and recording nervous activities were almost the same as those used by Beidler (1954) and others. The action potentials which were recorded from the chorda tympani nerve with a pair of platinum wire electrodes were amplified through a.c. amplifier and were integrated by means of an electronic summator whose time constant was about 10 sec. Integrated responses were recorded on a pen-recorder.

Stimulating solutions were usually prepared one day before experiments. In each stimulation 10 ml of stimulus solution was flowed over the tongue which was rinsed with tap water after a stationary state of the response was attained. Chemicals used were sucrose, D-fructose, D-glucose, glycine, DL-alanine, L-alanine and DL-α-amino butyric acid.

RESULTS

Taste Responses to Sugars

As pointed out by Beidler (1955) the rat is not so sensitive as the hamster or the cat and the magnitude of integrated response to 1 M sucrose was about $\frac{1}{5}$ that to 0·05 M sodium chloride. At the beginning of stimulation integrated response to sugar showed a transient increase of the magnitude but a steady level was attained after that. The steady level of the response was maintained for more than 10 min, as shown in Fig. 1. The size of the steady level was used as a measure of the response to taste stimulus in the present paper. Figure 2 shows a series of the responses to various concentration of sucrose. The magnitude of the response is plotted against molar concentration of sucrose in Fig. 3a and in Fig. 3b concentration/response (C/R) is plotted against concentration (C). The linear relation in Fig. 3b may show that the response of the sugar receptor of the rat to sucrose is a monomolecular reaction (cf. Beidler, 1954).

The responses to monosaccharides, D-fructose and D-glucose, were, however, different from the response to sucrose in the high concentration range. The magnitude of the response was increased with increase in the concentration of stimulus and, then, a saturation level was attained in

the lower concentration range. If we increase the stimulus concentration the response magnitude is increased again. Integrated responses to various concentrations of D-glucose and D-fructose are shown in Fig. 4 and the

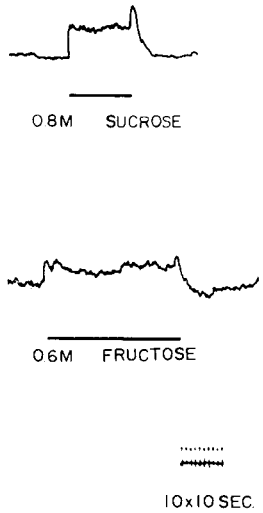


FIG. 1. Stationary state of the integrated response during prolonged stimulation. Upper trace is the response to 0·8 M sucrose. Lower trace is the response to 0·6 M D-fructose. Bar shows a period of stimulation.

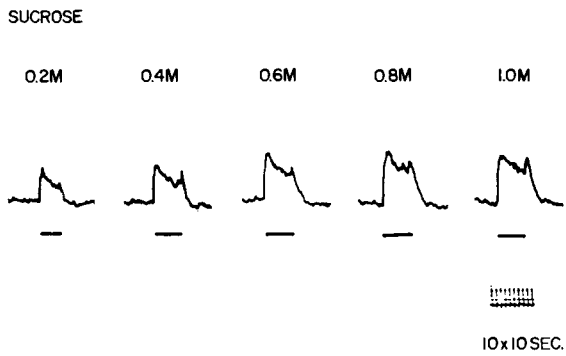


FIG. 2. Integrated responses to various concentrations of sucrose. Bar shows a period of stimulation.

magnitude of the response is plotted against the concentration of the stimulus in Fig. 5. The response to sucrose which was obtained from the same preparation is also plotted in Fig. 5. Since the characteristic of the curve of the response to sucrose is the same as that shown in Fig. 3a, the results on the D-fructose and D-glucose may show that the stimulating

mechanism of these substances differs from that of sucrose. When a very high concentration of fructose or glucose was used as stimulus, the second saturation level was attained, as shown in Fig. 9. Though such high concentration of stimulus sometimes affects the response to low concentration of stimulus, the data which showed reasonably good reproducibility of the response were used and the interval between stimuli was at least 15–20 min in the high concentration range.

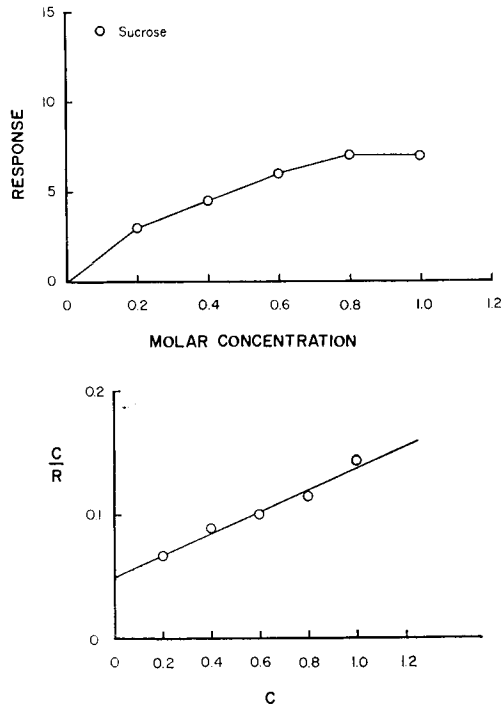


FIG. 3. a: The plot of the magnitude of the response to various concentrations of sucrose vs. the molar concentration of the stimulus.
b: The plot of the molar concentration of the sucrose/the magnitude of the response (C/R) vs. the molar concentration of sucrose.

Responses to α-Amino Acids

Halpern *et al.* (1962) studied on the response to glycine and alanine electrophysiologically, as well as psychologically. Results obtained in the present study were coincided with theirs. Figure 6a shows integrated responses to various concentration of glycine and the magnitude of the response is plotted against the concentration of glycine in Fig. 6b. Such an

S-shape relation between the magnitude of the response and the concentration of stimulus is far from the form of Langmuir's adsorption isotherm. Almost the same relation between the response magnitude and the

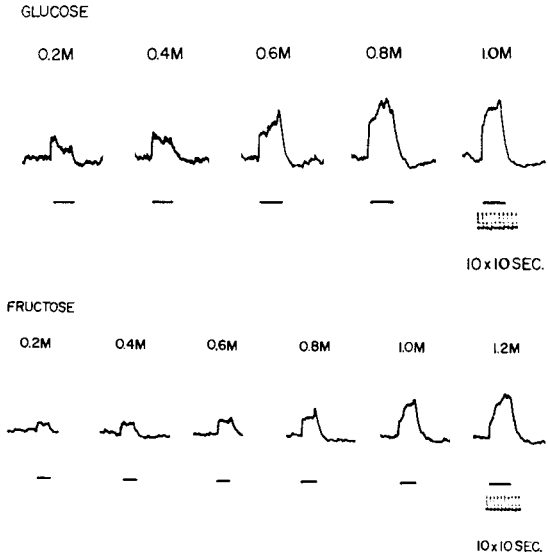


FIG. 4. a: Integrated responses to various concentrations of D-glucose. b: Integrated responses to various concentrations of D-fructose. Bar shows a period of stimulation.

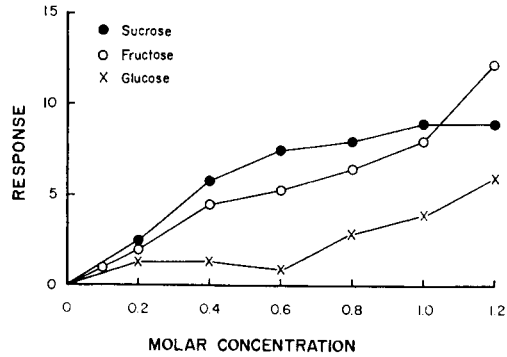


FIG. 5. The plot of the magnitude of the response vs. the molar concentration of stimulus obtained from the responses to sucrose, D-glucose, and D-fructose.

stimulus concentration was observed in responses to DL-alanine, as shown in Fig. 7.

A simple *S*-shape relation in the plot of response against the concentration obtained by α-amino acid stimulation suggests that one receptor

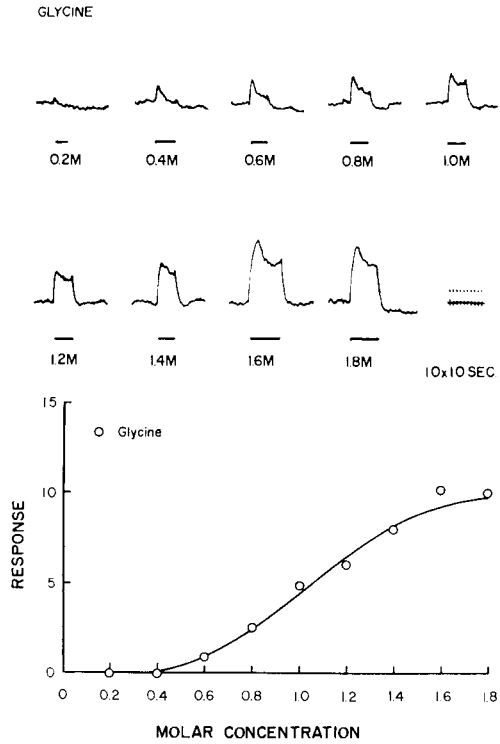


FIG. 6. a: Integrated response to various concentrations of glycine. Bar shows a period of stimulation.
b: The plot of the magnitude of the response to glycine vs. molar concentration of the stimulus.

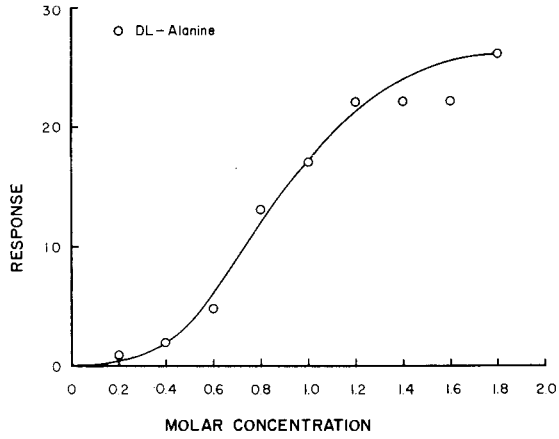


FIG. 7. The plot of the magnitude of the response to DL-alanine vs. molar concentration of the stimulus.

site may combine with several stimulus molecules simultaneously, instead of monomolecule adsorption as proposed by Beidler in the salt receptor (1954) (see Discussion).

Responses to Mixture of Sugar and α-Amino Acid

In 1961, Beidler reported that sodium chloride and sodium butyrate stimulated the same receptor sites and that competitive inhibition was observed. If Beidler's taste equation could be applicable to the excitation of the sugar receptor, competition between sugar and α-amino acid must be observed. In the present study the responses to α-amino acids could not be described quantitatively, as shown in the preceding section, so that it is not possible to describe competitive inhibition quantitatively, even if the competition between sucrose and α-amino acid occurred at the receptor sites. Therefore, only mutual effect of the mixture of sugar and α-amino acid was tried to observe qualitatively. If sugar and α-amino acid stimulated entirely different receptor sites independently, the response to a mixture must be a sum of the response to sugar and that to α-amino acid in the whole range of concentration of each component, while if both sugar and α-amino acid stimulate the same receptor site, the response to the mixture may, at least, not be a simple sum of the response to sugar and that to α-amino acid, especially in the high concentration range.

Sucrose was dissolved in glycine solution of a certain concentration and mixtures of various concentrations of sucrose and a certain concentration of glycine were prepared. If the appropriate concentration of glycine solution was used, responses to sucrose increased in the presence of glycine. Figure 8a shows records of such a synergistic effect of glycine and sucrose on the sugar receptor. The response to 0·6 M glycine was very small and the responses to 0·4 M or 0·8 M sucrose were not so large. Response to the mixture was three to five times larger than the sum of the response to glycine and sucrose. In Fig. 8b the magnitude of the response to various concentrations of sucrose in 0·6 M or 0·3 M glycine is plotted against the concentration of sucrose in mixture. The responses to zero mol of sucrose in 0·6 M or 0·3 M glycine mean the responses to 0·6 M or 0·3 M glycine. The response to the mixture was not a sum of the response to sucrose and to glycine present in the mixture but enhancement of the response was observed. Such an increase in the response to sucrose by the presence of glycine beyond the maximum of the sucrose response means that sucrose molecules and glycine molecules act synergistically on the same receptor site, even though their stimulating mechanisms

are somewhat different from each other. Such synergy is explained by the fact that the complex of the receptor site with one glycine molecule is unstable and easy to combine further with glycine molecules. That is,

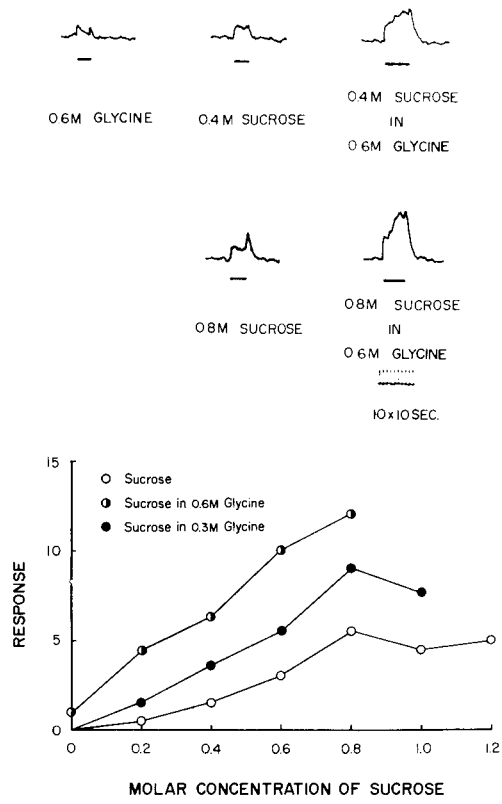


FIG. 8. a: Integrated responses to 0·6 M glycine, 0·4 M and 0·8 M sucrose, 0·4 M sucrose in 0·6 M glycine and 0·8 sucrose in 0·6 M glycine. Note synergy in responses to mixtures. Bar shows a period of stimulation.
b: The plot of the magnitude of the response to sucrose and mixture of sucrose and glycine, vs. molar concentration of sucrose. Note that the response to 0 M sucrose in 0·3 M glycine and that to 0 M sucrose in 0·6 M glycine show the response to 0·3 M glycine and that to 0·6 M glycine, respectively.

the glycine-receptor site complex may have a higher affinity with sucrose than the sucrose-receptor site complex, so that there are complexes of the receptor sites by some sucrose molecules and some glycine ones simultaneously and these complexes cause a large response (see Discussion).

The responses to glycine and D-fructose of high concentration were recorded from one and the same preparation and compared with those

to a mixture of glycine and fructose. The size of the maximum response attained by fructose was almost the same as that by glycine, though maximum response by fructose stimulation was attained in lower concentration than that by glycine, as shown in Fig. 9. The stimulation by fructose dissolved in 0·8 M glycine solution elicited almost the same size of maximum response but the concentration at which maximum response to the mixture was attained was much lower than in the case of fructose. The fact that the maximum of the response to the mixture was not more than that of the response to fructose may also show that glycine and fructose stimulate the same receptor site.

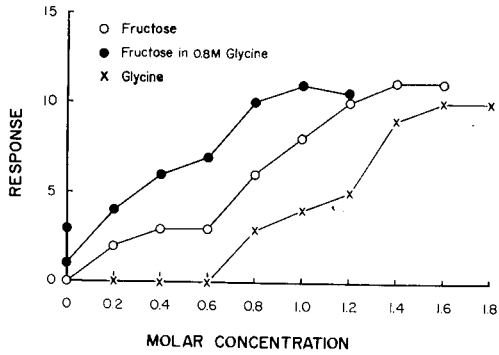


FIG. 9. Comparison of the stimulus–response curves to D-fructose, glycine and mixture of fructose and glycine. The magnitude of the response is plotted against the concentration of the stimulus for glycine and fructose but for mixture it is plotted against the concentration of fructose in 0·8 M glycine solution.

Response to High Concentration of Sucrose

It is possible to assume that the sugar receptor site combines with several molecules of stimulant, as shown in the response to glycine. If so, the second reaction between sucrose and sucrose-receptor site complex may start and make $(\text{sucrose})_2$-receptor site complex when a high concentration of sucrose is applied to the tongue. Such $(\text{sucrose})_2$-receptor sites may cause greater response than sucrose-receptor complex. In such a case, the affinity of sucrose-receptor site complex for sucrose must be smaller than that of the bare receptor site for sucrose. In fact, a second increase of the response was often observed in stimulation with sucrose from 1·0 M to 2·0 M as shown in Fig. 10. Such a high concentration of stimulation often depresses the response. In this experiment a series of stimulations by fructose was carried out after a series of sucrose stimulations, so that

the coincidence of the saturated level of the response to sucrose with that attained by fructose stimulation may be an incidental occurrence. It may be clear that the response to sucrose also does not fit Beidler's taste equation. This may be explained by multimolecular combination as assumed by glycine stimulation (see Discussion).

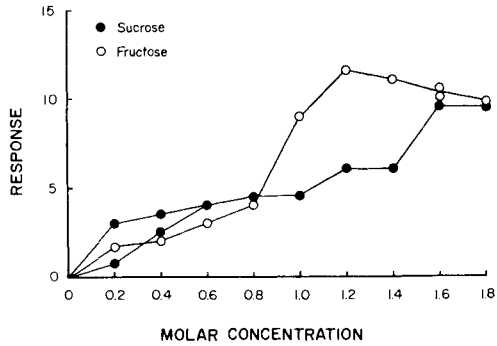


FIG. 10. The response to high concentrations of sucrose and D-fructose. The magnitude of the response is plotted against the concentration of the stimulus.

DISCUSSION

According to Beidler (1954) the taste stimulus combines with the receptor site by adsorption—described by a monomolecular reaction—and the magnitude of the neural response is proportional to the number of sites filled with stimulus molecules. The reaction may be written as follows:

$$\underset{C}{\text{Stimulus}} + \underset{N-A}{\text{Receptor Sites}} \rightleftharpoons \underset{A}{\text{Stimulus} - \text{Receptor Sites}}$$

where C is the concentration of stimulus, N is the total number of receptor sites and A is the number of receptor sites filled with stimulus at a concentration C. Applying the mass action law the equilibrium constant (K) is found to be:

$$K = A/C(N-A).$$

Since the magnitude of the response is proportional to the number of complex, A, ($R = kA$), and the maximum response occurs when all the sites are filled ($Rs = kN$), the above equation then becomes:

$$C/R = CR_s + 1/KR_s$$

where R is the magnitude of response to the taste stimulus at concentration C, and R_s is the maximum response.

If Beidler's taste equation describes all of the present data, then the plot of C/R vs. C will result in a straight line relation. Figure 11a shows such a plot obtained from the responses to sucrose and fructose and Fig. 11b shows the same plot obtained from the responses to sucrose and

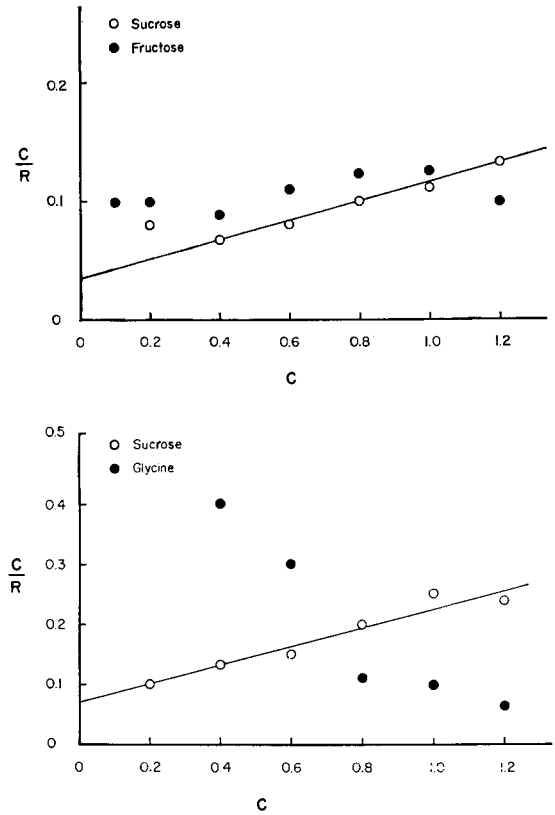


FIG. 11. a: Comparison of the response to sucrose with that to D-fructose in the plot of C/R vs. C. The linear relation is shown for sucrose alone.
b: Comparison of the response to sucrose with that to glycine in the plot of C/R vs. C. The linear relation is shown for sucrose alone.

glycine. It must be noted that the plots obtained from the responses to fructose and glycine do not show a straight line relation, while the plot from the sucrose response shows such a relation. This illustrates the fact that the responses to glycine or fructose do not fit Beidler's taste equation.

Since sucrose, fructose and glycine showed mutual competition (cf. Fig. 8 and 9), we have to consider that these may stimulate the same receptor sites. From what kind of difference in stimulating mechanism

does the difference among stimulus–response curves of these substances result? Difference in affinity of these substances with the bare receptor site alone cannot explain the present data. The results may be explained by assuming that a limited number of molecules combine with the receptor site simultaneously. The reaction may be written as follows:

$$\begin{array}{lll} \text{Stimulus} & - \text{ Receptor Site} & \rightleftharpoons \text{ Stimulus-Receptor Site} \\ C & N-(A_1+A_2+\ldots+A_n) & A_1 \end{array}$$

$$\begin{array}{lll} \text{Stimulus} & - \text{ Stimulus-Receptor Site} & \rightleftharpoons (\text{Stimulus})_2\text{-Receptor Site} \\ C & A_1 & A_2 \end{array}$$

$$\begin{array}{lll} \text{Stimulus} & - (\text{Stimulus}_2\text{-Receptor Site} & \rightleftharpoons (\text{Stimulus})_3\text{-Receptor Site} \\ C & A_2 & A_3 \end{array}$$

$$\cdots\cdots\cdots\cdots\cdots\cdots$$

$$\begin{array}{lll} \text{Stimulus} & - (\text{Stimulus})_{n-1}\text{-Receptor Site} & \rightleftharpoons (\text{Stimulus})_n\text{-Receptor Site} \\ C & A_{n-1} & A_n \end{array}$$

where A_1, A_2, ..., A_n are numbers of the receptor sites filled with one, two ... and n molecules, respectively. Applying the mass action law, the equilibrium constants are

$$K_1 = \frac{A_1}{C\{N-(A_1+A_2+\ \ldots\ +A_n)\}},$$

$$K_2 = \frac{A_2}{CA_1}, \quad K_3 = \frac{A_3}{CA_2}, \ldots, K_n = \frac{A_n}{CA_{n-1}}.$$

In order to simplify the relation, we assume that the magnitude of the response is proportional to a sum of the receptor sites combined with the stimulus ($R = kA_1+\alpha kA_2+\beta kA_3+\ldots+\nu kA_n$) and that $K_2 = K_3 = \ldots = K_n = K_e$. The above equation, therefore, becomes

$$R = \frac{R_m K_1 C\{1+\alpha K_e C+\beta(K_e C)^2+\ \ldots\ +\nu(K_e C)^{n-1}\}}{1+K_1 C\{1+K_e C+(K_e C)^2+\ldots+(K_e C)^{n-1}\}},$$

where R is the magnitude of the response to the taste stimulation at concentration C, R_m is the magnitude of the response when all the receptor sites are filled with one molecule alone, and α, β, ..., ν are constants which are larger than one. In the case where K_e is much smaller than K_1 ($K_e \ll K_1$), Beidler's taste equation is obtained from the above equation as follows:

$$R = \frac{R_m K_1 C}{1+K_1 C}.$$

This may be the case of sucrose stimulation. It must be expected, therefore, that the response to sucrose increases in size if one uses so high a concentration of sucrose that the second reaction cannot be neglected. This was obtained in the present study, as shown in Fig. 10.

If K_2 is much larger than K_1, K_3 is larger than K_2 and so on, the plot of R vs. C will give an S-shape relation. The plot obtained from the response to glycine in the present paper was similar to this relation. In this case, however, R_m cannot be assumed. Moreover, there are so many constants, K_1, K_2, ..., K_n, α, β, ..., ν, and n, that it is difficult to obtain reliable values for them. Instead of such theoretical approach, we assume a reaction roughly as:

$$n(\text{Stimulus}) + \text{Receptor Site} \rightleftharpoons (\text{Stimulus})_n\text{-Receptor Site}$$

In other words, the receptor sites saturated partially with glycine are unstable and easy to combine further with glycine. This assumption may not be so far from the fact in the case where K_n is much larger than K_{n-1}. Then a rough expression is as:

$$R = \frac{R_s' K' C^n}{1 + K' C^n} \quad \text{or} \quad \frac{R}{R_s'} = \frac{K' C^n}{1 + K' C^n},$$

where R_s' is the maximum response obtained in high concentration of stimulation, K' is the equilibrium constant and n is a whole number. The latter expression is similar to Hill's empirical relation used for combination of haemoglobin with oxygen. By rearranging the above equation we obtain

$$\log \frac{R}{R_s' - R} = n \log C + \log K'.$$

In the plot $\log \dfrac{R}{R_s' - R}$ against $\log C$, the linear relation must be obtained and n must also be assumed from the plot, if this assumption is to fit the present case. It is clear from the results shown in Fig. 12 that this expression describes the response to glycine with a very high degree of accuracy. We, however, obtained $n = 4{\cdot}4$. As in the combination of haemoglobin with oxygen, n did not turn out to be a whole number. This may be due to the fact that when the receptor sites combine with glycine molecules they make a whole series of different complexes, as assumed before.

Synergy observed in the response to sucrose–glycine mixture is also explained by the assumption mentioned above. A complex of glycine-receptor site is unstable. Sucrose may, therefore, combine more easily with the glycine-receptor site complex than with the sucrose-receptor site complex so that the sucrose–glycine-receptor site is newly made and elicits as large a response as the $(\text{glycine})_2$-receptor site.

In the present paper only the response of the stationary state was studied but the response observed in the beginning of the stimulation was not

taken into consideration. It must be noted, however, that the behavior of the early phase of the response is sometimes different from that of the stationary state of the response. For instance, it was often observed that the rising phase to the stationary state started so late after the peak of the fast phase that the change in the magnitude of each phase could be measured separately within a relatively wide concentration range as shown in Fig. 4b.

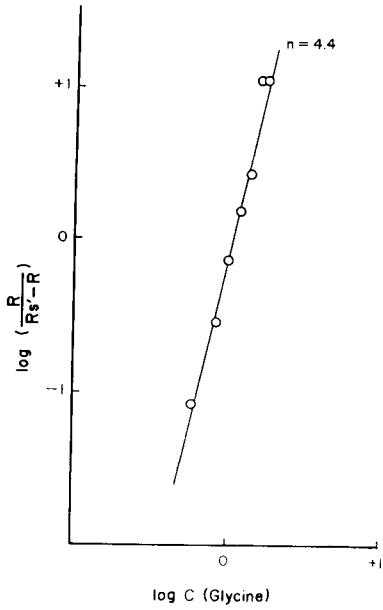


FIG. 12. The plot of $\log\left(\frac{R}{R_s'-R}\right)$ vs. $\log C$ obtained from the responses to glycine. See text for explanation.

In such case, the fast phase did not increase so much with increase of the concentration as that of the stationary state did in the higher concentration range. It is, therefore, possible that the response of the fast phase occurs by excitation of different receptors from those which elicit the response of the stationary state. It is, however, clear from the present study that, either by sugars or by α-amino acids, an entirely different type of the response from the monomolecular reaction type of the response is elicited in the stationary state. This can be explained by assuming multimolecular combination of the stimulus with the receptor site.

SUMMARY

Taste responses to sugars and α-amino acids were studied electrophysiologically, recording from the chorda tympani nerve of the rat.

Stimulus–response curves obtained from the responses to various concentrations of D-fructose and D-glucose did not conform to Beidler's taste equation, while that obtained from the response to sucrose did.

The stimulus–response curve obtained from the responses to glycine or DL-alanine showed an *S*-shape relation.

The response to a mixture of sucrose and glycine was not a sum of the response to sucrose and that to glycine, but synergistic increase of the response was observed.

Similarly the response to a mixture of fructose and glycine was not in linear relation with that to fructose and that to glycine.

In order to explain the present data, multimolecular combination of stimulus with the sugar receptor site was considered as the primary process of the excitation.

REFERENCES

BEIDLER, L. M. 1954. A theory of taste stimulation. *J. Gen. Physiol.*, **38**, 133–139.

BEIDLER, L. M. 1955. Species differences in taste responses. *Amer. J. Physiol.*, **181**, 235–239.

BEIDLER, L. M. 1961. Taste receptor stimulation. *Progress in Biophysics and Biophysical Chemistry*, Pergamon Press, New York, pp. 107–151.

DIAMANT, H., M. FUNAKOSHI, L. STRÖM and Y. ZOTTERMAN. 1963. Electrophysiological studies on human taste nerves in *Olfaction and Taste*. Ed. ZOTTERMAN, Pergamon Press, New York, pp. 193–213.

HALPERN, B. P., R. A. BERNARD and M. R. KARE. 1962. Amino acids as gustatory stimuli in the rat. *J. Gen. Physiol.*, **45**, 681–701.

HILL, A. V. 1913. The combinations of haemoglobin with oxygen and with carbon mono-oxide. I. *Biochem, J.*, **7**, 471–480.

MONCRIEFF, R. W. 1946. *The Chemical Senses*, John Wiley and Sons, Inc. New York, pp. 102.

PATTERNS OF IMPULSES PRODUCED BY MSG AND 5′-RIBONUCLEOTIDES IN TASTE UNITS OF THE RAT

MASAYASU SATO, SATORU YAMASHITA and HISASHI OGAWA

Department of Physiology, Kumamoto University Medical School, Kumamoto, Japan

SOME of the 5′-ribonucleotides are known to show flavor-enhancing ability in men especially when they are given with monosodium glutamate (MSG). The experimental approach to the effect of four kinds of 5′-ribonucleotides, i.e. sodium 5′-ionosinate (5′-IMP), sodium 5′-guanylate (5′-GMP), sodium 5′-uridylate (5′-UMP) and sodium 5′-cytidylate (5′-CMP), on the gustatory receptors of the rat has been undertaken by Sato and Akaike (1965). By recording the integrated response of the chorda tympani nerve to stimulation of the tongue by MSG, 5′-ribonucleotides and their mixtures, they have shown that the 5′-ribonucleotides of a very low concentration produce a response in the chorda tympani, characterized by a marked sustained phase, that the effectiveness is in the order of 5′-GMP > 5′-IMP > 5′-UMP > 5′-CMP and that a marked enhancement in the response magnitude is produced when a small amount of 5′-GMP or 5′-IMP is added to MSG while the enhancement by the addition of 5′-UMP or 5′-CMP is small compared with the former.

ENHANCEMENT OF THE CHORDA TYMPANI RESPONSE BY 5′-RIBONUCLEOTIDES

The enhancement in the chorda tympani response produced by the addition of a small amount of 5′-GMP to MSG is clearly shown in Fig. 1. Addition of 5′-GMP or 5′-IMP of 1/100 of the amount of MSG produced a response magnitude significantly greater than the magnitude produced by MSG alone.

A similar enhancement, observed when varying parts of 0·3 % MSG were substituted for 5′-GMP or 5′-IMP, is shown in Fig. 2, where the magnitude of the enhancement becomes greater with an increase in the amount of

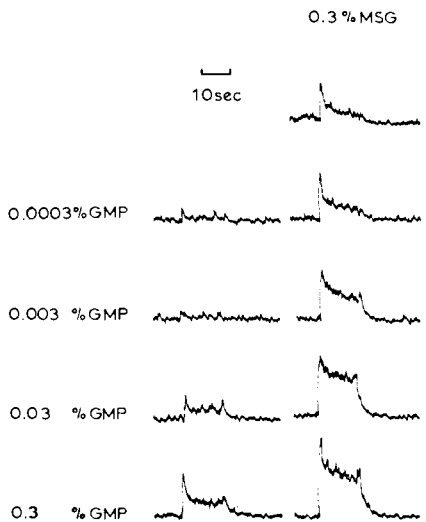


FIG. 1. The integrated chorda tympani response to stimulation of the rat tongue by 0·3% MSG (top record), 0·0003–0·3% 5′-GMP (records at the left) and 0·3% MSG solutions to which 5′-GMP of 0·0003–0·3 g/100 ml was added (records at the right). From Sato and Akaike, *Jap. J. Physiol.*, **15** (1965), 53–70.

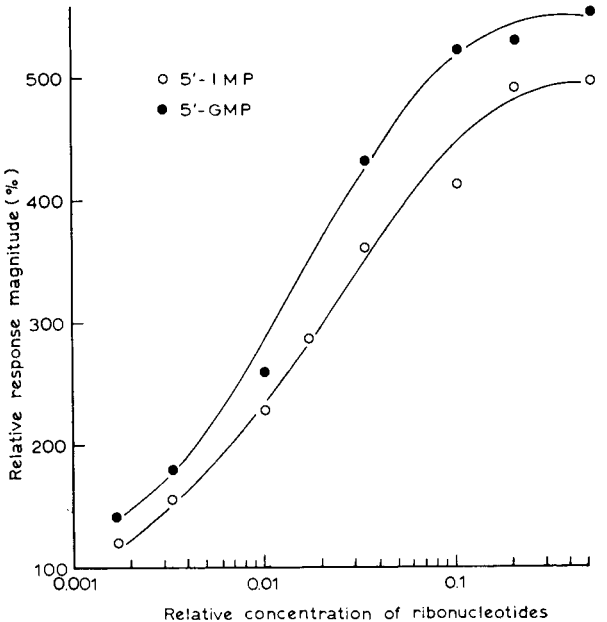


FIG. 2. Enhancement of the magnitude of the chorda tympani response of rat, obtained when a part of 0·3 g/100 ml MSG was substituted for equal amount of 5′-GMP or 5′-IMP. Abscissa; the relative concentration of 5′-ribonucleotide to the total concentration of the mixture, ordinate; the magnitude of response to mixtures of MSG and 5′-ribonucleotides, expressed relative to the magnitude of response to 0·3% MSG. The magnitude of response was measured 10 sec after the onset of stimulation. Redrawn from Sato and Akaike, *Jap. J. Physiol.*, **15** (1965), 53–70.

the substitution and attains a saturated value. The ability of four kinds of 5′-ribonucleotides to produce the enhancement when mixed with MSG is in the order of 5′-GMP > 5′-IMP > 5′-UMP > 5′-CMP. As shown in Fig. 2, the magnitude of the response to mixtures of 5′-GMP or 5′-IMP with

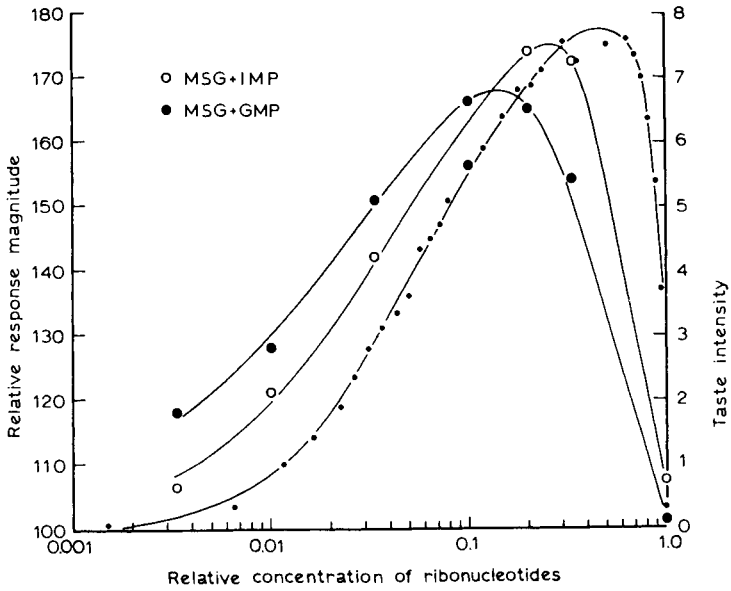


FIG. 3. Enhancement of the chorda tympani response of rat obtained when a part of 0·3 g/100 ml MSG in 0·1% NaCl solution was substituted for equal amount of 5′-GMP or 5′-IMP. Abscissa; the ratio of substitution of MSG for 5′-ribonucleotide, ordinate (left); magnitude of response to mixtures of MSG and 5′-GMP or 5′-IMP in 0·1% NaCl solution, expressed relative to the magnitude of response to 0·3 g/100 ml MSG in 0·1% NaCl solution. The magnitude of response was measured 10 sec after the onset of stimulation. Redrawn from Sato and Akaike, *Jap. J. Physiol.*, **15** (1965), 53–70.

Small filled circles indicate human taste intensity (ordinate at the right), obtained when varying parts of 0·05% MSG in 1% NaCl solution were substituted for equal amount of 5′-IMP. Reproduced with the courtesy of Mr. S. Ikeda from Ikeda, S., Furukawa, H. and Yamaguchi, S., *Statistical Quality Control*, **13** (1962), 76–79.

MSG, when the enhancement is maximal, is greater by about 5 times than that for MSG alone. However, when mixtures of MSG and 5′-GMP or 5′-IMP, dissolved in NaCl solution, were used as stimuli, the magnitude of enhancement shown in Fig. 3 is markedly small as compared with that shown in Fig. 2, suggesting that NaCl inhibits the potentiation produced by MSG and 5′-ribonucleotides. The small filled circles in Fig. 3 represent human taste intensity, measured by Ikeda, Furukawa and Yamaguchi (1962)

when the mixing ratio of 5′-IMP and MSG was varied while the total concentration was maintained at 0·05%, the mixture being dissolved in 1% NaCl solution. The results obtained on the rat chorda tympani response and those on the human taste sensation have yielded a similar relationship between the relative concentration of the ribonucleotides and the response. This indicates that the flavor-ennancing ability of the ribonucleotides is attributed entirely to the receptor mechanism and that the psychological event is reflected in the informations in the chorda tympani nerve.

RESPONSE PATTERNS OF TASTE UNITS

In order to elucidate the mechanism by which addition of a small amount of 5′-ribonucleotides to MSG produces a prominent potentiating effect on the chorda tympani nerve response, impulse discharges by four basic kinds of taste stimuli (0·1 M NaCl, 0·5 M sucrose, 0·01 N HCl and 0·02 M quinine), 0·3% MSG, 0·3% 5′-ribonucleotides and mixtures of 0·27% MSG and 0·03% 5′-ribonucleotides were recorded from a few nerve fibers in the chorda tympani of the rat. MSG, 5′-ribonucleotides and

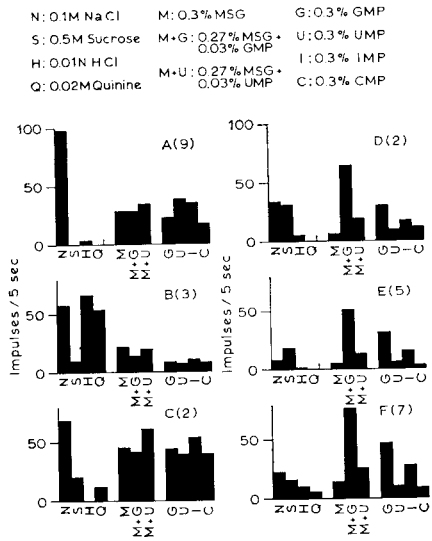


FIG. 4. The response spectrum of single taste units, the stimuli being 0·1 M NaCl, 0·5 M sucrose, 0·01 N HCl, 0·02 M quinine, 0·3% MSG, 0·27% MSG+0·03% 5′-GMP, 0·27% MSG+0·03% 5′-UMP, 0·3% 5′-GMP, 0·3% 5′-UMP, 0·3% 5′-IMP and 0·3% 5′-CMP (from the left). The ordinate represents number of impulses in each unit (*A–F*) during the first 5 sec after stimulation. Number of units in each group is shown in the parenthesis.

their mixtures were dissolved in either deionized water or in 0·1 % NaCl solution.

The taste units showed a response to more than one kind of stimuli, as would be expected from the previous works (Pfaffmann, 1955; Fishman, 1957). Examples of the response spectrum, obtained from 28 units are shown in Fig. 4. Among 14 units, which showed a prominent response to 0·1 M NaCl, 11 units showed a poor response to HCl and quinine (*A* and *C* in Fig. 4), while three units a relatively large response to HCl and quinine

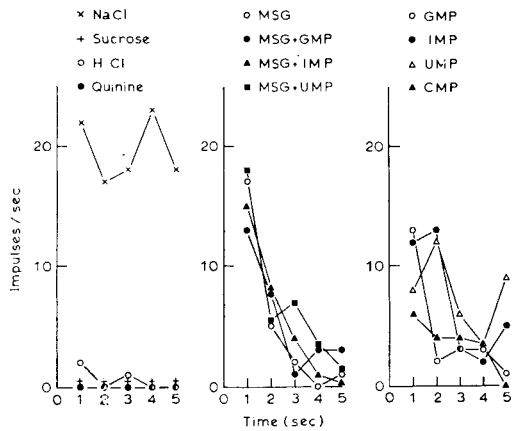


FIG. 5. Patterns of the impulse frequency in a single taste unit during the first 5 sec after stimulation. Note that the unit showed a marked response to NaCl, but little potentiation by MSG and 5′-ribonucleotides.

(*B*). They either did not respond to sucrose (*A*) or responded to it very little (*B* and *C*). Fourteen units showed a response to sucrose, which is greater than or as great as the one to 0·1 M NaCl (*D–F*). As shown in Fig. 4, the units responding to sucrose relatively well showed a highly enhanced response to mixtures of MSG and 5′-ribonucleotides, while the enhancement was small in the units which were sensitive to NaCl. This is also clearly illustrated in Figs. 5 and 6, where the impulse frequency during the first 5 sec after the onset of stimulation is shown.

The ratio of the response to the mixture of MSG and one of the 5′-ribonucleotides against that to MSG alone *(potentiation ratio)* is shown in Table 1, where it is clear that the ratio is far greater in the sucrose-sensitive units than in the NaCl-sensitive units. The table also reveals that addition of 5′-GMP to MSG produces a greater effect than that of 5′-UMP and the potentiation ratio obtained with the mixture dissolved in 0·1 % NaCl is about half of the one with the mixture dissolved in water.

The last column in Table 1 indicates the mean potentiation ratio for 26 units. These mean ratios are approximately equal to those obtained by Sato and Akaike (1965) on the whole chorda tympani nerve response (see Figs. 10 and 11 in Sato and Akaike's paper).

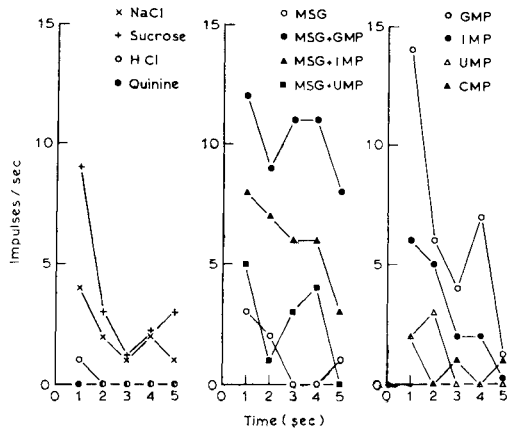


FIG. 6. Patterns of the impulse frequency in a single taste unit during the first 5 sec after stimulation. Note that the unit responded to sucrose well and showed a marked potentiation by MSG and 5'-ribonucleotides.

TABLE 1. POTENTIATION RATIO FOR MSG AND 5'-RIBONUCLEOTIDES

	NaCl-sensitive units	Sucrose-sensitive units	Mean
0·27% MSG+0·03% GMP	1·93 (12)	8·24 (14)	5·33 (26)
0·27% MSG+0·03% UMP	1·49 (12)	3·13 (14)	2·41 (26)
0·27% MSG+0·03% IMP	1·26 (3)	6·32 (7)	4·80 (10)
0·27% MSG+0·03% GMP*	1·08 (12)	6·15 (12)	3·62 (24)
0·27% MSG+0·03% UMP*	0·84 (12)	1·94 (12)	1·39 (24)
0·27% MSG+0·03% IMP*	0·70 (3)	4·67 (7)	3·48 (10)

*Dissolved in 0·1% NaCl solution.

The effect of the mixture of MSG with 5'-IMP was not examined in all the experiments.

ANALYSIS OF THE UNIT RESPONSE

Relationship between the Response to the Mixture of MSG and 5'-Ribonucleotides and that to Sucrose

The above results indicate that there is a definite relationship between the response to sucrose and the potentiation of the chorda tympani re-

sponse by the addition of 5′-ribonucleotides to MSG. Therefore relationships between the response to sucrose and those to other test solutions were investigated in all the units obtained. They are shown in Fig. 7, in which the response to the mixture of MSG and 5′-GMP seems to be lin-

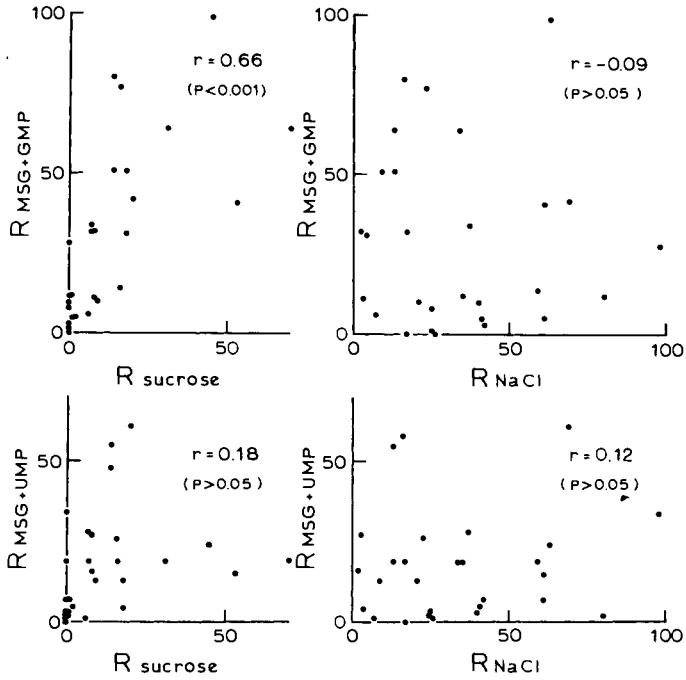


FIG. 7. The relationships between the response to the mixture of MSG with 5′-GMP or 5′-UMP and that to 0·5 M sucrose or to 0·1 M NaCl. The magnitude of all responses is expressed by number of impulses during the first 5 sec after stimulation.

early related to the sucrose response. The correlation between these two responses is highly significant ($r = 0{\cdot}66$, $P < 0{\cdot}001$). However, no significant correlation was obtained between the response to the mixture of MSG and 5′-UMP and that to sucrose ($r = 0{\cdot}18$).

In Fig. 8 the relationship between the potentiation ratio and the sucrose response is shown. It is also clear that the potentiation ratio obtained for the mixture of MSG and 5′-GMP appears to be related to the magnitude of the sucrose response ($r = 0{\cdot}48, 0{\cdot}01 < P < 0{\cdot}05$), but that no significant correlation exists between the potentiation ratio for MSG and 5′-UMP and the response to sucrose ($r = 0{\cdot}21$). Therefore these results indicate that the

response to the mixture of MSG and 5′-GMP is related to the response to sucrose, and that in this respect the response is different from that to the mixture of MSG and 5′-UMP.

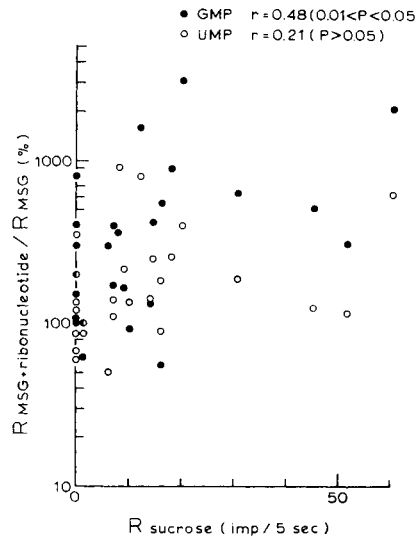


FIG. 8. The relationship between the potentiation ratio for MSG and 5′-GMP or 5′-UMP and the magnitude of response to 0·5 M sucrose. The potentiation ratio is expressed as per cent and shown in logarithmic scale.

Relationship Between the Response to the Mixture of MSG and 5′-Ribonucleotides and that to NaCl

The relationship between the response to the mixture of MSG with 5′-GMP or 5′-UMP and the response to 0·1 M NaCl is also shown in Fig. 7. Although the former appears to decrease as the latter increases, the data are scattered very much and do not yield any significant correlation between these two parameters. However, when the correlation between the potentiaton ratio for MSG and 5′-GMP or that for MSG and 5′-UMP and the respose to 0·1 M NaCl was calculated (Fig. 9), a significant negative correlation was obtained between these two parameters ($r = -0{\cdot}41$, $0{\cdot}01 < P < 0{\cdot}05$ for 5′-GMP and $r = -0{\cdot}53$, $0{\cdot}001 < P < 0{\cdot}01$ for 5′-UMP). Therefore the potentiation produced by the addition of one of 5′-ribonucleotides to MSG is negatively correlated with the response to 0·1 M NaCl; this is consistent with the observation that the potentiation ratio is smaller when the mixture of MSG and 5′-ribonucleotides is dissolved in NaCl solution than when it is dissolved in water (Table 1). The results indicate

that NaCl inhibits the potentiation by MSG and 5′-ribonucleotides. The mechanism of the inhibitory action of NaCl on the potentiation is not clear at present, but a kind of competition between MSG and NaCl may be assumed.

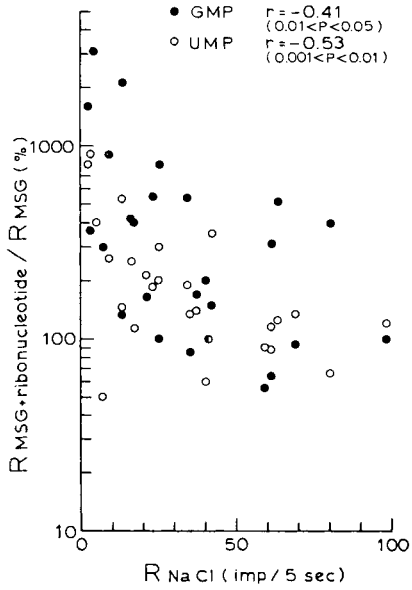


FIG. 9. The relationship between the potentiation ratio for MSG and 5′-GMP or 5′-UMP and the magnitude of response to 0·1 M NaCl. The potentiation ratio is expressed as per cent and shown in logarithmic scale.

Potentiation Ratio as a Function of the Ratio, Sucrose-Response/NaCl-Response

It has now become clear that the potentiation produced by MSG and 5′-ribonucleotides is positively correlated with the response to sucrose but negatively correlated with the response to NaCl. Therefore, the potentiation ratio was plotted in Fig. 10 against the ratio, sucrose-response/NaCl-response. As is clear in Fig. 7, the potentiation ratio for MSG and 5′-GMP or 5′-UMP shows a highly significant correlation to the sucrose/NaCl response ratio ($r = 0{\cdot}87$ for 5′-GMP and $r = 0{\cdot}76$ for 5′-UMP).

The Response to MSG and to 5′-Ribonucleotides

In general, the units responding to 5′-ribonucleotides showed a marked response to the mixture of MSG and 5′-ribonucleotides (Fig. 11), i.e. a highly significant correlation exists between the response to the mixture of

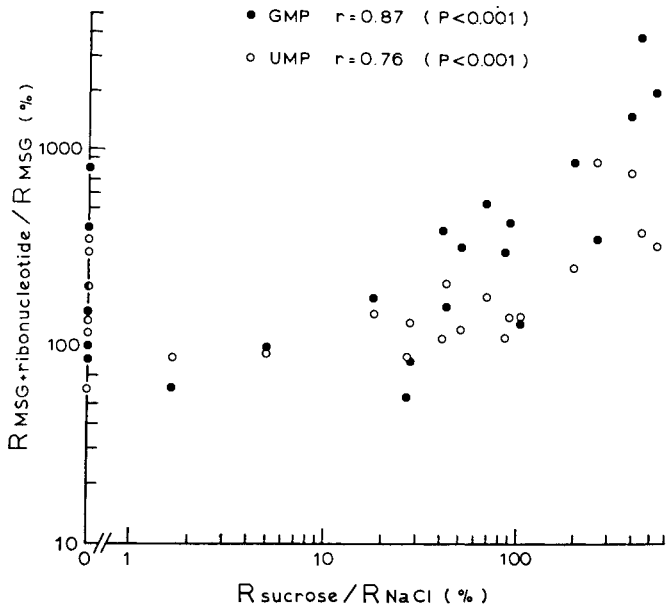


FIG. 10. The relationship between the potentiation ratio for MSG and 5′-GMP or 5′-UMP and the ratio, sucrose-response/NaCl-response. The potentiation ratio is expressed as per cent and shown in logarithmic scale.

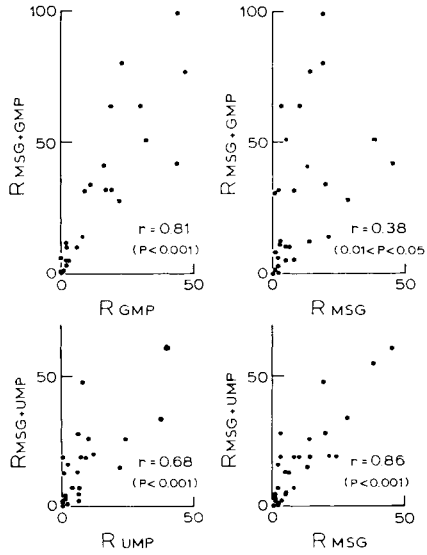


FIG. 11. The relationship between the response to the mixture of MSG with 5′-GMP or 5′-UMP and the response to 5′-ribonucleotide or MSG. The magnitude of response is expressed by number of impulses during the first 5 sec after stimulation.

MSG and 5′-GMP (or 5′-UMP) and the response to 5′-GMP (or 5′-UMP) alone ($r = 0{\cdot}81, P < 0{\cdot}001$ for 5′-GMP and $r = 0{\cdot}68, P < 0{\cdot}001$ for 5′-UMP).

In addition the units showing a marked response to the mixture of MSG and 5′-ribonucleotides responded relatively well to MSG alone ($r = 0{\cdot}38$, $0{\cdot}01 < P < 0{\cdot}05$ for 5′-GMP and $r = 0{\cdot}86, P < 0{\cdot}001$ for 5′-UMP).

This would mean that the potentiation by MSG and 5′-ribonucleotides is not due to the activation of the units which do not have a sensitivity to 5′-ribonucleotides or to MSG, but it is an increase of the magnitude of response in the units which possess a certain sensitivity to both chemicals.

The Response to the Mixture of MSG and 5′-IMP

The response to the mixture of MSG with 5′-IMP was not recorded in all the taste units studied, but it was approximately the same as the response to the mixture of MSG with 5′-GMP. The potentiation ratio obtained for the mixture of MSG and 5′-IMP is, on the average, 4·80 (10 units), which is a little smaller than the one for MSG and 5′-GMP (see Table 1). This is in close agreement with the results by Sato and Akaike (1965) on the whole chorda tympani recording.

CONCLUSION

It has become clear from the present experiment that the response to the mixture of MSG and 5′-GMP (or 5′-IMP) is positively correlated to the response to sucrose and 5′-GMP and probably to that to MSG. On the other hand, the response to the mixture of MSG and 5′-UMP is positively correlated to the response to MSG and 5′-UMP, but not to that to sucrose. If we make an assumption that the discrimination between stimuli is related to the profiles of the impulse frequency and highly correlated stimuli taste alike but uncorrelated ones taste differently (Erickson, 1963; Pfaffmann, 1964), then the mixture of MSG and 5′-GMP (or 5′-IMP) should produce a taste sensation, which is to some extent similar to that by sucrose but is entirely different from the taste by the mixture of MSG and 5′-UMP.

The potentiation between MSG and 5′-ribonucleotides has been found to be related to the sucrose/NaCl response ratio. The physicochemical mechanism for this significant relationship is unexplained, but needs a further investigation.

ACKNOWLEDGEMENT

The authors aknowledge their indebtedness to Takeda Chemical Industries, Ltd. for providing many facilities in carying out the experiment.

REFERENCES

ERICKSON, R. P. 1963. Sensory neural patterns and gustation. In *Olfaction and Taste*, ed. Y. ZOTTERMAN. Pergamon Press, Oxford.

FISHMAN, I. Y. 1957. Single fiber gustatory impulses in rat and hamster. *J. cell. comp. Physiol.* **49**, 319–334.

IKEDA, S., FURUKAWA, H. and YAMAGUCHI, S. 1962. An attempt to establish scales of taste. Measurement of delicious taste intensity by Thurstone-Mosteller's method. *Statistical Quality Control*, **13**, 76–79 (in Japanese).

PFAFFMANN, C. 1955. Gustatory nerve impulses in rat, cat and rabbit. *J. Neurophysiol.* **18**, 429–440.

PFAFFMANN, C. 1964. Taste, its sensory and motivating properties. *Amer. Scientist.* **52**, 187–206.

SATO, M. and AKAIKE, N. 1965. 5′-ribonucleotides as gustatory stimuli in rats. Electrophysiological studies. *Jap. J. Physiol.* **15**, 53–70.

NEUROPHYSIOLOGICAL STUDIES ON TASTE EFFECTIVENESS OF CHEMICAL TASTE ENHANCERS

A. ADACHI, M. FUNAKOSHI and Y. KAWAMURA

Department of Oral Physiology, Osaka University Dental School, Kitaku, Osaka, Japan

SEVERAL reports have been published on effectiveness of various chemical taste enhancers mostly in the level of integrated response of the taste nerves.

In the present experiment, effectiveness of chemical taste enhancers was studied in the level of functional single taste fiber of the chorda tympani nerve in the cat.

As the test solutions, monosodium glutamate (0·05 M, about 1%), sodium inosinate (0·005 M, about 0·2%) and sodium guanylate (0·005 M, about 0·2%) were used.

As shown in Fig. 1, some of the salt fibers responded to these chemical taste enhancers. The mixture of monosodium glutamate and sodium inosinate or mixture of monosodium glutamate and sodium guanylate always enhancement the nerve responses; however, the mixture of sodium inosinate and sodium guanylate did not induce any enhancement of the response comparing with individual solutions.

As shown in Fig. 2, when monosodium glutamate, sodium inosinate or sodium guanylate was added respectively to salt solution, the activity of the salt fiber was increased; while the activity of the quinine fiber was depressed when these chemicals were blended to quinine solution (Fig. 3).

REFERENCES

ADACHI, A. 1962. Study on the taste mechanisms of Na-glutamate and Na-inosinate. *J. Physiol. Soc. Japan*, **24**, 607–613.

ADACHI, A. 1964. Neurophysiological study on taste effectiveness of seasoning. *J. Physiol. Soc. Japan*, **26**, 347–355.

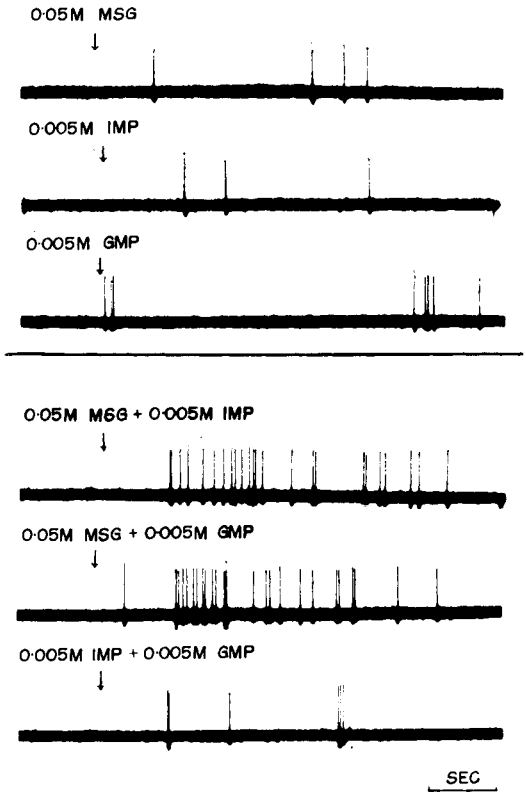


FIG. 1. Responses of salt fiber to chemical taste enhancers and their mixtures.

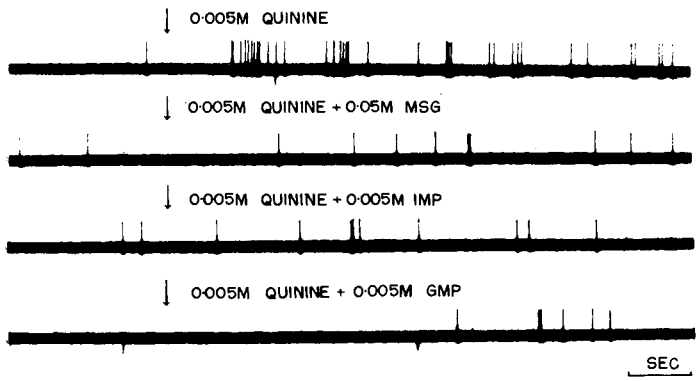


FIG. 2. Effect of chemical taste enhancers on activity of salt fiber.

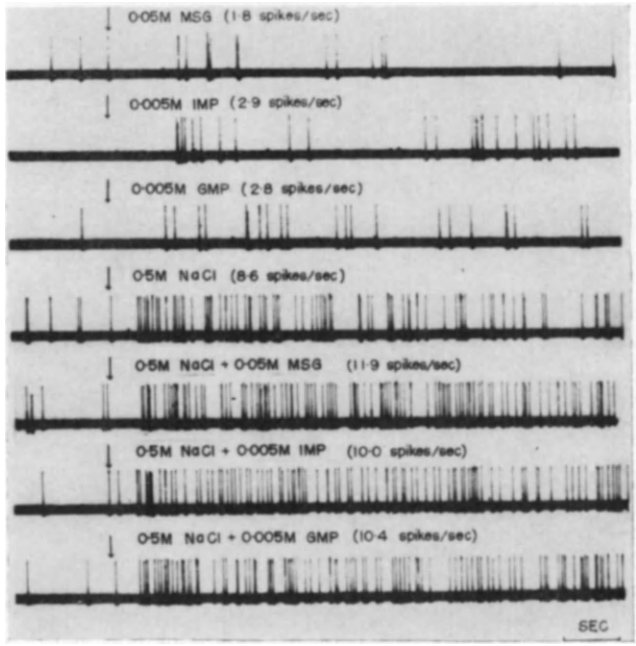


FIG. 3. Effect of chemical taste enhancers on activity of quinine fiber.

KAWAMURA, Y., A. ADACHI, M. OHARA and S. IKEDA. 1964. Neurophysiological studies on taste effectiveness of flavor enhancing substances. *Amino Acid. Nucleic Acid*, **10**, 168–178.

KAWAMURA, Y. and A. ADACHI. 1965. Single taste nerve responses to the chemical taste enhancers. *J. Physiol. Soc. Japan*, **27**, 279–284

SATO, M. and N. AKAIKE. 1965. 5′-ribonucleotides as gustatory stimuli in rats; Electrophysiological studies. *Jap. J. Physiol.* **15**, 53–70.

THE INNATE APPETITE FOR SALT EXHIBITED BY SODIUM-DEFICIENT SHEEP*

ELSPETH BOTT, D. A. DENTON and SIGRID WELLER

Howard Florey Laboratories of Experimental Physiology,
University of Melbourne, Parkville, N.2., Australia

THIS paper sets out a physiological investigation of the appetite for salt exhibited by sodium-deficient ruminant animals. Over a period of 7 years, investigations have been made into the nature of the extero- and intero-receptor system responsible for evocation of appetite in a sodium-deficient animal, and the mechanisms constituting satiation during the consummatory act.

There is long-standing recognition of the avid appetite for salt shown by ruminant animals under some natural conditions. Bunge (1873) stated that stags, deer, chamois and other plant-eaters seek out salty pools and rocks to lick salt, and hunters lie in wait for them in such places, or they put out salt to attract them. There are places in the Altai Mountains where animals have licked clean entire caves in the salty, soft argillaceous slates. This appetitive behaviour has never been observed in beasts of prey. Ruminant animals in mountain areas, where sodium content of plants and foliage is very low, show endocrine and metabolic evidence of sodium-deficiency and an appetite for salt (Bott, Denton, Goding and Sabine, 1964). Herds of reindeer in the Arctic region are reported to drink sea water with relish (Wolf, 1958) and salt is used to attract wild ruminant species to observation points in Africa. Kangaroos in central Australia and in southern mountain regions have been observed to be attracted to salt licks put out for cattle, and studies on sodium-deficient sheep with oesophageal fistula by Arnold (personal communication) have revealed selective grazing of plants which have a high sodium content. Coincident

* Assisted by grants from the National Health and Medical Research Council, research grants Nos. HE 06284 and AM 08701 from the National Institutes of Health, Washington, The Wool Industry Fund of the Commonwealth and The Rural Credits Fund of the Reserve Bank of Australia.

to the observations on cattle reported above, Myers and colleagues (1965) have observed that following the thaw of the winter snow, rabbits on the Snowy Mountains in southern Australia have a very wide zona glomerulosa of the adrenal gland, a low urinary sodium concentration, and manifest a specific appetite for sodium salts. Whereas there has been considerable debate as to whether or not there is an innate appetite for salt in man (Dahl, 1960; Richter, 1956; Denton, 1965; Haldane, 1956), the widely observed occurrence of salt appetite of ruminant animals in circumstances where sodium-deficiency is likely or has been demonstrated, is strongly suggestive—though not conclusive—that the appetite is innate.

The particular features of digestive fluid organization which have evolved in the Ruminantia and Tylopodae, and the consequent importance of sodium-balance and endocrine mechanisms of sodium-regulation, have been reviewed recently from the comparative viewpoint (Denton, 1965). This large circulation of sodium-rich fluids in ruminant digestion highlights the potential survival value of salt appetite in the instance of ecological circumstances causing sodium-deficiency. Such ecological circumstances occur in many areas of the world (Denton, 1965; Denton, Goding, Sabine and Wright, 1961).

Knowledge on salt appetite in animals has been advanced greatly as a result of the brilliant studies of Richter (1956). He showed that young rats, for whom the sole source of nourishment had been mother's milk, consistently had an appetite for salt when first confronted with it. The appetite for sodium in rats was greatly augmented by adrenalectomy. Richter believes that the salt appetite of higher mammals is a genetically-determined behaviour pattern. The ability to taste salt, which is a primary component of the appetite, is innate in all mammals, and natural selection has favoured those animals with a capacity to taste nutritious substances. Epstein and Stellar (1955) have studied voluntary ingestion of 3 per cent NaCl solution by adrenalectomized rats and have shown that intake is quantitatively related to body deficit. Previous experience is not necessary for development of post-operative salt appetite of adrenalectomized animals—the reaction to deficit being innate and Pfaffmann's studies (1959) have shown that it is not dependent on any change in the electrophysiological threshold of the salt taste receptors.

There are some considerable advantages in the use of sheep as experimental animals for studies on salt appetite. They are docile, easily habituated to laboratory condition recover rapidly from major surgery, and, in the instance of Merino breeds, have a large amount of neck skin which facilitates plastic surgical procedures associated with exteriorization of

vessels or organ transparation. However, the great advantage lies in the ease of preparation. of a permanent unilateral parotid fistula from which the animal loses 1–5 l./day of alkaline saliva containing 150–750 mEq of sodium. The digestive system functions normally with the secretion of only one parotid gland and the animal will live in good health indefinitely provided the large sodium loss from the fistula is replaced.

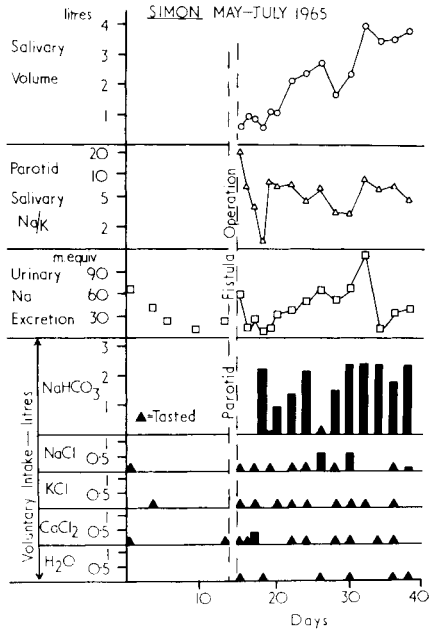


FIG. 1. Merino wether (Simon) aged 8 months, known not to have had access to salt licks or saline water since birth. The effect of making a permanent unilateral parotid fistula on the voluntary intake of solutions of $NaHCO_3$, KCl, NaCl, and $CaCl_2$ (300 mEq/1.) and water is shown when these were presented each day for an hour, and, after 20th day, every second day for an hour. The salivary volume, the salivary Na/K ratio and the urinary sodium excretion are also shown.

Metabolism cages are used, which easily separate saliva, urine and faeces, and make balance studies practical. Large sodium deficits can be produced rapidly, and voluntary ingestion of sodium salts can be measured accurately. Furthermore, the behaviour of an animal with no previous experience of salt or saline waters can be observed following rapid onset of sodium deficiency produced by the parotid fistula operation. Experimental studies on a large number of animals have given the following results:

(1) Sheep known not to have had access to saline or bore waters, or mineral licks since birth, were studied for some weeks under conditions of

constant diet (sodium content approximately 100 mEq/day and free access to water). Solutions of NaCl, $NaHCO_3$, KCl, $CaCl_2$ and $MgCl_2$ (all 300 mEq/l.) were presented along with water in random positions around the front and side of the cage for an hour each day. The majority of the sheep after tasting the solutions a few times, showed very little interest and lay down during most of the experimental period. A minority did drink a substantial amount, usually NaCl. However, when a permanent unilateral

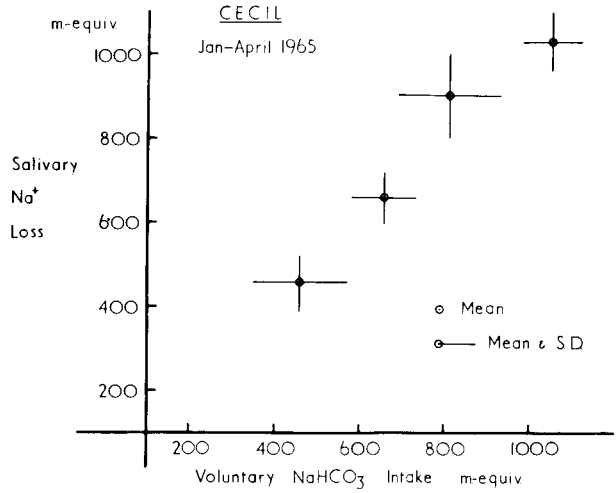


FIG. 2. Sodium appetite in relation to deficit for the sheep Cecil. The ordinate shows the different degrees of deficit achieved by allowing saliva to be lost for 1–3 days. The abscissa shows the amount of sodium drunk during 15 min that the sodium solution was presented. Each point is a mean of 10–24 observations. Each line represents one S.D. from the mean. Two separate experimental periods, several weeks apart, were involved in the one-day depletion series and the salivary loss in the second period was greater than in the first thus warranting a separate grouping.

parotid fistula was made, and the animal lost a large amount of sodium in the saliva, a specific appetite for sodium solutions developed over 1–4 days postoperatively with the intake of $NaHCO_3$ usually predominating, which was interesting in view of the alkaline ($NaHCO_3$) salivary loss (Fig. 1). The animals became very restless over the period of 1–4 days after the fistula operation and exploratory behaviour included much more tasting (Denton, 1965) which is consistent with the appetitive phase of an innate behaviour pattern described by Tinbergen (1951). As this regime continued over weeks, the behaviour diversified with clear evidence of superimposition of conditioned reflexes (Denton and Sabine, 1963). The usual labor-

atory events indicating imminent presentation of sodium solutions were associated with increased salivation, rise of blood pressure, respiration and cardiac rate, cage pawing and bleating. In some animals the motor behaviour eventually became manifest when they were sodium-deficient, whether laboratory events were related to preparation or presentation of solutions or not. If these animals are given each day sufficient to replenish Na deficit by intraruminal tube and are then offered sodium bicarbonate 23 hours later, they show little or no interest in the solution presented.

(2) The amount of sodium drunk is related to the extent of the deficit, although individual variation is observed between sheep. For example, under experimental conditions where the animal has access to $NaHCO_3$ solution for 15–30 min each 24–48 hours, some sheep are remarkably accurate in replenishing their deficit, whereas others tend to "hunt" around the deficit amount. Figure 2 is an example where sodium deficit was varied in an animal by withholding sodium for 1–3 days and it is observed that the voluntary sodium intake over 15 min was adjusted accurately to the deficit.

(3) The act of ingestion of sodium solution is rapid, and in the majority of instances, is complete in 3–10 minutes. There is usually a sharp decline in motivation as evidenced by lack of interest and cessation of sampling.

(4) If the concentration of sodium bicarbonate solution offered to sodium-deficient sheep is varied over a wide range (100–900 mEq/l.), the volume drunk varies inversely so that intake approximates deficit. This is observed in animals given continuous access to $NaHCO_3$ solutions, and also in many animals given access to solutions of varying concentration for 15–30 min once every 48 hours (Fig. 3). This latter observation indicates that the adjustment of behaviour is not dependent on retro-active sense of benefit, but involves a central integration of taste impulses signalling concentration, with pharyngo-oesophageal impulses metering volume swallowed. In effect, the animal may multiply concentration by volume during the course of the consummatory act. The results in Fig. 3 (Satan) which show variation from 0·7 to 7·0 l. of $NaHCO_3$ ingested in 15 min, also illustrate that the intake was usually less than the deficit in the experiments when the sodium concentration was 100 mEq/l. This may reflect the effect of a number of factors including distension of the rumen by the rapid ingestion of a large amount of fluid, and more rapid adaptation of the taste sense at low concentrations than at high (Pfaffmann, 1959).

These four experimental observations (above) made on a large number of animals indicate that salt appetite in the sheep is innate and that a substantial neural organization is involved in the behaviour manifest. It is

evident that the innate behaviour may become extensively conditioned, and learning may be involved in the increasing accuracy of the satiation process which is seen over a period of time in some animals. Type II conditioned responses to environmental events develop in many of the animals. The basic elements of behaviour are consistent with the contemporary ethological theories (Lorenz, 1950; Tinbergen, 1951; Thorpe, 1956).

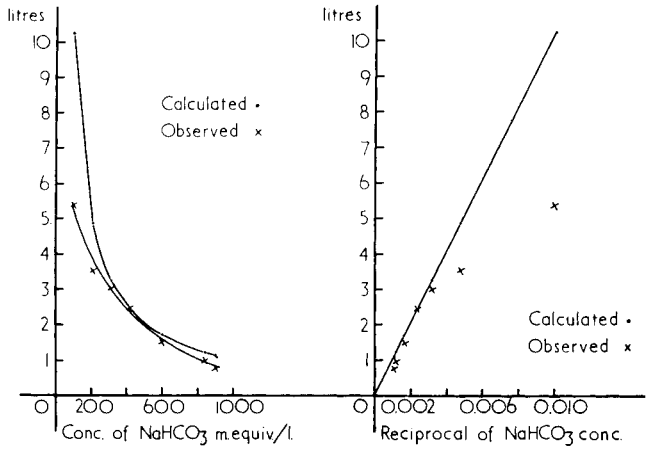


FIG. 3. Variation in volume of $NaHCO_3$ drunk with variation in concentration of the solution offered for 30 min after 48 hours of sodium depletion. The concentration most commonly offered to the sheep was 420 mEq/l. Using the sheep's intake at this concentration as starting point (intake being very close to loss at this concentration) a curve showing the volumes theoretically expected to be drunk to equal the deficit at the various concentrations was calculated. This is shown on the left (curve drawn between circles). The results actually observed are shown also (curve drawn between crosses). On the right, both curves are linearized by plotting the independent variable concentration of $NaHCO_3$ solutions, as the reciprocal form.

The graphs show clearly that there is good agreement between observed and expected at all except the most dilute concentrations.

Appetitive behaviour develops commensurate with the extent of body deficit and the smell and/or taste of salt is the innate releasing mechanism of a consummatory act which rapidly discharges the actionspecific energy. At present there is no evidence of peripheral stimulation causing or contributing to central excitation of salt appetite in a manner similar to the dry mouth factor in thirst, or the gastric contractions in hunger.

Two questions which stand out for physicochemical analysis in the behaviour described above are:

(i) What change (or changes), which occur in the body as a result of sodium deficiency are operative in causing salt appetite, and what is the specific site of action of the stimulus?

(ii) How does the consummatory act of ingestion of an amount of sodium approximating deficit discharge the action-specific energy before the sodium could be absorbed and reverse any change in the milieu interieur or intracellular fluid which might be postulated as stimulating appetitive behaviour?

Considering experimental findings bearing on (i):

(a) It is unlikely that increased aldosterone concentration in blood acts in the brain to evoke salt appetite. Adrenalectomized animals without circulating steroid have an appetite for sodium. Similarly, changed composition of saliva in the mouth is not a stimulus since parotid salivary Na^+/K^+ ratio is normal in sodium-deficient adrenalectomized animals, whereas in sodium-deficient normal animals the Na^+/K^+ ratio is greatly reduced (Denton, 1956, 1957). The failure of increased sodium concentration in lingual and parotid arterial blood to influence appetite greatly (Beilharz, Bott, Denton and Sabine, 1965) is also evidence against a local receptor system in this region.

(b) The possibility exists that the mechanism of humoral stimulation of aldosterone secretion in Na deficiency evokes salt appetite. Decrease of sodium concentration of blood, which is also considered below, stimulates aldosterone secretion, but it is not the main mechanism of aldosterone control (Blair-West, Coghlan, Denton, Goding, Wintour and Wright, 1963). Cross circulation experiments made concurrently and independently on sodiumdeficient sheep in Melbourne and on dogs with inferior vena cava constriction in Bethesda, U.S.A., indicated the existence of a non-ionic humoral stimulus of aldosterone secretion (Denton, Goding and Wright, 1959; Yankopoulos, Davis, Kliman and Peterson, 1959). Present evidence indicates that this stimulus may be the renin-angiotensin pressor system (Gross, 1958; Davis, 1963; Tobian, 1960) and thus the concentration of either of these substances in the cerebral circulation might evoke salt appetite. It has been shown that intravenous infusion of pressor amounts of angiotensin has little effect on drinking in sodium-deficient sheep. Hypertension induced by the Goldblatt procedure does not significantly alter salt appetite of sodium-deficient sheep (Fig. 4) and sodium-deficient sheep will drink sodium solutions 12–24 hours after nephrectomy, when angiotensin and renin would have been cleared from the blood. Hence, this pressor system does not appear to be the stimulus of salt appetite.

(c) A critical consideration in sodium deficiency is whether the reduction

of the sodium concentration of the peripheral blood causes appetite—a proposal made initially by Richter (1956), who suggested that this stimulus acted at the hypothalamic level to activate the cerebral cortex. In sodium-deficient sheep, the plasma sodium concentration falls. If the sheep are depleted concurrently of water and sodium so that plasma sodium concentration increases, instead of the usual fall, the sheep show a normal

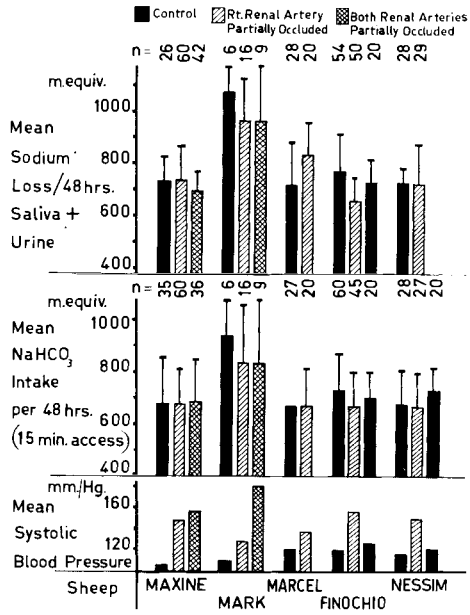


FIG. 4. Effect of production of renal hypertension by chronic partial occlusion of one or both renal arteries on the voluntary intake of $NaHCO_3$ solution (300 mEq/l.) during 15 min. The solution was offered after 48 hours of sodium deprivation. Mean blood pressure is recorded on the lowest block. In Finochio and Nessim, the clamped kidney was eventually removed and a subsequent series of control observations were made.

sodium appetite. They corrected one deficiency and then immediately ingested the alternative fluid. This is interesting in that some proprioceptor elements are common to the process of satiation of either deficit. If hypertonic sodium solution was encountered first it was drunk first, although the body fluids were hypertonic (Beilharz, Denton and Sabine, 1962). It has been pointed out that water depletion affects both extracellular and intracellular fluid, and the result does not preclude a relative change of sodium concentration between the outside and inside of receptor cells.

(d) The introduction of 300–600 mEq of $NaHCO_3$ in 1·5 l. of water into the rumen via a tube 10 minutes before offering the sodium-deficient sheep $NaHCO_3$ to drink after 24 hours sodium depletion did not reduce voluntary intake significantly, and any small effect was no greater than when the same volume of water was given (Beilharz and Kay, 1963). If the intrarumenal dose of sodium was given 120 minutes before offering $NaHCO_3$ solution some reduction of intake (*ca.* 25 per cent) occurred. The results suggest that any intrarumenal receptors do not modify voluntary sodium intake immediately in a manner analagous to the mechanism described by Kassil, Ugolev and Chernigovsky (1959) in the dog, or Stellar, Hyman and Samet (1954) or Mook (1963) in the rat.

(e) The sodium concentration of blood passing through the tissues of the head and brain was increased by 10–20 mEq/l. for 7–8 minutes before and for 7–8 minutes during the time $NaHCO_3$ solution was offered to sheep which had been depleted of sodium for 48 hours. This did not significantly reduce the voluntary sodium intake or the characteristic appetitive behaviour (Beilharz, Bott, Denton and Sabine, 1965). The experimental method employed was to infuse 4 M NaCl into one carotid loop at 1·5–2·0 ml/minute while the other carotid loop was occluded by a pneumatic cuff inflated to 300 mmHg, so that sodium concentration was increased in the blood on both sides of the head. By making the infusion into the carotid artery, this large change in composition of blood in the head continued for 15 min with infusion of less than 10 per cent (60 mEq) of the animal's sodium deficit. A further implication of these experiments is that increased sodium concentration of blood in the tongue during the consummatory act did not abolish appetitive behaviour or modify voluntary intake.

(f) Over a period of 30–40 min, 115 ml of 2·6–4·4 M NaCl containing 290–511 mEq of NaCl was infused intravenously to sodium-deficient sheep and 10 min later $NaHCO_3$ solution and water were offered for 30 min (Beilharz and Kay, 1963). The mean increase of plasma sodium concentration was 22 mEq/l. which was a rise well above the normal range. Appetitive behaviour and voluntary intake were not abolished despite the large rise of plasma sodium. However, there was a significant reduction of voluntary sodium intake, which was not observed in the control experiments when 0·16 M NaCl was infused (Fig. 5). The mean reduction of voluntary intake over the series (480 mEq) approximated the amount of sodium infused. There was considerable variation in that on some days intake was reduced much more than the amount of the intravenous infusion, and on others there was little or no reduction. The

infusion of an equal volume of 2·7 M glucose in the same animals caused a small reduction of sodium intake which was not statistically significant. The fact that the glucose infusion caused a mean decrease of 9 mEq/l. in plasma sodium concentration, with no increase of sodium intake was

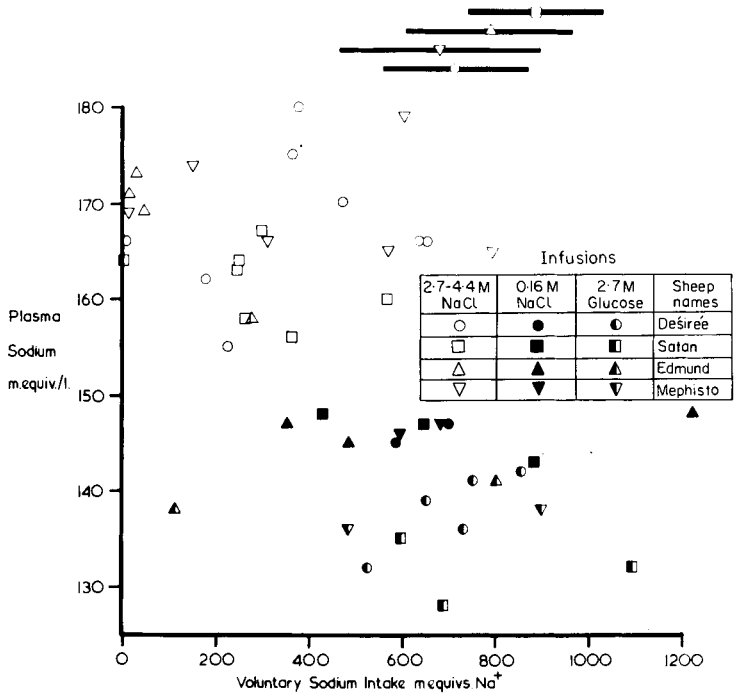


FIG. 5. Sodium appetite and plasma sodium concentration after intravenous infusions in Na deficient sheep. The ordinate shows the concentration of sodium in the plasma 5 min after completion of various intravenous infusions (i.e. 5 min before the beginning of the 30 min sodium-drinking period). The abscissa shows the amount of sodium drunk during the sodium-drinking period. The mean and S.D. of the amount of sodium drunk on control days is shown at the top.

Open symbols — 2·7–4·4 M NaCl
Closed symbols — 0·16 M NaCl
Shaded symbols — 2·7 M glucose

further evidence that voluntary intake was largely independent of rapid change of plasma sodium concentration.

(g) Rapid intravenous infusion of 2·0–2·5 l. of balanced saline solution (Na^+ 150, Cl^- 110, HCO_3^- 25 mEq/l.) providing 300–375 mEq of sodium was made over 40–50 min in sodium-deficient sheep (Bott, Denton and Weller, unpublished). The sodium solution was presented immediately

afterwards, and the results of 24 experiments showed that either intake was not reduced, or the extent of reduction was less than half the amount of sodium infused. There was no change in plasma sodium concentration. Similar infusions in sodium-deficient sheep, with measurement of plasma volume made by Dr. G. Boyd in these laboratories (Blair-West, Boyd, Coghlan, Denton, Goding, Wintour and Wright, unpublished), showed that plasma volume was expanded to sodium-replete level. Infusion of 500–750 ml of 6 per cent dextran in normal saline, which expands plasma volume of sodium-deficient sheep to the sodium-replete range or above, did not influence voluntary sodium intake or appetite in the deficient animals (Bott, Denton and Weller, unpublished).

(h) Intravenous infusions of 2·0–2·5 l. balanced saline or 80 ml of 4 M NaCl were made in sodium-deficient sheep over 45–60 min and the presentation of sodium solution was delayed until 120 min after the infusion. In the instance of isotonic saline infusion, voluntary intake was not reduced on a number of occasions, or else reduction was less than the amount of sodium infused. With the infusion of a small volume of 4 M NaCl (80 ml) intake was reduced by an amount approximating the amount infused.

Experimental findings relating to the consummatory act—(ii) above—were:

(A) Sheep with oesophageal fistulae were studied. When water depleted for 48 hours in control experiments the animals drank an amount which accurately repaired body deficit in 1–5 min. With the oesophageal fistula open the animals sham-drank continuously over a 120 min period, with intake exceeding deficit by 2·5–9 times (Fig. 6). The sheep were observed for a further 120 min after the fistula was closed, during which time they usually drank enough to repair the body deficit (Bott, Denton and Weller, 1965). When the sheep were sodium-depleted for 48 hours, the mean amount of 300 mEq sodium bicarbonate solution sham-drunk was 2·7 times greater than deficit. When the concentration of the sodium solution offered was varied, the volume sham-drunk varied inversely with concentration on many occasions (Fig. 7). During the 120 min period after the fistula was closed voluntary sodium intake was not adequate to repair deficit in the majority of the experiments. The results (Bott, Denton and Weller, unpublished) show that the act of ingestion of saline solution through the pharynx and upper third of the oesophagus does not constitute a consummatory act, and results in discharge of the action-specific energy of sodium appetite. The sensory impulses arising from passage of the fluid through the lower two-thirds of the oesophagus and

entry into the rumen represent an essential component in the satiation process. However, the observations indicate that sham-drinking in sodium deficiency more nearly approaches a satiating effect than sham-drinking water in water-deficiency.

(B) Some preliminary observations have been made in which the lingual, glossopharyngeal and buccal nerves have been sectioned with

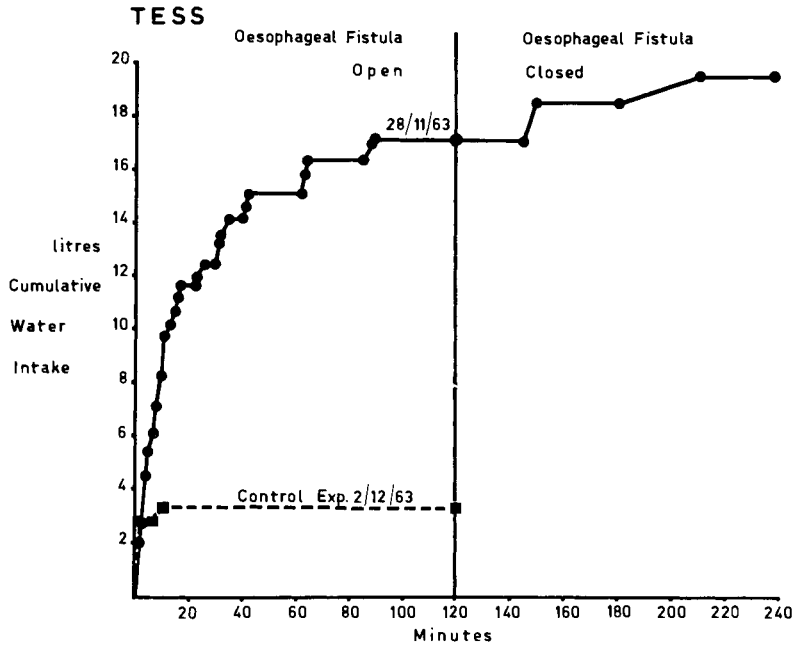


FIG. 6. Merino ewe (Tess). The cumulative voluntary water intake after 2 days' water deprivation when drinking with the oesophageal fistula open for 2 hours, and then closed for 2 hours. The interrupted line designated "Control Exp." is the water intake after 2 days' water deprivation when the oesophageal fistula was closed.

some small reduction in sodium intake during deficiency (Beilharz and Kay, 1963). As observed with normal sodium-depleted sheep, they were able to indentify the sodium solution by smell. These results obtained using sheep require further investigation before any definitive statement is possible, particularly in the light of Richter's observations (1956) on the difficulty of surgical elimination of taste.

(C) Some very interesting observations have been made recently on the question of the relation of the mechanisms of salt appetite with those

regulating the secretion of aldosterone. In sodium-deficient sheep with an adrenal transplant it has been observed that following the consummatory act of drinking sodium solution, aldosterone secretion may decline abruptly over 15–30 min and rise again subsequently before falling as a result of absorption of the ingested sodium (Denton, 1965; Blair-West

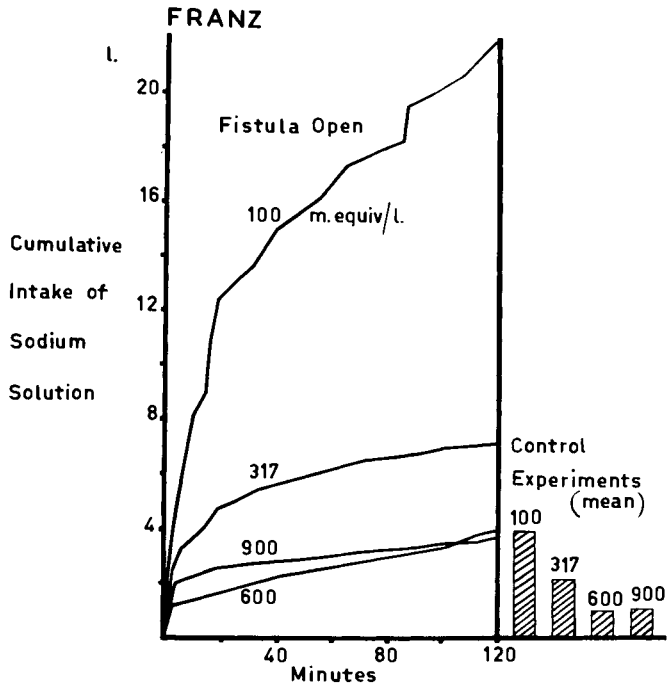


FIG. 7. Merino wether (Franz). The cumulative volumes of 100, 317, 600 and 900 mEq/l. $NaHCO_3$ solution "sham-drunk" over 120 min after the sheep had been depleted of sodium for 48 hours. The mean control intake of these four sodium solutions when the sheep was drinking with the oesophageal fistula closed is shown on the extreme right.

et al., unpublished). This effect varied from animal to animal, some showing a large decline, others showing little or no effect. The fall of aldosterone secretion was more rapid than in experiments where the sodium was given by rumen tube, or by rapid systemic infusion. As the rate of entry of sodium into the circulation is much faster with rapid systemic infusion than in voluntary drinking, the results suggest that discharge of the action-specific energy of sodium appetite evokes a temporary inhibition of aldosterone secretion by pathways which are yet to be defined.

SUMMARY

The main fact emerging is that in sodium-deficient sheep an immediate substantial rise of sodium concentration of blood perfusing the brain to normal or above, did not abolish the sodium appetite. Systemic administration of a large amount of isotonic sodium solution did not commensurately reduce appetite immediately, or reduce it considerably 2 hours later. However, the same amount of sodium given as a small volume of hypertonic NaCl which increased plasma sodium concentration did reduce voluntary intake within 30 min to an extent approximating the amount given. If a change in intracellular sodium of brain cells is involved in the stimulatory process (Denton and Sabine, 1961), delay in the rate of penetration of sodium across the blood-brain barrier and into brain cells (Davson and Pollay, 1963) may be reflected in the delay of translation of change in sodium balance into reduced appetite (Beilharz, Bott, Denton and Sabine, 1965). The fact that the change is reflected more rapidly with hypertonic than with isotonic infusion may be consistent with this process. These findings at least suggest a different type of mechanism to a simple receptor system reacting directly and immediately to plasma sodium concentration or some haemodynamic parameter altered by sodium-deficiency, such as has been proposed in the instance of aldosterone control. The work discussed in this paper suggests that a physicochemical system involving some change of composition of neurones in the brain is involved in genesis of salt appetite.

REFERENCES

ARNOLD, G. Personal communication.

BEILHARZ, S., BOTT, E., DENTON, D. A. and SABINE, J. R. (1965). *J. Physiol. (Lond.)*, **178**, 80.

BEILHARZ, S., DENTON, D. A. and SABINE, J. R. (1962). *J. Physiol. (Lond.)*, **163**, 378.

BEILHARZ, S. and KAY, R. N. B. (1963). *J. Physiol. (Lond.)* **165**, 468.

BLAIR-WEST, J. R., BOYD, G. W., COGHLAN, J. P., DENTON, D. A., GODING, J. R., WINTOUR, M. and WRIGHT, R. D. Unpublished data.

BLAIR-WEST, J. R., COGHLAN, J. P., DENTON, D. A., GODING, J. R., WINTOUR, M. and WRIGHT, R. D. (1963). *Recent Progr. Hormone Res.* **19**, 311.

BOTT, E., DENTON, D. A., GODING, J. R. and SABINE, J. R. (1964). *Nature*, **202**, 461.

BOTT, E., DENTON, D. A. and WELLER, S. Unpublished data.

BOTT, E., DENTON, D. A. and WELLER, S. (1965) *J. Physiol. (Lond.)*, **176**, 323.

BUNGE, G. (1873). *Zeits. Biol.* **9**, 104.

DAHL, L. K (1960). In *Essential Hypertension*. Eds. K. D. BOCK and P. T. COTTIER. Springer Verlag: Berlin. p. 53.

DAVIS, J. O. (1963). In *Hormones and the Kidney*. Ed. P. C. Williams. Academic Press: London. p. 325.

DAVSON, H. and POLLAY, M. (1963). *J. Physiol. (Lond.)*, **167**, 247.

DENTON, D. A. (1956). *J. Physiol. (Lond.)*, **131**, 516.
DENTON, D. A. (1957). *Quart. J. exp. Physiol.* **42**, 72.
DENTON, D. A. (1965). *Physiol. Rev.* **45**, 245.
DENTON, D. A., GODING, J. R., SABINE, J. R. and WRIGHT, R. D. (1961). *Proc. Teheran Symposium.* Unesco: Paris. p. 193.
DENTON, D. A., GODING, J. R. and WRIGHT, R. D. (1959). *Brit. Med. J.* **ii**, 447 and 522.
DENTON, D. A. and SABINE, J. R. (1961). *J. Physiol. (Lond.)*, **157**, 97.
DENTON, D. A. and SABINE, J. R. (1963). *Behaviour*, **20**, 364.
EPSTEIN, A. N. and STELLAR, E. (1955). *J. Comp. Physiol. Psychol.* **45**, 167.
GROSS, F. (1958). *Klin. Woch.* **36**, 693.
HALDANE, J. (1956). In Discussion of C. P. Richter, in *L'Instinct dans le Comportement des Animaux et de l'Homme.* Masson et Cie: Paris, p. 630.
KASSIL, V. G., UGOLEV, A. M. and CHERNIGOVSKY, V. N. (1959). *C. R. Acad. Sci. U.S.S.R.* **126**, 692.
LORENZ, K. Z. (1950). In *Symposia of the Society for Experimental Biology, No. 4. "Physiological Mechanisms in Animal Behaviour"*. University Press: Cambridge, p. 221.
MOOK, D. G. (1963). *J. Comp. Physiol. Psychol.* **56**, 645.
MYERS, K. (1965). Personal communication.
PFAFFMANN, G. (1959). In *Handbook of Physiology, Section I: Neurophysiology.* Vol. 1, p. 507. American Physiological Society: Washington.
RICHTER, C. P. (1956). In *L'Instinct dans le Comportement des Animaux et de l'Homme.* Masson et Cie; Paris. p. 577.
STELLAR, E., HYMAN, R. and SAMET, S. (1954). *J. Comp. Physiol. Psychol.* **47**, 220.
THORPE, W. H. (1956). *Learning and Instinct in Animals.* Methuen: London.
TINBERGEN, N. (1951). *The Study of Instinct.* Oxford University Press: Oxford.
TOBIAN, L. (1960). *Physiol. Rev.* **40**, 280.
WOLF, A. V. (1958). *Thirst—Physiology of the Urge to Drink and Problems of Water Lack.* Charles C. Thomas: Springfield, Illinois.
YANKOPOULOS, N. A., DAVIS, U. O., KLIMAN, B. and PETERSON, R. E. (1959). *J. Clin. Invest.* **38**, 1278.

ELECTROPHYSIOLOGICAL ANALYSIS OF TASTE EFFECTIVENESS OF SODA WATER AND CO_2 GAS

YOJIRO KAWAMURA and AKIRA ADACHI

Department of Oral Physiology, Osaka University Dental School,
Kitaku, Osaka, Japan

PHYSIOLOGIC mechanisms of taste effectiveness of unsweetened soda water still remain obscure in details. In addition, soda water is a simple chemical substance with weak acidity and it releases carbon dioxide as a bubble. Therefore, the physiologic mechanism by which soda water stimulates the oral sensory receptors is an interesting problem.

In the present experiment, taste effectiveness of soda water was analyzed in animals by means of the neurophysiological method. It revealed that soda water applied to the tongue surface stimulated not only the salt and acid taste receptors, but also cold receptors of the tongue. The mechanisms of this effectiveness of soda water are discussed.

RESPONSE FROM THE WHOLE CHORDA TYMPANI TO BEER

In the first step of this experiment, responses of the chorda tympani nerve to carbonated and non-carbonated alcoholic beverages were recorded in cats, dogs and rats.

The response patterns of the chorda tympani nerve of these animals, cat, dog, and rat, to these drinks were almost similar.

The responses to fresh beer consisted of two types of the response elements like Fig. 1. Iinitial marked transient discharges were elicited immediately after an application of fresh beer to the surface of the tongue and diminished within about 5 sec. This response was tentatively named *A*. Other weak and long lasting discharges were sustained for about 20 sec or more. This latter element was named *B*. The *A* response was also obtained by simple soda water, but it was not elicited by flat beer. On the contrary, 5 per cent ethanol, which is similar in alcohol content to beer,

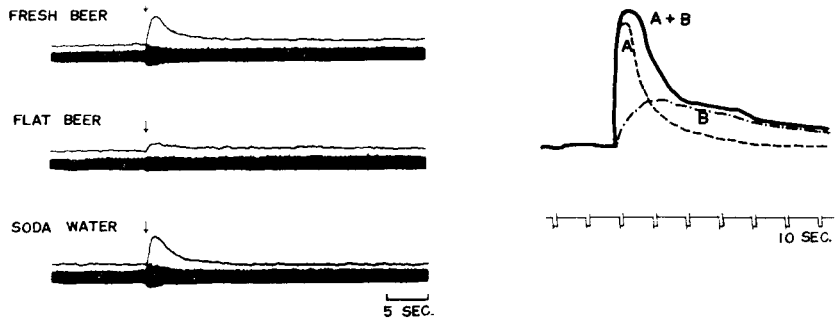


FIG. 1. Difference of integrated response between beer and soda water. Schema indicating elements of the response.

A: soda water.
B: flat beer.
A+*B:* fresh beer.

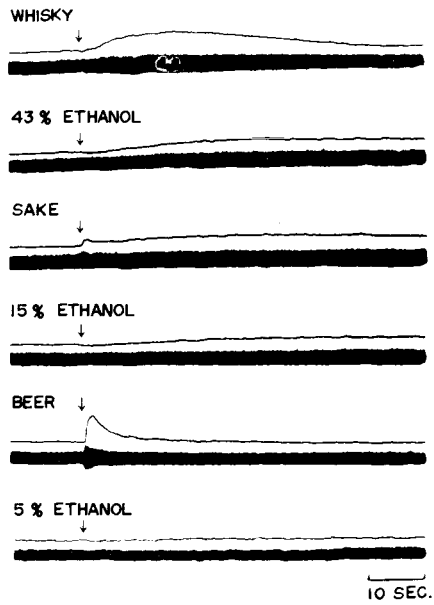


FIG. 2. Relative taste effectiveness of different drinks.

did not elicit any recognizable discharges of the nerve. In addition, as shown in Fig. 2, whisky, sake, 15 per cent, 43 per cent ethanol, all of these did not also elicit any transient response of *A*-type and they induced only *B*-type responses.

For these reasons, the *A*-type response of the nerve is assumed to come from supersaturated carbonic acid or its gas in the beer.

RESPONSE OF A SINGLE TASTE FIBER TO SODA WATER

We used pure soda water as the test solution in the second step of this experiment. As shown in Fig. 3 some of the acid or salt single fibers in the chorda tympani nerve of the cat or dog showed remarkable discharges to soda water when it was applied to the surface of the tongue. Thus,

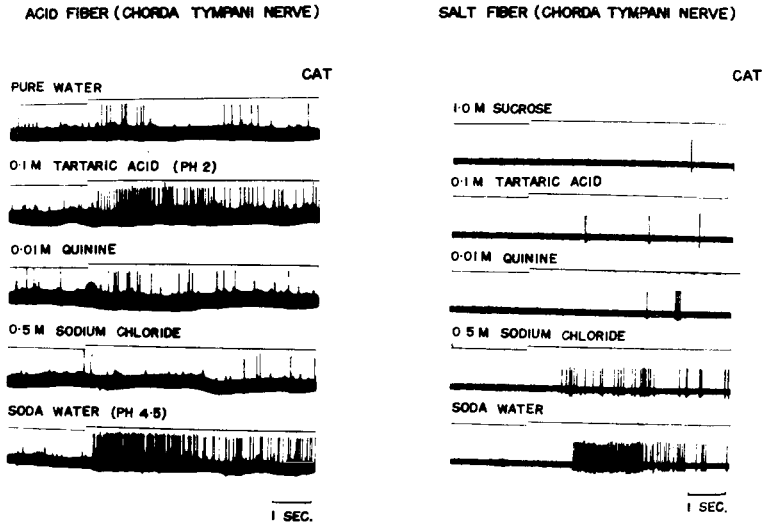


FIG. 3. Responses of acid fiber and salt fiber to soda water.

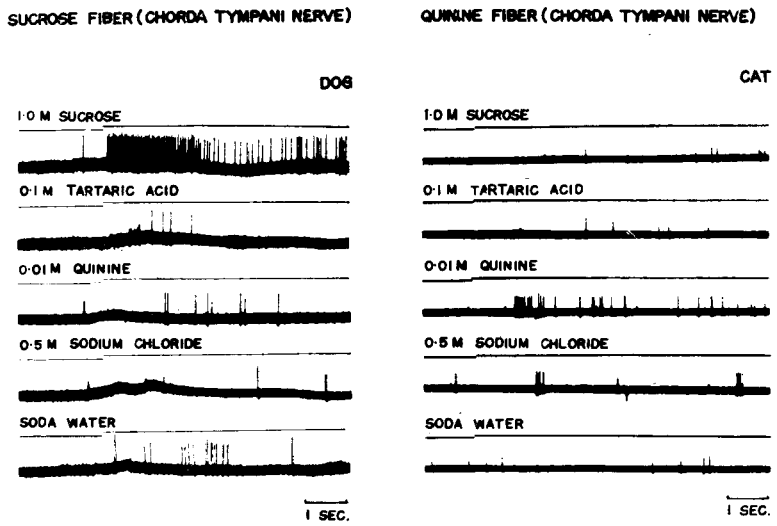


FIG. 4. Responses of sucrose fiber and quinine fiber to soda water.

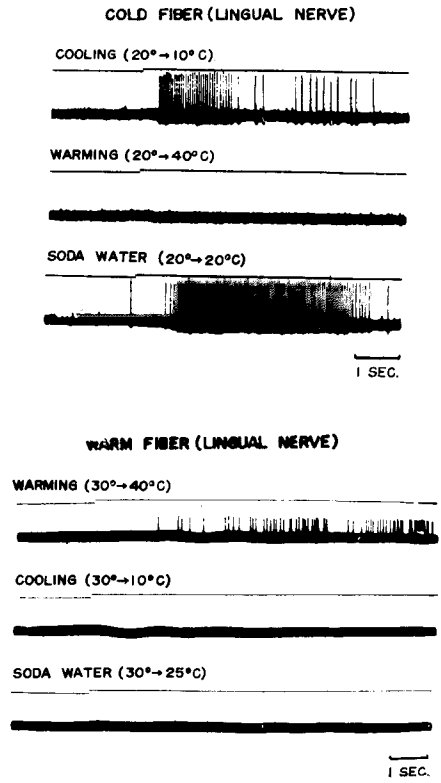


FIG. 5. Responses of cold fiber and warm fiber to soda water.

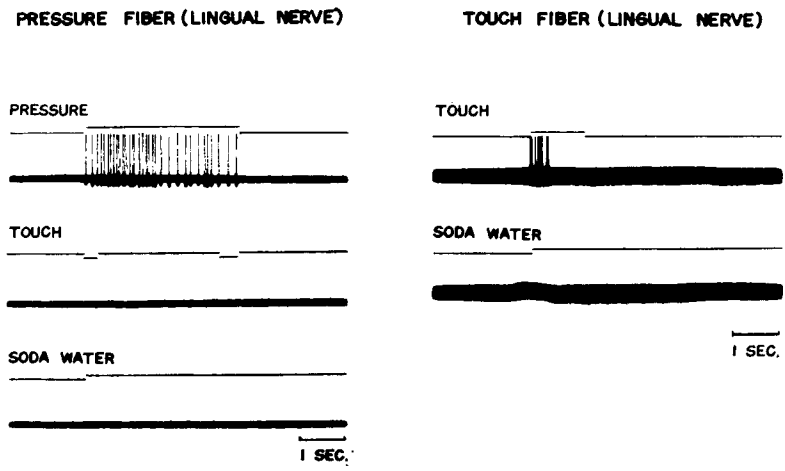


FIG. 6. Responses of pressure fiber and touch fiber.

soda water stimulated acid and salt taste receptors. Further, response of soda water (about pH 4·5) was more dominant than that of tartaric acid solution (about pH 2–3) or 0·5 M sodium chloride solution.

A sucrose fiber and a quinine fiber did not show any remarkable responses to soda water (Fig. 4).

Soda water also stimulated the cold receptors of the tongue. For example, as shown in Fig. 5, soda water (around 20°C) induced remarkable discharges of the single lingual nerve fiber which responded to cooling, but it did not stimulate warm (Fig. 5), pressure and touch receptors of the tongue (Fig. 6). These results mean that an interplay of cold and taste information from oral sensory receptors will strongly participate to make up taste sensation of the soda water and fresh beer.

EFFECT OF CO_2 AND O_2 GAS ON THE SENSORY RECEPTORS OF THE TONGUE

Then, in the third step of the experiment, we tried to solve the mechanisms by which soda water stimulates cold and taste receptors of the tongue. When we applied soda water to the tongue, it induced many bubbles. Therefore, at first, effects of bubbles on the cold receptor of the tongue were tested. To make CO_2 bubbles, sodium bicarbonate was applied first to the surface of the tongue of the cat, and then hydrochloric acid (0·01 M) was poured on it.

$$(NaHCO_3 + HCl = NaCl + H_2O + CO_2)$$

To make O_2 bubbles, $KMnO_4$ (potassium permanganate) was applied to the surface of the tongue and H_2O_2 (hydrogen peroxide) was successively added.

$$(2KMnO_4 + 3H_2O_2 = 2KOH + 2MnO_2 + 3O_2 + 2H_2O)$$

As shown in Fig. 7, when we applied CO_2 bubbles to the tongue we could record remarkable discharges from the single cold fiber. However, O_2 bubbles did not induce any detectable discharges of the nerve. This fact suggests to us that CO_2 gas itself is a strong stimulant to the cold receptor and to the taste receptor. The bubble itself does not play any role.

For such reasons, we tried to apply pure CO_2 gas to the surface of the tongue, and responses of the single cold fiber and single acid fiber were recorded. Pure CO_2 or O_2 gas at nearly room temperature and at 50–80 ml/sec was applied to the surface of the tongue by tubing. When we applied CO_2 gas to the surface of the tongue, we could record obvious responses from

the single cold fiber and single acid fiber as indicated in Figs. 8 and 9. In Fig. 8, the upper trace in each record is a temperature curve of the tongue surface electrically recorded by a thermistor, and the lower record is a dis-

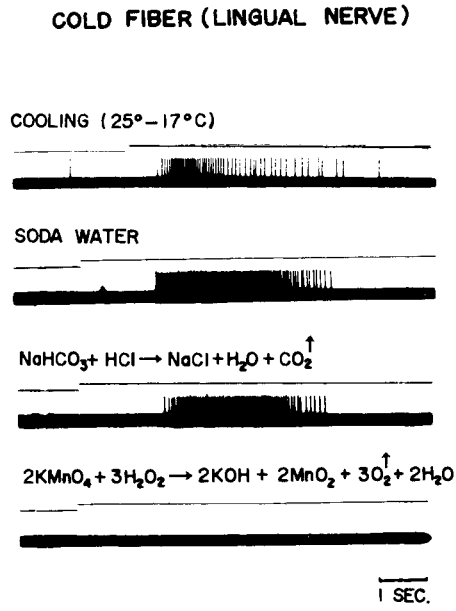


FIG. 7. Responses of cold fiber to bubbles.

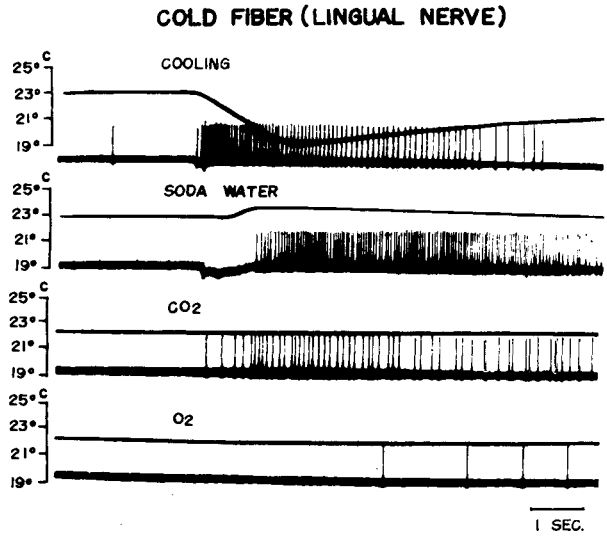


FIG. 8. Responses of cold fiber to CO_2 gas and O_2 gas.

charge of a single cold nerve fiber. The top record is a response of this nerve to cold water, the second is to soda water, the third is to CO_2 gas and the bottom trace is to O_2 gas. O_2 gas did not induce any discharges in the cold fiber, but conversely CO_2 gas produced conspicuous discharges of the nerve.

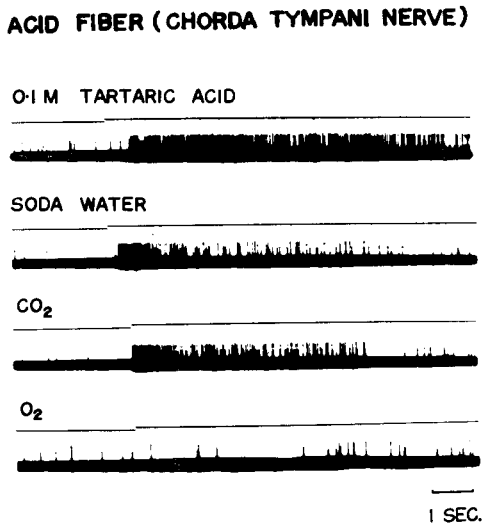


FIG. 9. Responses of acid fiber to CO_2 gas and O_2 gas.

The response of a single acid fiber to the CO_2 gas was shown in Fig. 9. The top record in the figure is a response to 0·1 M tartaric acid of the nerve fiber, the second is to soda water, the third is to CO_2 gas and the bottom trace is to O_2 gas. CO_2 gas induced remarkable discharge of the acid fiber, but O_2 gas did not effect at all.

When we applied CO_2 gas to the skin surface we did not get any responses from the innervated cutaneous sensory nerves.

SUMMARY

In summary, taste effectiveness of soda water is attributed not only to acidity of the carbonic acid, but also to CO_2 gas contained in it.

Further, CO_2 gas itself stimulates the taste receptors for acid and salt, and also stimulates a cold receptor of the tongue. Such sort of CO_2 gas' effects on the receptors of the tongue come from the gas itself and not the effect of bubbling.

HORMONAL REGULATION OF THE SPONTANEOUS SODIUM CHLORIDE APPETITE OF RATS*

MELVIN J. FREGLY and IRVING W. WATERS

Department of Physiology, University of Florida,
College of Medicine, Gainesville, Florida

REMOVAL of the adrenal glands has been known to increase the spontaneous NaCl appetite of rats since the classical studies of Richter published in 1936.[1] Furthermore, transplantation of adrenal cortical tissue to adrenalectomized rats reduces NaCl intake to normal levels.[2] Thus, it can be assumed that a hormone secreted by the adrenal cortex exerts an inhibitory effect on the spontaneous NaCl appetite of rats. Indeed, Richter[3] showed that administration of the synthetic mineralocorticoid, desoxycorticosterone acetate, at a dose level of 0·5–1·0 mg/rat/day to adrenalectomized rats reduced NaCl appetite to that of intact controls. This observation contrasts with those of others in that similar dose levels of desoxycorticosterone acetate, administered to either normal or renal hypertensive rats, increased their spontaneous NaCl intake when given choice between water and 0·15–0·17 M NaCl solution to drink.[4–6] That the same dose level of this steroid could either decrease or increase NaCl intake, depending upon either the presence or absence of adrenal glands, seemed worthy of explanation. The experiments described here were performed to study the dose–response relationship between intake of NaCl solution and dose of desoxycorticosterone acetate (DOCA) administered to adrenalectomized rats. Similar studies were also performed utilizing the naturally occurring mineralocorticoid, aldosterone, which has recently become available. In addition, the specificity of mineralocorticoids for reduction of spontaneous NaCl intake of adrenalectomized rats was tested by administration of certain other naturally occurring hormones. The results suggest, but do not prove, that compounds containing mineralocorticoid-like activity affect the spontaneous NaCl intake of adrenalectomized rats.

* Supported by grant HE-03503-08 from the National Institutes of Health, Bethesda, Maryland.

METHODS AND RESULTS

All experiments utilized male albino rats of either the Holtzman or Carworth CFN strains. The rats were caged individually and maintained in a windowless room at 26 ± 1°C. The room was illuminated from 8 a.m. to 6 p.m. Fluid containers consisted of Evenflo nursing bottles with cast aluminum spouts as described by Lazarow.[7] Food containers were spill-proof and have been described in detail.[8]

Treatments were assigned at random so that a Randomized Statistical Design and Analysis of Variance could be used to aid in interpretation of results.[9] Six separate experiments were performed and are described below:

Experiment 1. Effect of Desoxycorticosterone Acetate on Spontaneous NaCl Intake

Twenty-one male rats weighing initially from 190 to 230 g were divided into 3 equal groups. Group 1 was anesthetized with ether and sham-operated while Groups 2 and 3 were anesthetized with ether and both adrenals removed. One day was allowed for recovery after which measurements of intakes of water, 0·15 M NaCl solution and a synthetic sodium deficient diet* were made daily for 6 days. In addition to the intakes, urine was collected by means of a funnel and a graduated cylinder containing paraffin oil to prevent evaporation of urine. A small screen placed in the funnel separated faeces from urine.

Figure 1 shows mean salt, water and food intake, urinary output and change in body weight of each group for the 6 day period. One of the two adrenalectomized groups received 200 μg DOCA/day (100 μg/100 g BW/day) dissolved in peanut oil (0·2 ml) and injected subcutaneously. The other two groups received 0·2 ml peanut oil subcutaneously daily. Treatment with DOCA reduced intake of 0·15 M NaCl solution significantly ($P < 0{\cdot}01$) below that of adrenalectomized controls and to the level of the sham-operated group. Water intake of the adrenalectomized group was less than that of the sham-operated group (second panel) while administration of DOCA increased significantly ($P < 0{\cdot}01$) water intake to the level of the sham-operated group. Food intakes were not affected significantly by treatment (third panel). DOCA-treated rats reduced urinary output significantly ($P < 0{\cdot}01$) below that of adrenalectomized controls and to the level of the sham-operated rats (panel 4). Mean change in body weight

* Sodium Deficient Test Diet, Nutritional Biochemicals Corp., Cleveland, Ohio.

(shown at right of first panel) revealed a gain of 6 g for DOCA-treated rats while controls lost 7 g. Sham-operated rats gained 1 g. Since food intake was not affected by treatment with DOCA, it is suggested that the

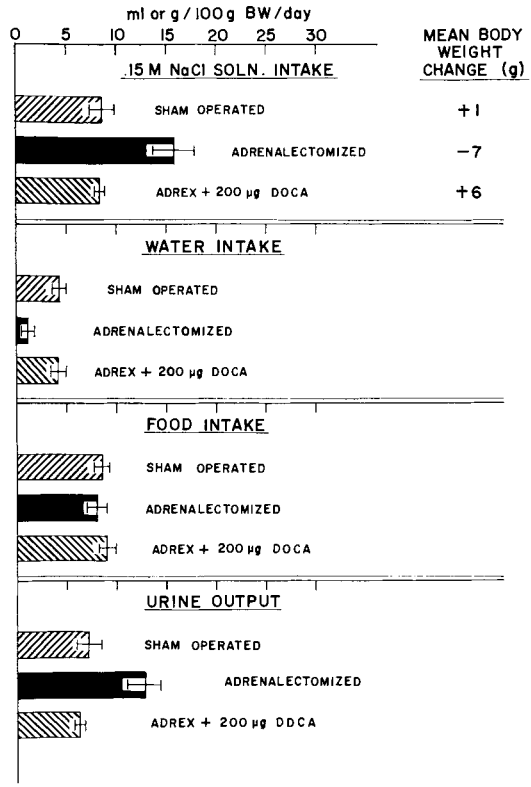


FIG. 1. Spontaneous intakes of 0·15 M NaCl solution (upper panel), water (2nd panel) and food (3rd panel) are shown for sham-operated, adrenalectomized rats treated daily with 200 μg desoxycorticosterone acetate. Urinary output of each group is given in the 4th panel. ± 1 standard error is set off at each mean. Change in body weight of each group is given in the upper right of the figure. Intakes were measured for 6 days.

greater weight gain of the DOCA-treated rats is associated with the sodium and water retaining effects of this steroid.

Administration of 200 μg DOCA/day to adrenalectomized rats reduced their NaCl intake 52 per cent below that of adrenalectomized controls.

Experiment 2. Effect of Desoxycorticosterone Acetate on Spontaneous NaCl Intake

Twelve male rats ranging in body weight from 387 to 427 g were used. They were adrenalectomized 2 weeks prior to the experiment and were divided randomly into 4 equal groups. Each rat was caged individually and

TABLE 1. EFFECT OF DESOXYCORTICOSTERONE ACETATE ON PERCENT CHANGE IN SPONTANEOUS INTAKES OF 0·15 M NaCl SOLUTION, WATER AND FOOD BY ADRENALECTOMIZED RATS

Daily drug dose	Days given drug	Measurements made	Mean % change from pre-drug control period
Adrex + oil	4	0·15 M NaCl Soln. Intake	− 18·8
		Water Intake	− 36·7
		Food Intake	− 14·0
		Δ Body Weight (g/4 days)	+ 0·5
Adrex + 100 μg Doca	4	0·15 M NaCl Soln. Intake	− 15·7
		Water Intake	+ 12·4
		Food Intake	+ 1·7
		Δ Body Weight (g/4 days)	+ 2·4
Adrex + 200 μg Doca	4	0·15 M NaCl Soln. Intake	− 28·4
		Water Intake	+119·0
		Food Intake	+ 3·1
		Δ Body Weight (g/4 days)	+ 2·3
Adrex + 400 μg Doca	4	0·15 M NaCl Soln. Intake	− 44·2
		Water Intake	+255·6
		Food Intake	+ 4·7
		Δ Body Weight (g/4) days	+ 3·9

Analysis of variance (mean % change):

		0·15 M NaCl Soln.			Water			Food		
Source	df	MSS	F	P	MSS	F	P	MSS	F	P
Treatment	3	490·73	2·94	>·05	50319·68	1·51	>0·05	226·09	3·11	>0·05
Linear 1		1179·93	7·07	<·05	145110·50	4·35	>0·05	497·09	6·84	<0·05
Error	8	166·91			33396·45			72·72		
Total	11									

given choice between tap water and 0·15 M NaCl solution to drink. The food was finely ground Rockland Rat Diet. Food and fluid containers and other experimental conditions were the same as those described in Experi-

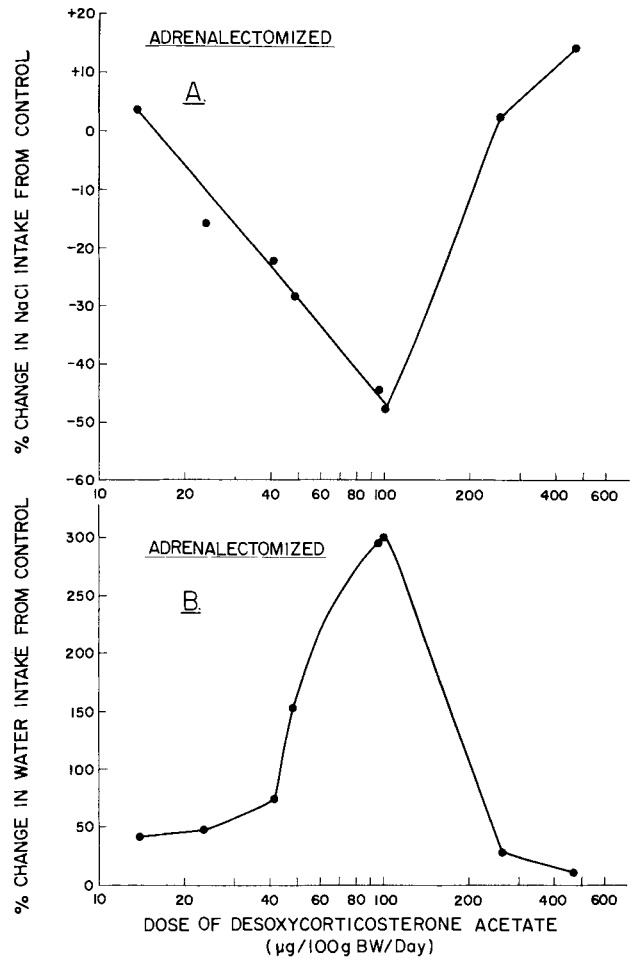


FIG. 2A. Composite graph to summarize data from three separate experiments. Percent change in intake of 0·15 M NaCl solution of DOCA-treated adrenalectomized rats from control, adrenalectomized rats at each dose level of DOCA administered.

FIG. 2B. Similar data are graphed for percent change in simultaneous water intake.

ment 1. During a 4 day control period, fluid and food intakes and body weights were measured daily. At the end of the control period, the four treatments were assigned randomly and included daily subcutaneous injec-

tions of 0·2 ml peanut oil, 100, 200 or 400 μg DOCA in 0·2 ml peanut oil. Treatment continued during the next 4 days after which the experiment was terminated. Intakes of NaCl solution, water and food were measured daily during treatment. The mean percent change in intake of fluid and food from the control, pre-injection period was calculated for each rat and the data analyzed by analysis of variance.

Table 1 shows that percent change in intake of 0·15 M NaCl solution from predrug control period decreased as dose of DOCA increased. There is a significant ($P<0.05$) linear relationship between the two. Water intake increased with increasing dose of DOCA but the increase was not significant. Treatment with DOCA increased food intake linearly ($P<0.05$) as drug dose increased.

Figure 2 is a composite graph made up of data from Experiments 1 and 2 as well as from data reported elsewhere.[10, 11] Percent change in both NaCl intake (part A) and water intake (part B) are plotted against logarithm of daily dose of DOCA administered (μg/100 g body weight/day). This figure shows that a U-shaped dose–response relationship exists between percent change in NaCl intake from untreated controls and log dose of DOCA administered. Administration of increasing doses of DOCA to adrenalectomized rats was accompained by a decrease in NaCl intake compared with oil-treated controls until a dose level of 100 μg/100 g body weight/day was reached. Further administration increased salt intake such that at the highest dose level (470 μg/100 g body weight/day) the treated rats ingested more NaCl solution than controls. The percent change in water intake (part B) was approximately the mirror image of the percent change in NaCl intake of part A.

Experiment 3. Effect of d-Aldosterone on Spontaneous NaCl Intake

Two groups each containing 5 adrenalectomized rats were used. The rats were adrenalectomized 3 weeks prior to the study. Each rat was kept in an individual cage and given finely ground Rockland Rat Diet to eat and choice between distilled water and 0·15 M NaCl solution to drink. The experiment consisted of 4 periods each lasting 5 days. During the first period, food and fluid intakes of both groups were measured; during the remaining periods, the first group received daily subcutaneous injections of peanut oil (0·20 ml) while the second group received 0·5 μg (period 2); 5·0 μg (period 3) or 50·0 μg (period 4) of *d*-aldosterone in peanut oil (0·20 ml) subcutaneously daily. Food and fluid intakes and body weight of each rat were measured throughout the four periods.

The results of this study are shown in Fig. 3. Intakes of 0·15 M NaCl solution and water were similar for the two groups during period 1 (Fig. 3A). Administration of either 0·5 or 5·0 μg aldosterone daily reduced intake of

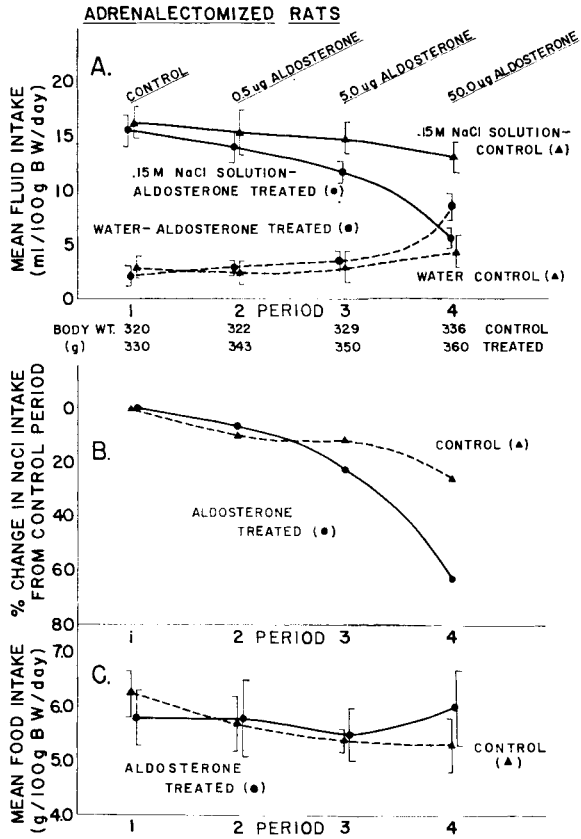


FIG. 3A. Effect of administration of *d*-aldosterone on spontaneous intakes of 0·15 M NaCl solution by treated (solid line, dot) and control adrenalectomized rats (solid line, triangle). Simultaneous water intakes of treated (broken line, dot) and control rats (broken line, triangle) are shown at the bottom of the figure. Each period lasted 4 days.

FIG. 3B. Effect of administration of *d*-aldosterone on percent change in NaCl intake from control period for aldosterone-treated and control rats.

FIG. 3C. Effect of treatment on mean food intake of the two groups. ± 1 standard error is set off at each mean. Mean body weights of the two groups at each period are given at the bottom of part A.

0·15 M NaCl solution and increased water intake slightly. In neither case was intake increased significantly ($P > 0.05$). However, administration of 50·0 μg aldosterone reduced significantly ($P < 0.01$) intake of 0·15 M NaCl

solution and increased significantly ($P < 0.01$) water intake above the level of controls. Food intakes of the two groups did not differ significantly throughout the experiment. Mean body weight of control, oil-treated rats increased 16 g during the experiment while aldosterone-treated rats gained 30 g. Since the gain in body weight is not accompanied by a commensurate increase in food intake, it seems probable that the sodium and water retaining effect of aldosterone accounts for the greater increase in weight of treated rats. The percent change in NaCl intake from period 1 is graphed in Fig. 3B. Administration of peanut oil only was accompanied by a gradual decrease in NaCl intake and amounted to about 22 per cent by period 4. Administration of aldosterone resulted in a greater percent change in NaCl

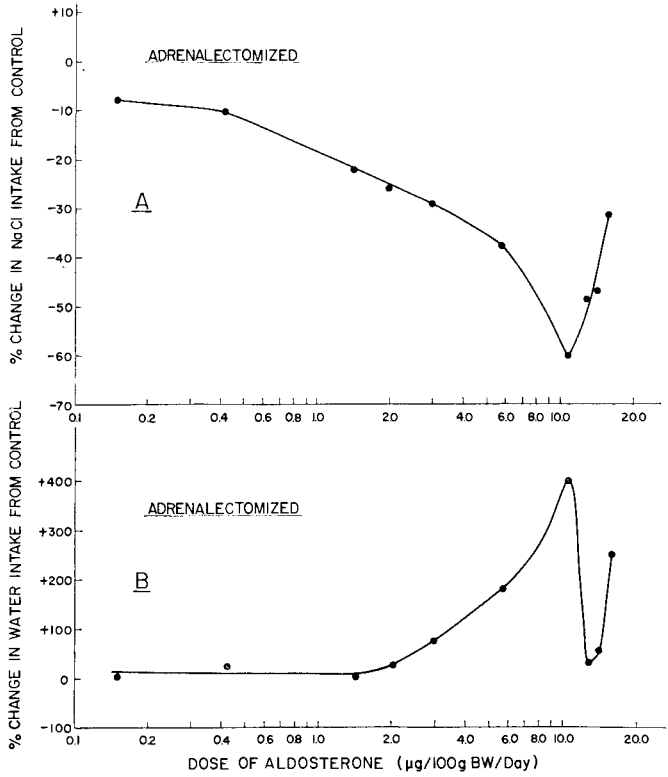


FIG. 4A. Composite graph to summarize data from three separate experiments. Percent change in intake of 0·15 M NaCl solution of aldosterone-treated, adrenalectomized rats from control, adrenalectomized rats at each dose level of aldosterone administered.

FIG. 4B. Similar data are graphed for percent change in simultaneous water intake.

intake from control period and amounted to 63 per cent by period 4. Since 22 per cent of this can be accounted for by injection of oil only, apparently the 50 μg injection of aldosterone reduced NaCl intake 41 per cent.

Figure 4 is a composite graph made up of data from Experiment 3 as well as from similar data reported elsewhere.[11, 12] Percent changes in both NaCl (part A) and water (part B) intake are plotted against logarithm of daily dose of aldosterone administered (μg/100 g body weight/day). This figure also shows a U-shaped dose–response relationship. Administration of increasing doses of aldosterone to adrenalectomized rats is accompanied by a decrease in NaCl intake compared with oil-treated controls until a dose level of 10·8 μg/100 g body weight/day is reached. Doses of aldosterone higher than this maximally effective dose tend to return NaCl intake toward that of untreated, adrenalectomized rats. The percent change in water intake (part B) is approximately the mirror image of the percent change in NaCl intake of part A.

Experiment 4. Effect of Corticosterone Acetate on Spontaneous NaCl Intake

Sixteen male rats weighing from 381–494 g were used. They were adrenalectomized 6 weeks prior to the experiment. The rats were divided into 4 equal groups and caged individually. Each rat was given choice between tap water and 0·15 M NaCl solution. Other experimental conditions were the same as those described in Experiment 1. During a 4 day control period, body weights and intakes of water and 0·15 M NaCl solution were measured daily. At the end of the control period, one group of rats was injected subcutaneously with 0·2 ml peanut oil while the 2nd, 3rd and 4th groups were injected respectively with 64, 128 and 256 μg corticosterone acetate in 0·2 ml oil daily for 4 days. The percent change in intake of NaCl solution and water from the pre-drug control period was calculated and is shown in Table 2.

Administration of corticosterone acetate significantly affected the percent change in NaCl intake from pre-drug control period ($P < 0.05$). The relationship between the two will fit a cubic regression ($P < 0.01$). The lowest dose level of corticosterone acetate (64 μg) reduced intake of NaCl solution to its lowest level. The relationship between hormone dose and percent change in NaCl intake bears a rough U-shape as observed above for DOCA and aldosterone but the maximal reduction in NaCl intake was less than that observed with either DOCA or aldosterone.

TABLE 2. EFFECTS OF CORTICOSTERONE ACETATE ON SPONTANEOUS INTAKES OF 0·15 M NaCl SOLUTION AND WATER BY ADRENALECTOMIZED RATS

Daily drug dose	Days given drug	Measurements made	Mean % change from pre-drug control period
Adrex + oil	4	0·15 M NaCl Soln. Intake	− 3·1
		Water Intake	− 48·8
		△ Body Weight (g/4) days)	+ 1·7
Adrex + 64 μg corticosterone acetate	4	0·15 M NaCl Soln. Intake	− 16·1
		Water Intake	− 56·4
		△ Body Weight (g/4) days	+ 1·7
Adrex + 128 μg corticosterone acetate	4	0·15 M NaCl Soln. Intake	+ 0·7
		Water Intake	− 52·6
		△ Body Weight (g/4 days)	+ 4·3
Adrex + 256 μg corticosterone acetate	4	0·15 M NaCl Soln. Intake	− 8·1
		Water Intake	− 55·5
		△ Body Weight (g/4 days)	+ 2·2

Analysis of variance (mean % change):

	0·15 M NaCl Soln.				Water		
Source	df	MSS	F	P	MSS	F	P
Treatment	3	209·84	4·64	<0·05	46·76	0·05	>0·05
Cubic 1		611·62	13·51	<0·01	21·84	0·02	>0·05
Error	12	45·26			999·62		
Total	15						

Experiment 5. Effect of Thyroxine on Spontaneous NaCl Appetite

Ten male adrenalectomized rats weighing 350–380 g were used. The rats were divided randomly into two equal groups. During the initial 5 day control period, all rats were caged individually and given choice between 0·15 M NaCl solution and tap water. Ground Rockland Rat Diet was used. Other experimental conditions were the same as those described in Experiment 1. Daily measurements of intakes of water, 0·15 M NaCl solution and

food were made. At the end of the control period, one of the two groups was given 7·5 μg thyroxine daily by the intraperitoneal route, while controls received the vehicle used to dissolve the thyroxine. This period lasted 5 days during which all the above measurements were made. During subsequent 5 day periods, 15 and 30 μg thyroxine/day was administered to the treated group.

Intake of NaCl solution was not affected significantly by treatment with thyroxine (Table 3). NaCl intake decreased by 12–13 per cent as a result of treatment with thyroxine regardless of the dose level used. Water intake increased as dose level of thyroxine increased. The percent change in water intake is linearly related to dose of thyroxine administered. Food intake of treated rats increased when the two higher dose levels of thyroxine were administered. Thus, thyroxine failed to affect significantly NaCl intake of adrenalectomized rats but increased water intake in a near linear fashion with increase in dose level.

Experiment 6. Effect of Synthetic Angiotensin on Spontaneous NaCl Intake

Two separate studies were performed. The first study utilized 6 male rats that had been adrenalectomized 7 weeks prior to the experiment. The rats were caged individually and given choice between tap water and 0·15 M NaCl solution to drink. The food was finely ground Rockland Rat Diet. Food and fluid containers and other conditions were as described in Experiment 1. Intakes of water, NaCl solution and food were measured daily for 10 days. During this time all rats were injected subcutaneously daily with 0·2 ml peanut oil. At the end of the control period all rats were injected subcutaneously with 20 μg synthetic angiotensin in 0·2 ml peanut oil daily for 3 days. Following this, all rats were injected with 0·2 ml peanut oil daily for 8 days and finally with 50 μg angiotensin in 0·2 ml peanut oil daily for 3 days. Daily measurements of fluid and food intakes continued throughout the period.

The second study utilized 15 male adrenalectomized rats ranging in body weight from 240 to 280 g. The rats were adrenalectomized 2 weeks prior to the study and were randomly assigned to 5 equal groups. Each animal was caged individually and given choice between tap water and 0·15 M NaCl solution to drink. The food was finely ground Rockland Rat Diet. All experimental conditions were as described in Experiment 1.

During a 3 day control period, daily intakes of water, 0·15 M NaCl solution and food were measured. The following treatments were then initiated: Group 1 peanut oil (0·2 ml) subcutaneously daily; Groups 2, 3, 4

TABLE 3. EFFECT OF THYROXINE ADMINISTRATION ON SPONTANEOUS SALT INTAKE OF ADRENALECTOMIZED RATS

(ml or g/100 g BW/Day)

	Control†	Control†	Control	7·5 μg Thyroxine	Control	15 μg Thyroxine	Control	30 μg T Thyroxine
0·15 M NaCl Soln.	12·6 ± 0·7*	12·6 ± 1·2	13·3 ± 1·2	11·6 ± 1·5	12·4 ± 1·1	10·8 ± 1·9	13·6 ± 1·7	12·0 ± 0·9
Water	1·6 ± 0·2	1·9 ± 0·6	1·3 ± 0·5	2·3 ± 0·6	1·1 ± 0·5	3·0 ± 1·4	0·9 ± 0·3	3·7 ± 1·3
Food	5·0 ± 0·1	5·6 ± 0·4	5·4 ± 0·2	5·0 ± 0·5	4·5 ± 0·5	6·2 ± 0·7	4·8 ± 0·1	5·8 ± 0·4
% change in intake from simultaneous control:								
0·15 M NaCl Soln.		0		−12		− 13		− 12
Water		+19		+77		+117		+311
Food		+12		− 7		+ 38		+ 21

* ± one standard error of mean.

† 5 rats per group.

and 5; 8, 16, 32 and 64 μg angiotensin respectively in 0·2 ml peanut oil. Treatment lasted 3 days during which measurement of intakes continued.

The results of the first study (Table 4) show that administration of angiotensin reduced intake of NaCl solution and increased intake of water. Food intake was reduced slightly but body weight was unaffected.

TABLE 4. EFFECT OF SYNTHETIC ANGIOTENSIN ON SPONTANEOUS FLUID AND FOOD INTAKE OF SIX MALE ADRENALECTOMIZED RATS

Intakes (ml or g/100 g BW/day)	Periods: Control	20 μg Angiotensin	Control	50 μg Angiotensin
Water	3·0 ± 1·1*	4·5 ± 1·0	3·1 ± 0·6	5·8 ± 2·1
0·15 M NaCl solution	13·7 ± 2·2	12·0 ± 2·0	18·1 ± 2·4	14·3 ± 3·0
Food	4·8 ± 0·1	4·5 ± 0·5	5·4 ± 0·4	4·9 ± 0·4
Mean body wt. (g)	397	399	422	434
Days per period	10	3	8	3
	% change from control period			
Water	0	+50	0	+87
0·15 M NaCl solution	0	−12	0	−21
Food	0	− 6	0	− 9
Mean body wt.	0	+ 1	0	+ 3

* ± 1 standard error of mean.

The results of the second study (Table 5) confirm those of the first and reveal a significant ($P < 0·05$) effect of angiotensin on intake of NaCl solution with a highly significant ($P < 0·01$) linear reduction as dose of angiotensin increased. Water intake was affected significantly ($P < 0·05$) by treatment and was increased linearly as dose of angiotensin increased. Food intake was not affected by treatment.

The highest dose level of angiotensin administered (26·4 μg/100 g BW/day) reduced NaCl intake 35 per cent below that of controls. This compares favorably with DOCA (Fig. 2) but is less potent than aldosterone (Fig. 4). A 35 per cent reduction in NaCl intake occurred when either 63 μg DOCA/100 g BW or 4·8 μg aldosterone/100 g BW was administered. This suggests that angiotensin is about 2·4 times more effective than DOCA but only about 1/6 as effective as aldosterone. However, a comparison of the effectiveness of these compounds in reduction of NaCl intake on the basis of mole fractions would certainly reveal angiotensin to be the most potent of the three since this compound is an octapeptide.

TABLE 5. EFFECT OF ANGIOTENSIN ON SPONTANEOUS INTAKES OF 0·15 M NaCl SOLUTION, WATER AND FOOD BY ADRENALECTOMIZED RATS

Daily Drug dose	Days given drug	Measurements made	Mean % change from control period
Adrex + oil	3	0·15 M NaCl soln. Intake	− 6·1
		Water intake	− 52·8
		Food intake	− 18·1
		△ Body weight (g/3 days)	+ 14
Adrex + 8 μg angiotensin	3	0·15 M NaCl soln. Intake	− 4·3
		Water intake	− 40·7
		Food intake	− 3·5
		△ Body weight (g/3 days)	+ 12
Adrex + 16 μg angiotensin	3	0·15 M NaCl soln. Intake	− 7·7
		Water intake	− 45·6
		Food intake	− 4·6
		△ Body weight (g/3 days)	+ 15
Adrex + 32 μg angiotensin	3	0·15 M NaCl soln. Intake	− 23·5
		Water intake	+ 21·7
		Food intake	− 3·6
		△ Body weight (g/3 days)	+ 7
Adrex + 64 μg angiotensin	3	0·15 M NaCl soln. Intake	− 35·1
		Water intake	+163·2
		Food intake	− 9·9
		△ Body weight (g/3 days)	+ 12

Analysis of variance (mean % change):

	0·15 M NaCl soln.				Water			Food		
Source	df	MSS	F	P	MSS	F	P	MSS	F	P
Treatment	4	587·60	3·45	<0·05	49,512·08	5·03	<0·05	118·18	0·77	>0·05
Linear 1		1,942·46	11·78	<0·01	51,203·74	5·20	<·005	81·02	0·53	>0·05
Error	10	164·91			9,837·32			154·29		
Total	14									

DISCUSSION

The mineralocorticoids, desoxycorticosterone acetate and aldosterone, maximally reduced the spontaneous NaCl intake of adrenalectomized rats at 100 μg/100 g BW/day and 10·8 μg/100 g BW/day respectively (Figs. 2 and 4). Doses higher than these returned NaCl intake toward that of untreated adrenalectomized rats. Thus, a U-shaped dose–response curve exists between percent change in NaCl intake from control and dose of mineralocorticoid administered. The shape of the curve may have physiological significance. We suggest that the minimum approximates the area of regulation of NaCl intake for normal rats since this dose level of aldosterone (10·8 μg/100 g BW/day) is nearly the same as the aldosterone secretion rate of normal rats measured directly from left adrenal vein blood using the double isotope dilution-derivative technique (9·8 μg/100 g BW/day).[12] If normal rats regulate NaCl intake at a near minimal level, it is clearer why similar dose levels of DOCA, injected into adrenalectomized rats reduced NaCl intake while the same dose levels administered to intact rats, increased NaCl intake.[4–6] Whether mineralocorticoids increase or decrease NaCl intake may depend on the initial position of the rat on the dose–response curve.

Mineralocorticoids affect water intake in a fashion that is roughly a mirror image of the NaCl intake. The greater percentage change in water intake than in NaCl intake reflects the fact that water represents only about 5–10 per cent of the total fluid intake of an adrenalectomized rat. Hence, small changes in water intake produce large percentage changes.

The specificity of the mineralocorticoid effect was tested by administration of corticosterone acetate to adrenalectomized rats. This compound reduced NaCl intake; however, the reduction was small when compared with that of either DOCA or aldosterone. Although corticosterone acetate is a potent glucocorticoid, it has about 15 per cent of the mineralocorticoid activity of DOCA.[13] Other studies showed that cortisone acetate, a glucocorticoid with less mineralocorticoid activity than corticosterone acetate, had no effect on NaCl intake of adrenalectomized rats.[10] Furthermore, thyroxine had no significant effect on intake of NaCl solution but increased water intake roughly linearly as dose of thyroxine increased. The dissociation between intakes of water and NaCl solution is not explained by these studies although the increased water intake may be associated with the increased food intake accompanying thyroxine administration. Although not reported here, the specificity of mineralocorticoid effect on NaCl intake is also suggested by the fact that estrone and testo-

sterone propionate, administered in graded dose levels, failed to affect NaCl intake of adrenalectomized rats.[10]

Wolf[14] recently published results similar to those reported here for DOCA. Using a two bottle choice, administration of single doses of desoxycorticosterone trimethyl acetate (DTMA) to adrenalectomized rats resulted in a U-shaped dose–response relationship between drug dose and intake of either 0·154 or 0·333 M NaCl solution. The minimal intake occurred when 11·0 mg DTMA/kg body weight was administered. Since about 2 to 4 per cent of the injected dose is absorbed per day, the minimal intake of NaCl solution occurred between 22 and 44 μg 100 g BW/day. This is about half the dose level reported here for multiple doses of DOCA. Wolf also observed that administration of corticosterone with DOCA increased the NaCl intake above that observed with DOCA alone while administration of 0·5 and 2·5 mg corticosterone alone had no effect on NaCl intake. The latter finding differs from the results of Experiment 4 shown in Table 2 although lower dose levels of corticosterone acetate were used.

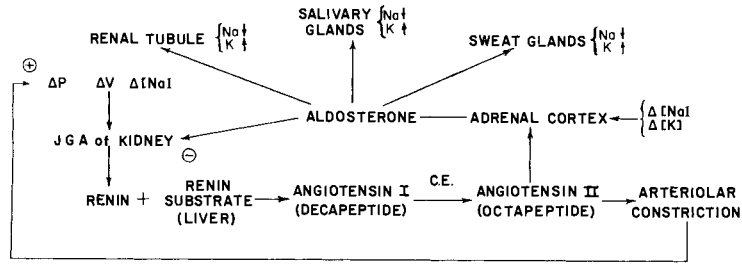


FIG. 5. Schema to illustrate feedback mechanisms affecting aldosterone secretion rate. A decrease in arterial pressure, in arterial volume or in plasma sodium concentration increases rate of renal production of renin and of formation of angiotensin II. Angiotensin II both increases adrenal cortical secretion of aldosterone and induces arteriolar constriction. The latter forms one limb of a negative feedback loop which will return to normal a reduced blood pressure. An increase in plasma sodium or decrease in plasma potassium concentration may increase aldosterone secretion by direct action on the adrenal cortex. Increase in blood level of aldosterone may also reduce renal secretion of renin and act as a second negative feedback loop. Aldosterone appears to reduce sodium loss from kidneys, salivary and sweat glands and to increase potassium loss from these same organs.

One mechanism currently thought to influence aldosterone secretion rate is shown in Fig. 5. This schema is based largely on studies from the dog[15] and sheep.[16] Although there is reason to believe that angiotensin II may not be the trophic hormone stimulating aldosterone secretion in the rat,[17, 18] there is, nevertheless, a considerable body of evidence linking the juxtaglomerular apparatus of rat kidneys with control of aldosterone

secretion.[19] We assume the possibility that this species may produce another octapeptide different in structure from angiotensin II but specific for the rat. With this assumption in mind, the schema shown in Fig. 5 may also hold for the rat. Synthetic angiotensin II was used in Experiment 6 to determine whether it affected the NaCl intake of adrenalectomized rats. The unexpected finding was that this octapeptide is more potent than DOCA and about 1/6 as potent as aldosterone on an equal weight basis in reduction of the NaCl intakes of adrenalectomized rats. Table 5 reveals a near linear reduction in NaCl intake as dose of angiotensin increases. Whether the U-shaped dose–response curve typical of DOCA and aldosterone would have been seen with higher doses of angiotensin remains speculative. The significance of this observation may lie in the fact that physiological situations inducing secretion of aldosterone also induce increases in angiotensin secretion, both of which could either reduce or increase NaCl intake depending on the initial position of the animal on the dose–response curve (Figs. 2 and 4).

The mechanism by which intake of NaCl solution is affected by mineralocorticoids may be related to changes in either sodium concentration or in the ratio of sodium to potassium concentration of saliva. Blair-West *et al.*[20] have shown that graded increases in rate of infusion of aldosterone into sheep reduce Na/K ratio of saliva in a graded fashion whereas the glucocorticoids, cortisol and corticosterone, were without effect in physiological doses. In addition, several investigators report a reduction of salivary Na/K ratio in patients treated with mineralocorticoids.[21, 22] McBurney and Pfaffmann[23] and Bartoshuk *et al.*[24] showed recently that adaptation of the human tongue to certain concentrations of NaCl solutions resulted in either a salty or sweet taste of test concentrations of NaCl solution above adapting concentration and sour or bitter tastes of test concentration below that of the adapting concentration. Tests using the adapting concentration showed it to be tasteless. Thus, the taste of both water and NaCl solution are specific and apparently depend on the concentration of sodium in the saliva bathing the taste receptors. It is tempting to suggest that the common denominator for "taste" of NaCl in both humans and rats is either the concentration of sodium bathing taste receptors or the ratio of the concentrations of sodium and potassium in saliva. In the case of humans described by McBurney and Pfaffmann[23] alterations in salt taste were accomplished by bathing the tongue with NaCl solutions of varying concentration. In our experiments, blood level of mineralocorticoid would be expected to alter the sodium and potassium concentrations of saliva by its effect on salivary secretion of these ions (Fig. 5) and thereby

produce a condition similar to bathing the tongue with a salt solution.

The effect of angiotensin on NaCl intake is worthy of further comment since it may lend support to the notion that salivary concentration of sodium and/or potassium influences NaCl intake. This peptide has been shown to influence renal handling of sodium and potassium in normal rats and dogs[25–27] as well as in adrenalectomized dogs.[25] Infusions of angiotensin at levels of 0·06 μg/min into normal rats increased both sodium and potassium excretion rates.[26] In normal dogs infusion of angiotensin at rates varying from 0·025 to 0·100 μg/kg/min decreased sodium excretion rate at the lowest and increased it at the highest dose level.[27] Sodium excretion rate of adrenalectomized dogs was reduced by infusion of angiotensin at a rate of 2 μg/min.[25] Thus, angiotensin may affect salivary sodium and potassium concentration in the same fashion as the renal tubule. The similarity of response of renal and salivary tubules to hormones is striking.[20] Experiments are needed to determine the effect of graded doses of angiotensin on the salivary sodium and potassium concentrations of adrenalectomized rats.

SUMMARY

Administration of the mineralocorticoids, desoxycorticosterone acetate (DOCA) and aldosterone, to adrenalectomized rats reduced spontaneous NaCl intake maximally at dose levels of 100 and 11 μg/100 g BW/day respectively. Still larger doses of these hormones tended to return NaCl intake to the level of untreated, adrenalectomized controls. Thus, a U-shaped dose–response curve existed between log dose of mineralocorticoid administered and intake of NaCl solution. The shape of the curve suggests why a certain dose level of mineralocorticoid could decrease NaCl intake of adrenalectomized rats while increasing NaCl intake of intact rats. The different responses may be explained by their initial positions on the dose–response curve. Reduction in NaCl intakes of adrenalectomized rats appears to be a relatively specific response to administration of compounds with mineralocorticoid activity. Compounds tested that do not affect NaCl intake of adrenalectomized rats include cortisone acetate, testosterone propionate, estrone and thyroxine. Synthetic angiotensin reduced NaCl intake in a near linear fashion with increasing dose level. It its suggested that compounds affecting NaCl intake of adrenalectomized rats alter either the sodium concentration or the ratio of sodium to potassium in saliva. These changes initiate the behavioral response to drug administration by central nervous pathways that are incompletely known.

REFERENCES

1. RICHTER, C. P. Increased salt appetite in adrenalectomized rats. *Am. J. Physiol* **115,** 155–161, 1936.
2. RICHTER, C. P. and J. F. ECKERT. Mineral metabolism of adrenalectomized rats studied by the appetite method. *Endocrinology*, **22,** 214–224, 1938.
3. RICHTER, C. P. Sodium chloride and dextrose appetite of untreated and treated adrenalectomized rats. *Endocrinology*, **29,** 115–125. 1941.
4. BRAUN–MENENDEZ, E. and P. BRANDT. Aumento del apetito especifico para la sal provocada por la desoxicorticosterona. I. Caracteristicas. *Rev. Soc. Argent. Biol.* **28,** 15–23, 1952.
5. RICE, K. K., and C. P. RICHTER. Increased sodium chloride and water intake of normal rats treated with desoxycorticosterone acetate. *Endocrinology*, **33,** 106–115, 1943.
6. TOSTESON, D. C., A. I. C. DEFRIEZ, M. ABRAMS, C. W. GOTTSCHALK and E. M. LANDIS. Effects of adrenalectomy, desoxycorticosterone acetate and increased fluid intake of sodium chloride and bicarbonate by hypertensive and normal rats, *Am. J. Physiol.* **164,** 369–379, 1951.
7. LAZAROW, A. Methods for quantitative measurement of water intake. *Methods Med. Res.* **6,** 225–229, 1954.
8. FREGLY, M. J. A simple and accurate feeding device for rats. *J. Appl. Physiol.* **15,** 539, 1960.
9. SNEDECOR, G. W. *Statistical Methods*, 5th ed, Iowa State Univ. Press: Ames, p. 237, 1962.
10. FREGLY, M. J. and I. W. WATERS. Effect of mineralocorticoids on spontaneous sodium chloride appetite of adrenalectomized rats. *Physiol. Behavior* **1,** 65–74, 1966.
11. FREGLY, M. J. and I. W. WATERS. Effect of mineralocorticoids on spontaneous NaCl intake of adrenalectomized rats. *The Physiologist*, **7,** 134, 1964.
12. FREGLY, M. J., J. R. CADE, I. W. WATERS, J. A. STRAW and R. E. TAYLOR, JR. Secretion of aldosterone by adrenal glands of propylthiouracil-treated rats. *Endocrinology* **77,** 777–784, 1965.
13. RUSSELL, J. A. in *Medical Physiology and Biophysics*, 18th ed, Ed. T. C. RUCH and J. F. FULTON, Phila.: Saunders, p. 1092, 1960.
14. WOLF, G. Effect of desoxycorticosterone on sodium appetite of intact and adrenalectomized rats. *Am. J. Physiol.* **208,** 1281–1285, 1965.
15. URQUHART, J. and J. O. DAVIS, Role of the kidneys and the adrenal corltex in congestive heart failure II, Mod. Concepts Cardiovascular Dis. **32,** 787–792, 1963.
16. BLAIR-WEST, J. R., J. P. COGHLAN, D. A. DENTON, J. R. GODING, M. WINTOUR and R. D. WRIGHT. The control of aldosterone secretion, *Recent Prog. Hormone Res.* **19,** 311–383, 1963.
17. CADE, J. R. and T. PERENICH. Secretion of aldosterone by rats. *Am. J. Physiol.* **208,** 1026–1030, 1965.
18. SINGER, B., C. LOSITO and S. SALMON. Some studies on the effect of angiotensin II on adrenal hormone secretion in hypophysectomized rats with renal pedical ligation. *Endocrinology*, **75,** 325–332, 1964.
19. TOBIAN, L. Interrelationship of electrolytes, juxtaglomerular cells and hypertension. *Physiol. Rev.* **40,** 280–312, 1960,
20. BLAIR-WEST, J. R., J. P. COGHLAN, D. A. DENTON, J. R. GODING and R. D. WRIGHT. The effect of aldosterone, cortisol and corticosterone upon the sodium and potassium content of sheep's parotid saliva. *J. Clin. Invest.* **42,** 484–495, 1963.

21. LAULER, D. P., R. B. HICKLER and G. W. THORN. The salivary sodium-potassium ratio. *New Eng. J. Med.* **267,** 1136–1137, 1962.
22. FRAWLEY, P. F. and G. W. THORN. The relation of the salivary Na/K ratio to adrenal cortical activity, in J. R. MOTE, ed., *Proceedings Second Clinical ACTH Conference*, 1. Research, Blakiston: New York, 1951, pp. 115–122.
23. MCBURNEY, D. H. and C. PFAFFMANN. Gustatory adaptation to saliva and sodium chloride. *J. Exp. Psychol.* **65,** 523–529, 1963.
24. BARTOSHUK, L. M., D. H. MCBURNEY and C. PFAFFMANN. Taste of sodium chloride after adaptation to sodium chloride: implications for the "water taste". *Science,* **143,** 967–968, 1964.
25. LEVITIN, H., W. B. LEHMANN, G. PIGEON, Y. WARREN and I. S. GOLDENBERG. The effect of anesthesia and adrenalectomy on the renal response to angiotensin. *J. Clin. Invest.* **42,** 951, 1963.
26. BORKOWSKI, A., S. S. HOWARDS and J. H. LARAGH. Angiotensin and electrolyte excretion in renovascular hypertension. *Am. J. Physiol.* **208,** 1087–1092, 1965.
27. HEALY, J. K., C. BARCENA, J. M. B. O'CONNELL and G. E. SCHREINER. Renal and pressor action of angiotensin in the normal dog. *Am. J. Physiol.* **208,** 1093–1099, 1965.

THE GLOSSOPHARYNGEAL NERVE RESPONSE TO TASTE AND THERMAL STIMULI IN THE RAT, RABBIT, AND CAT

KAZUHIRO YAMADA

Department of Physiology, Kumamoto University
Medical School, Kumamoto, Japan

QUANTITATIVE electrophysiological studies of the chorda tympani nerve using the integrator recording method have been carried out by a number of investigators (Beidler, Fishman, and Hardiman, 1955; Pfaffmann, 1955; Sato and Yamashita, 1965). However, no detailed analysis on the glossopharyngeal nerve response has been made so far. In the present study, quantitative determination of the glossopharyngeal nerve response in the rat, rabbit, and cat has been carried out (Yamada, 1965).

The tongue was placed on a flow box to allow rapid stimulation of a defined and constant receptor area. After taste stimulation, the tongue was rinsed with water in the rat and with Ringer's solution in the rabbit and cat. The total electrical activity recorded from the glossopharyngeal nerve was averaged with an integrator having a time constant of 0·5 sec.

Typical responses of the rat glossopharyngeal nerve to water, 0·5 M NaCl, 0·02 M quinine hydrochloride, 0·5 M sucrose, and 0·01 M HCl are shown in Fig. 1. When the temperature of the rectum of an animal was kept at 37°C, no glossopharyngeal nerve response to water was observed at 37°C; consequently, the temperature of all taste stimuli was adjusted to 37°C. The temperature of animals was kept at 37°C throughout.

As seen in the figure, the response had a latency of a few seconds after the onset of stimuli and the latency of the response to 0·5 M NaCl was 2·5 sec, the latency decreasing with an increase in the concentration.

Since the stimulus must enter the deep grooves of the circumvallate papillae to stimulate the receptors, latency is thought to be much greater than that for the fungiform papillae (Beidler, 1962). By calculations using the formula derived from the diffusion equation (Jacobs, 1935, quoted by Beidler, 1962), 0·5 M NaCl requires 2·5 sec to reach 0·1 M in the layer of

380 μ beneath the surface, which is the average depth of the location of taste buds in the grooves of the circumvallate papillae.

The magnitudes of the integrated response, measured at the maximum and expressed relative to the response magnitude for 0·5 M NaCl, are presented in Table 1, where it will be noted that the relative magnitude of

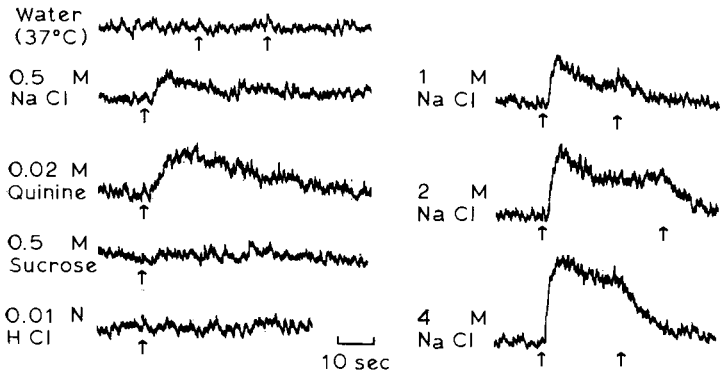


FIG. 1. Glossopharyngeal nerve response in a rat. The tongue was adapted to water at 37°C, and then stimulated. The first arrow indicates the onset of stimulation and the second one the rinse with water.

response to 0·02 M quinine is greater than that for 0·5 M NaCl. This is in contrast with the response of the chorda tympani, in which the ratio of the response magnitude for 0·02 M quinine to that for 0·1 M NaCl is 0·20 (Beidler *et al.*, 1955) or 0·48 (Sato and Yamashita, 1965).

TABLE 1. Glossopharyngeal nerve response magnitude in the rat, rabbit, and cat. Numerals in parentheses indicate the number of experiments.

Chemical stimuli	Rat (5) Circumvallate papillae	Rabbit (4) Foliate papillae	Cat (8) Circumvallate papillae
0·5 M NaCl	1·00	1·00	—
1 M NaCl	1·59	2·20	0·59
0·5 M KCl	1·44	1·93	—
1 M KCl	—	—	1·00
0·02 M quinine	1·22	1·17	1·63
0·005 N HCl	—	1·54	—
0·01 N HCl	0·01	—	1·57
0·5 M sucrose	0·17	2·51	0

The magnitude of response to NaCl increased linearly with the logarithm of NaCl concentration from 0·5 M to 4 M as shown in Fig. 1 and in Fig. 2. The response magnitude for 0·5 M KCl was greater than that for 0·5 M NaCl. These are other characteristic features of the glossopharyngeal nerve response different from the chorda tympani response.

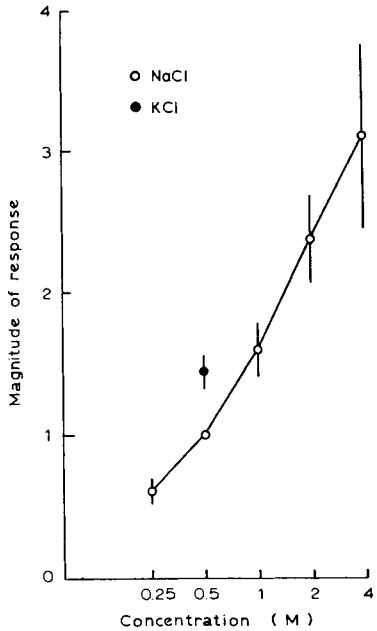


FIG. 2. Glossopharyngeal nerve response magnitude in the rat for NaCl of various concentrations, the magnitude being expressed relative to that for 0.5 M NaCl, with ± S.E. of the mean.

The glossopharyngeal nerve responded well to both cooling and warming of the tongue as shown in Fig. 3.

The glossopharyngeal nerve of the rabbit, when solutions were given to the tongue without rejecting the mandible, showed responses to KCl of more than 1 M and to NaCl of more than 2 M but not to 0·02 M quinine, 0·01 N HCl, and 0·5 M sucrose. The response showed a latency of several seconds and increased in magnitude gradually as in the case of the rat.

When the solutions were flowed over the foliate papillae after the mandible had been rejected, the glossopharyngeal nerve showed a marked response to solutions of the four taste qualities as well as to water, the response rapidly attaining a maximum (see Fig. 4). Responses to both cooling and warming were also observed.

The second type of response probably originates from the foliate papillae and the first type from the circumvallate papillae. Since the magnitude of the latter is negligibly small compared with that of the former, only the magnitude of the second type response is shown in the table, where the relative magnitudes of response to 0·02 M quinine, 0·01 N HCl,

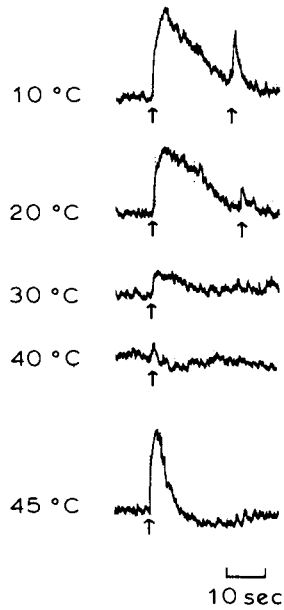


FIG. 3. The glossopharyngeal nerve response in a rat to thermal stimuli. The tongue was adapted to water at 37°C, then water of various temperature was applied (first arrow).
The second arrow, if present, shows the reapplication of water at 37°C. Note the decrease in the spontaneous discharge level after the application of warm water.

0·5 M sucrose, and 0·5 M KCl are greater than that for 0·5 M NaCl. This is different from the chorda tympani response, in which the magnitudes for 0·02 M quinine, 0·01 N HCl, 0·5 M sucrose, and 0·5 M KCl are smaller than that for 0·5 M NaCl (Beidler *et al.*, 1955).

The response of the glossopharyngeal nerve to NaCl increased in magnitude linearly with the logarithm of NaCl concentration from 1 to 4 M and rapidly attained a maximum, gradually decaying after the rinse with Ringer's solution. The response to water was very small, as noted previously (Appelberg, 1958; Ishiko and Amatsu, 1964). Since the response to NaCl was poor, the response magnitude for 1 M KCl was employed as

a standard response. The table shows that the response magnitudes for 0·02 M quinine and 0·01 N HCl are greater than that for 1 M KCl. This is in contrast with the chorda tympani response, in which the response magnitudes for 0·02 M quinine and 0·01 N HCl are about the same as that for 0·5 M KCl (Beidler *et al.*, 1955). Cats have several circumvallate papil-

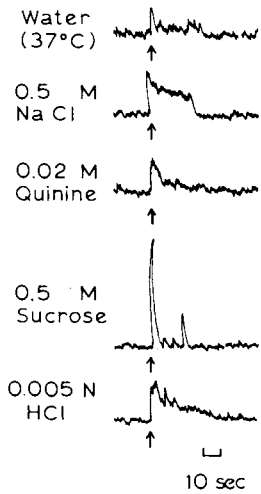


FIG. 4. Glossopharyngeal nerve response in a rabbit. The solutions were flowed over the foliate papillae.

lae and foliate papillae in the region of the tongue innervated by the glossopharyngeal nerve. After the foliate papillae had been extirpated without damaging the surrounding tissues, the glossopharyngeal nerve response to taste stimuli was not modified after the operation. This suggests that the foliate papillae in the cat respond little to taste stimuli.

ACKNOWLEDGEMENTS

The author wishes to express his thanks to Professor M. Sato for his guidance and much helpful advice, and to Professor C. Pfaffmann for offering the author the opportunity to present this paper at the symposium. It was read as a discussion of Professor Pfaffmann's work.

REFERENCES

APPELBERG, B. 1958. Species differences in the taste qualities mediated through the glossopharyngeal nerve. *Acta Physiol. Scand.* **44**, 129–137.

BEIDLER, L. M. 1962. Taste receptor stimulation. *Progress in Biophysics and Biophysical Chemistry*, **12**, 109–151.

BEIDLER, L. M., FISHMAN, I. Y. and HARDIMAN, C. W. 1955. Species differences in taste responses. *Am. J. Physiol.* **181,** 235–239.

ISHIKO, N. and AMATSU, M. 1964. Effects of stretch of the tongue on taste responses in glossopharyngeal and chorda tympani nerves of cat. *Kumamoto Med. J.* **17,** 5–17.

JACOBS, M. H. 1935. Diffusion processes. *Ergebn. Biol.* **12,** 1.

PFAFFMANN, C. 1955. Gustatory nerve impulses in rat, cat and rabbit. *J. Neurophysiol.* **18,** 429–440.

SATO, M. and YAMASHITA, S. 1965. The effect of temperature on the taste response of rats. *Kumamoto Med. J.* **18,** 41–43.

YAMADA, K. 1965. The glossopharyngeal nerve response to taste and thermal stimuli in the rat, rabbit, and cat. *Kumamoto Med. J.* **18,** 106–108.

ACTION AND INTERACTION OF ETHYL ALCOHOL AND SOME OTHER SUBSTANCES ON THE RECEPTORS OF THE TONGUE

GÖRAN HELLEKANT

Department of Physiology, Veterinärhögskolan,
Stockholm 50, Sweden

THE first study which included the taste of alcohol was probably that by Grützner (1894). A few authors since then have been scientifically interested in the taste of alcohol. The first studies were carried out exclusively or partly with psychophysical methods (Raether 1905, Hallenberg 1914 and Renqvist 1920). These psychophysical methods have since been supplemented with other methods as the determination of the rejection threshold used by, e.g. Dethier and Chadwick (1947) or the preference threshold used by, e.g. Myers (1962). The electrophysiological methods were also extended to this special field of research when Beidler (1953) recorded the effect of alcohol on the gustatory receptors. He reported that the electrical response to alcohol in the chorda tympani nerve of the rat was "barely perceptible". Later Diamant, Funakoshi, Ström and Zotterman (1963) tested the effect of alcohol on the tongues of two human subjects and one dog by recording the electrical response from the whole chorda tympani nerve just before the First Symposium on Olfaction and Taste. The gustatory effect of alcohol was further studied in a recent paper by Diamant, Oakley, Ström, Wells and Zotterman (1965).

This report presents a summary of results, published elsewhere *(Acta physiol. scand.)*, of three series of experiments on cat, dog and rat on the effect of alcohol on the receptors of the tongue, as well as results from some quite recent experiments on the interaction of alcohol on the neural response in the cat to other taste substances.

METHODS

The chorda tympani nerves of the different species were dissected according to the technique of Zotterman (1935). For recording, the whole nerve or the nerve bundle was placed across a platinum electrode which was fixed in a micromanipulator. A second indifferent electrode was put in contact with the animal. The amplified impulses were first summated, when recording from a whole nerve, or directly recorded on an oscilloscope, when recording from a filament. The alcohol solutions were applied to the tongue by means of a burette. Its two-way stop cock was connected to a tap water container and to the column of the burette. A signal recorded on the film revealed the position of the cock. The temperature of the water and the solutions was kept equal (29–33°C) and the temperature of the tongue surface was recorded on the film with a thermocouple.

The alcohol solutions were made with 95 per cent ethanol and tap water and had the concentrations 1·7, 2·5, 3·3, 4·1, 4·9, 5·7, and 6·5 M. Half molar sodium chloride and sucrose and 0·05 M quinine (mono) hydrochloride hydrate and acetic acid were used as test solutions.

RESULTS AND DISCUSSION

A. The Summated Response of the Whole Chorda Tympani Nerve in the Cat, Dog and Rat

The observed summated response to alcohol was in several respects similar in the three species investigated. The general pattern of the alcohol response distinguished it from the response observed to other taste stimuli. The pattern, shown in Fig. 1, was characterized by an initial depression

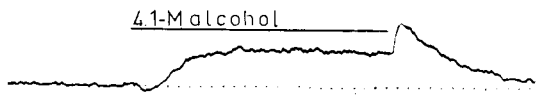


FIG. 1. The recording shows the summated response to 4·1 M alcohol in the chorda tympani nerve of the cat. The signal indicates the flow of alcohol. Observe the initial decrease of the nervous activity and the strong response to the water rinse following the alcohol exposure. Time marker 1 per second.

of the neural activity, a slow onset of the discharge and then a more or less strong burst of activity in response to a water rinse. In several details, however, the response to alcohol differed in different species. The thresh-

old concentration, defined as the alcohol concentration which first caused a visible change in the recorded nervous activity, differed. The threshold was lower in the cat than in the dog and the strongest solutions were required in the rat as may be seen in Fig. 2. The response to alcohol attained a maximum magnitude at 5·0 M alcohol in the cat and at 6·5 M in the rat. A further increase of alcohol concentration gave no increase or decrease of the response.

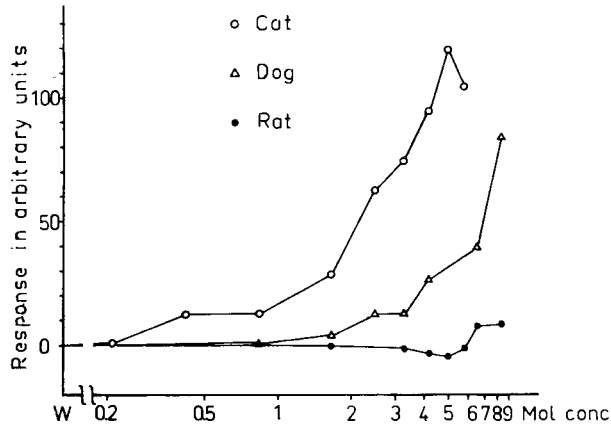


FIG. 2. Curves of the summated response in the chorda tympani nerves of the cat, dog and rat to different alcohol concentrations. The responses were measured 10 sec after the onset of alcohol flow.

As mentioned above, a depression of the nervous activity was observed initially during the alcohol flow. This period of decreased activity was observed in all species but not in all animals. In the rat 1·6–4·9 M alcohol caused a decrease of activity as long as the tongue was exposed to alcohol. The depression observed in the dog and cat lasted only a few seconds in contrast to its long duration in the rat. A similar initial depression in the dog was reported by Diamant *et al.* (1963).

A burst of neural activity was constantly observed when the tongues of the cat and dog were rinsed with water after alcohol. A similar increase of activity to water was also observed in several rats, though the increase was much smaller. The strongest response to water after alcohol was, however, observed in the cat. The response was therefore studied somewhat more closely in this animal.

B. A Single Fibre Analysis of the Chorda Tympani Nerve in the Cat

It was observed that all fibres which responded to at least one of the conventional taste solutions represented by 0·5 M NaCl, 0·5 M sucrose, 0·05 M acetic acid and 0·05 M quinine hydrochloride also responded to alcohol. About one-third of the fibres tested responded with an increase of activity to a concentration of 1·7 M alcohol and almost all responded to 2·5 M alcohol. They were divided into two principle groups with regard to their response pattern to alcohol and to a subsequent water rinse.

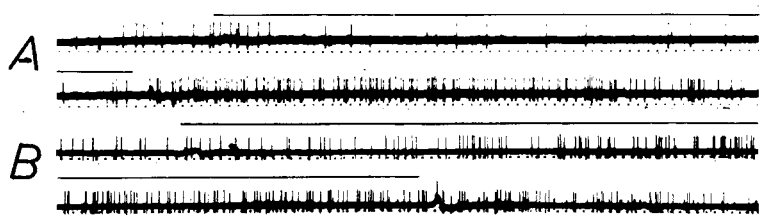


FIG. 3. Responses from two single chorda tympani units to a flow of 3·3 M alcohol which was preceded and followed by a constant flow of water. The horizontal line indicates a flow of alcohol over the tongue. Note in fibre A the initial depression to alcohol and the very strong response to water rinse. Eight seconds of the recording were excluded in A. Note that fibre B did not show any initial depression to alcohol but a constant increase of frequency, which decreased immediately when the tongue was rinsed with water. Time marker 10 per second.

The chorda tympani fibres of the first group, the "water" fibres, included 18 of 33 fibres. They usually discharged during the water rinse with a frequency below 5 imp/sec. When the tongue was rinsed with alcohol this spontaneous activity was at first substantially reduced (Fig. 3A). The period of reduced activity was then gradually followed by a period of increased discharge. The discharge was moderate, however, and usually did not reach the same frequency as the discharge elicited by acetic acid or quinine or both. Further there was always observed a strong burst of activity, when the tongue was rinsed with water after alcohol. Figure 4, gives a better demonstration of the moderate increase of the activity in two such fibres, represented by the circles and the dots, and the very intense response to water after alcohol. The fact that water never produced such a strong effect after other sapid solutions suggests that alcohol increases the sensitivity to water of these fibres. The discharge during the first second of the water rinse usually had a frequency higher than that observed during the exposure to any of the conventional taste solu-

tions, though acetic acid and quinine usually elicited a strong activity in these fibres. They were not or only to a slight extent stimulated by salt (Fig. 5A–D).

The fibres of the second group, the "non-water" fibres, differed from those described above in their responses to alcohol and water as well as in their responses to the conventional taste solutions used. In this group alcohol produced an immediate increase of activity (Fig. 3B) which, however, developed slowly in comparison with the increase of

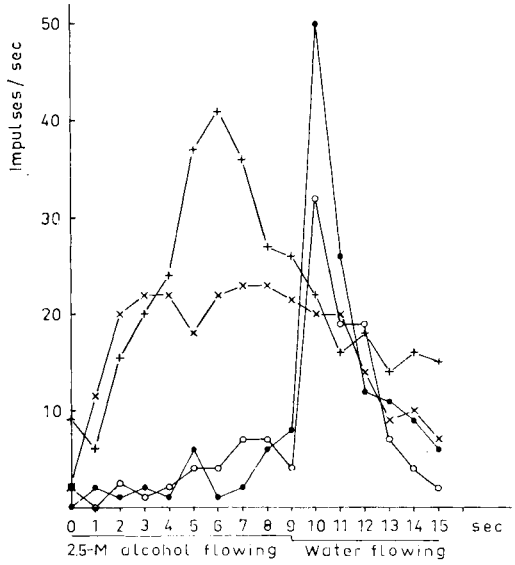


FIG. 4. Curves of the responses to 2·5 M alcohol and water in four different units. The frequency at time 0 sec was obtained during a constant flow of water. The signal below the abscissa indicated, when the flow was changed from alcohol to water.

activity elicited for instance by salt. Fibres of the second group usually responded at a higher rate to low alcohol concentrations. The frequency recorded (during the 6th sec) was maximal when the tongue was exposed to 4·1 M alcohol in the majority of these fibres. The frequency then decreased with increasing concentration. These fibres differed also in their behaviour to a subsequent water flow, as may be seen in Fig. 4. The water rinse after alcohol gave no burst of impulses in these fibres, but brought the nerve activity back to the value observed during the preceding water flow. The recordings revealed also that this group of fibres were predominantly stimulated by salt, e.g. Fig. 5, F–I. The sensitivity to salt was often associated with a sensitivity to sucrose, e.g. Fig. 5, G, H.

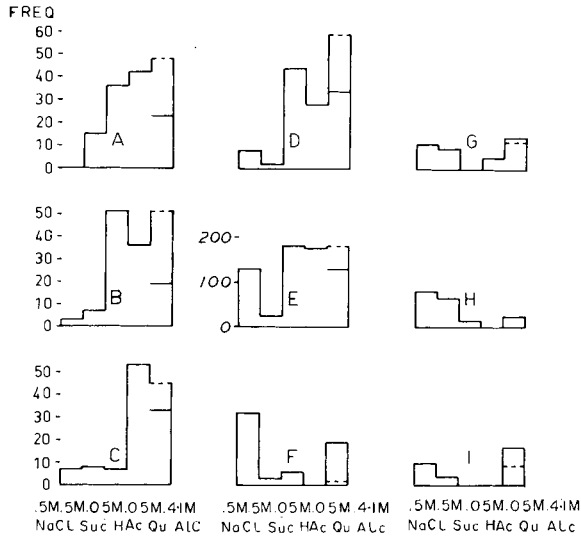


FIG. 5. Histograms summarizing frequency of responses during the first second to the standard solutions and the maximal impulse activity to 4·1 M alcohol in 8 different units. Histogram E shows the relative magnitude of the summated response to the test solutions in arbitrary units. The horizontal dotted line indicates the response to water during the first second after the 4·1 M alcohol rinse. Note the responses to water in A–D which were classified as "water" fibres, and that the summated response in E briefly speaking is a sum of A–D and F–I.

C. The Effect of Ethyl Alcohol on Non-gustatory Receptors of the Tongue of the Cat

The rather small response in the chorda tympani nerve to the application of alcohol on the tongue, in comparison with the strong response to the conventional taste solutions, raised the question of an alcohol effect on sensory receptors other than the gustatory cells in the taste buds. Further the application of alcohol on the tip of the tongue of the lightly anesthetized cat produced muscular reflexes. It was suspected that such reflexes caused by alcohol might be elicited via sensory fibres belonging to the trigeminal part of the lingual nerve, as similar reflexes were never observed during the exposure to conventional taste solutions. The third series was undertaken to study a possible response to alcohol in trigeminal fibres of the lingual nerve. The recordings were obtained in the majority of the animals from the trigeminal component of the lingual nerve after the branching off of the chorda tympani.

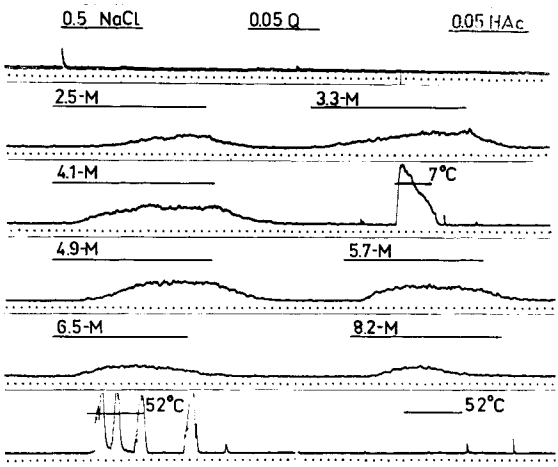


FIG. 6. The recording shows the summated response in the trigeminal part of the lingual nerve to the low of 0·5 M NaCl, 0·05 M quinine, 0·05 M acetic acid, a series of alcohol concentrations and water at 7° and 52°C. The signals indicate the flow. Time marker 1 per second.

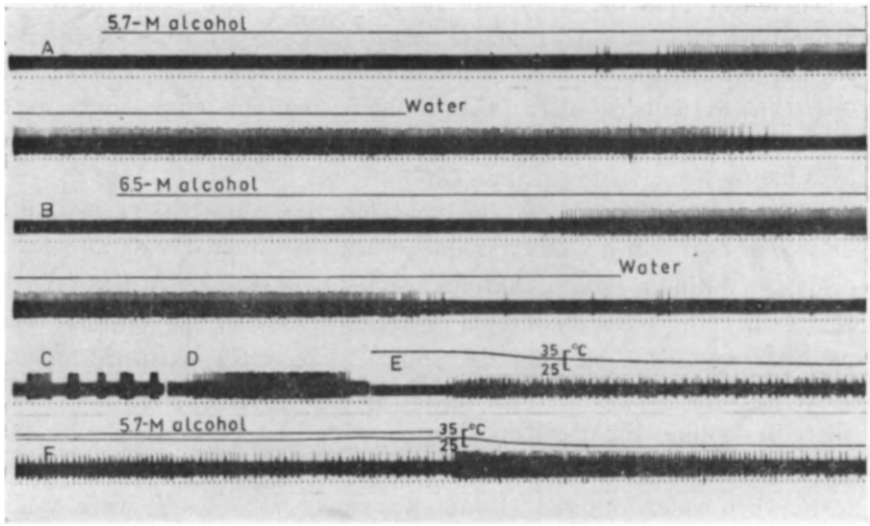


FIG. 7. Responses from a fine branch of the trigeminal part of the lingual nerve to: A 5·7 M alcohol flow, B 6·5 M alcohol flow, C repeated touch, D increasing and decreasing pressure, E cooling with water from 35 to 27°C, F cooling with water from 30 to 25°C after 35 sec flow of 5·7 M alcohol. A second smaller spike can be seen in E and F. The straight horizontal line indicates the flow of alcohol. The other line represents the temperature recording. Time marker 10 per second.

The recording in Fig. 6 of the summated response of the trigeminal part of the lingual nerve to alcohol shows clearly that the magnitude of this response at first increased with increasing alcohol concentration and attained a maximum at 4·9 M alcohol. The height of the response decreased then with increasing concentration. The conventional taste solutions produced no response whatever from these fibres.

An examination of the response pattern of single lingual nerve fibres revealed that those fibres which reacted positively to alcohol also responded to mechanical stimulation as well as to cooling of the tongue (Fig. 7). These fibres responded neither to moderate nor to more extreme heating (52°C) of the tongue surface. Their conduction velocity ranged from 5·7–18·0 m/sec, which indicated that they were myelinated. According to their responses to the above-mentioned stimuli, they were classified as unspecific mechanoreceptive fibres, previously described by Hensel and Zotterman (1951). Their behaviour to a flow of alcohol over the tongue showed several interesting characteristics. Their threshold values to alcohol were slightly higher than those observed in gustatory fibres. Thus only 6 out of 16 fibres had a threshold above 3·3 M alcohol. The average latency of the response to alcohol was about 10 sec, which is much longer than the latency of the gustatory fibres. Their discharge to alcohol was particularly characterized by its regularity (Fig. 7 A and B). An average maximum value of the impulse frequency was observed at 4·1 M alcohol concentration. This is interesting as similar values were also obtained in the chorda tympani nerve. No response to a water flow over the tongue after the alcohol exposure was observed.

It was noted that the alcohol concentration which first caused the fibre to discharge at maximum frequency usually caused a cessation of the discharge during a prolonged flow. The ability of the fibre to discharge to touch and cooling was diminished or abolished during this paralysis. The paralysis was reversible and the ability to discharge returned after a while when the tongue was rinsed with water. A stage of increased sensitivity to cooling and mechanical stimulation was observed before the paralysis. This may be seen in Fig. 7 E and F. The temperature decrease in F, when water followed alcohol, was smaller than in E, when water followed water. Despite this, the frequency elicited in F is 3 times that observed in E. This increase of sensitivity seems to be analogous with the strong response to water after alcohol in the gustatory fibres.

A second type of fibres was also observed. These responded to mechanical stimulation but not to cooling. Neither alcohol nor hot water elicited

any discharge in these fibres. They were characterized as specific mechanoreceptive fibres as described by Hensel and Zotterman (1951).

It might be expected that strong alcohol solutions also stimulate nociceptive endings, as concentrations above 3 M elicit smarting sensations in man (Diamant *et al.*, 1963). From the studies of earlier investigators it is known that the lingual nerve contains nociceptive myelinated fibres (Dodt, 1954). They react to temperatures above 47° C. In contrast to the "warm" fibres they were also stimulated by pinching. No response to alcohol could be obtained from such fibres.

The Electrophysiological Response to Mixtures of Alcohol and Conventional Taste Solutions in the Cat

The previous results described in this report indicated an immediate effect of alcohol on the taste receptors, though this effect was less evident when compared with the strong immediate response to the conventional taste solutions. The effect of mixtures of conventional taste substances and alcohol might therefore be of interest and the logical next step in such a series. It is further known that several gustatory stimuli interact (cf. Andersson *et al.*, 1950; Beidler, 1962). Observations obtained during the first series of experiments indicated such an interaction also for alcohol and some of the usual taste stimuli used.

METHOD

Half molar NaCl, 1 M sucrose, 0·02 M quinine (mono)hydrochloride and 0·05 M acetic acid were used as test solutions. Two sets of solutions were made, one in 2·5 M ethyl alcohol and one in distilled water. A 2·5 M alcohol in water solution was also used. The operating and recording techniques were the same as before.

RESULTS

Figure 8 is a histogram summarizing the results obtained from the whole chorda tympani nerve in 4 cats. The mean value of the summated respone within the first 2 sec is marked on the ordinate. The histogram shows a slight decrease of the response to salt and acid when these solutions were made up in 2·5 M alcohol. The quotient between the amplitude of the summated response to salt or acid in water and those in alcohol is 1·1. Alcohol caused

the most pronounced change of the gustatory response of quinine, which is evident in Fig. 8. The quotient between the summated response to quinine and alcoholic quinine is 2·5.

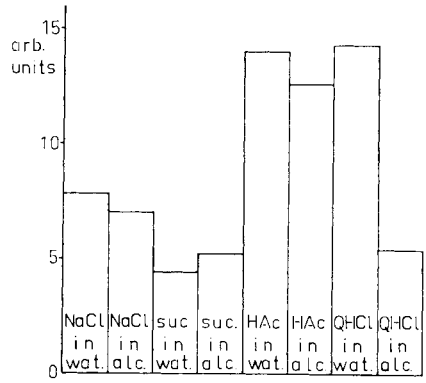


FIG. 8. Histogram summarizing the summated responses obtained during repeated exposure of the tongues of 4 cats.

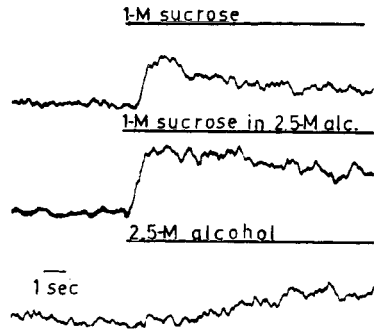


FIG. 9. The recordings show the summated response of the chorda tympani of one cat. Observe the increase of the immediate response when alcohol was added.

The response to sucrose, however, showed a small but significant increase when the sucrose was dissolved in alcohol. The quotient is 0·85. This increase was small in animals with a low gustatory sensitivity to sucrose, but it was particularly strong in one animal. A summated recording from this cat is shown in Fig. 9. These exposures were consecutive and repeated several times.

Single Fibres

The analysis was carried further by recording from thin filaments of the chorda tympani nerve. The impulse frequency of two fibres is plotted as histograms in Fig. 10. These fibres were classified as "water" fibres according to their responses to alcohol and water and to the criteria given in an earlier study (Hellekant, 1965b). The figure shows that the observed decrease of the summated response when alcoholic solutions were used corresponds with the observation obtained from single fibres. The relation between the responses to salt in water and to salt in alcohol is similar to that in Fig. 8 for the summated response. This relation is further illustrated in another fibre in Fig. 11 showing the response to salt, alcoholic salt and alcohol solutions.

The responses to sucrose in Fig. 10 do not show the same increase of activity when alcohol is added as was seen in the summated response. There are, however, good reasons to believe that alcohol only increases

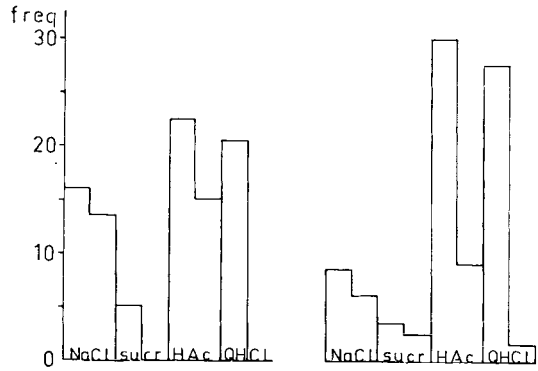


FIG. 10. Histograms summarizing frequency of responses in two different units. Each part of the columns shows the difference between the activity during the first 2 sec of stimulation and the spontaneous activity the last 2 sec before. The right part of each column shows the response to the alcohol containing solution.

the initial response to sucrose in fibres which, according to earlier studies (Hellekant, 1965*b*), can be classified as "non-water" fibres. Figure 11 supports this assumption, as the impulse activity plotted in this figure was obtained from a "non-water" fibre.

The difference between the responses to acetic acid and alcoholic acetic acid in Fig. 10 is greater than in Fig. 8. The difference is also greater in Fig. 12, which is another recording of a fibre responding to the application of acetic acid on the tongue.

The decrease of the initial response when alcohol was added to acetic acid was evident, but not so striking as the effect observed when alcohol was added to a quinine solution. This can be seen in each histogram of Fig. 10. The histogram of the left does not indicate any response at all and

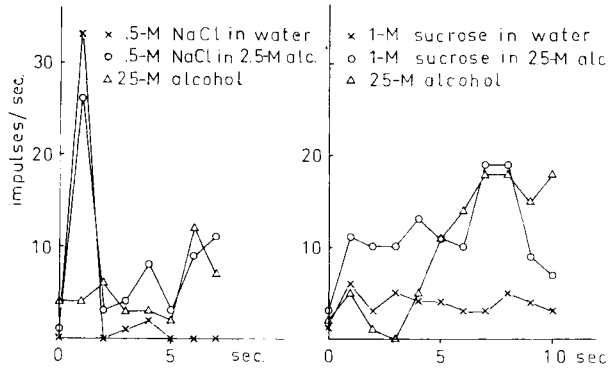


FIG. 11. Curves of the responses to salt, alcoholic salt and alcohol in one unit and to sucrose, alcoholic sucrose and alcohol in another unit. The frequency at time 0 sec was obtained during a constant flow of water.

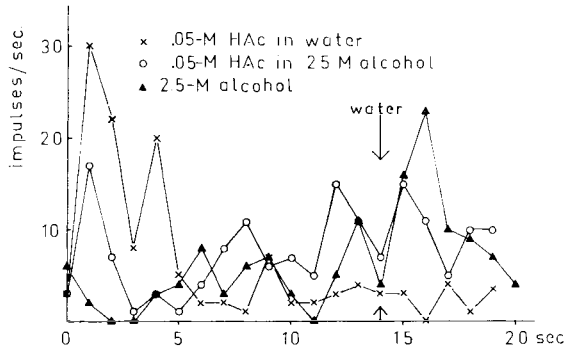


FIG. 12. Curves of the responses to acetic acid, alcoholic acetic acid and alcohol in one unit. The frequency at time 0 was obtained during a constant flow of water. The arrows indicate when the flow was changed to water.

the right histogram shows only a small response to alcoholic quinine solution. The complete reduction of the quinine response when alcohol was added is perhaps most evident in Fig. 13. The recordings of Figs. 12 and 13 were obtained from the same fibre.

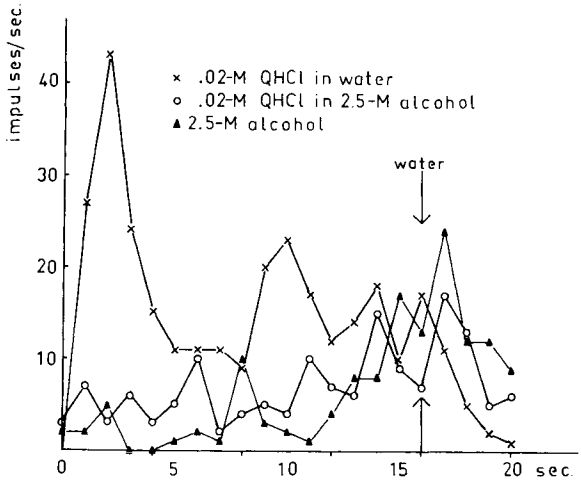


FIG. 13. Curves of the responses to quinine, alcoholic quinine and alcohol in one unit. The frequency at time 0 was obtained during a constant flow of water. The arrows indicate when the flow was changed to water.

DISCUSSION

The recordings of the summated response as well as the recordings from thin filaments of the chorda tympani nerve show that alcohol exerts an immediate effect on the taste receptors, though exposure to alcohol in water only slightly changes the number of impulses recorded during the first second (Hellekant, 1965*b*). It has earlier been proposed that this initial effect of alcohol which can be seen as a small decrease of the neural response in one type of fibres or as a slight increase in the other type, is related to the alcohol exposure of the microvilli serving as taste receptors (Hellekant, 1965*d*). This study shows that there is an immediate interaction between alcohol and the other sapid substances. This interaction is presumably not a chemical reaction between the different constituents of the mixed test solution occurring before the application on the tongue. Such a reaction may only be suspected in the alcoholic acid solution where an esterification may occur raising the pH of the solution. This is a slow reaction owing to the low acid concentration. Such a reaction may explain the observed decrease of the response to acetic acid. This explanation can not, however, be applied when discussing the effect of the other solutions on the tongue. The effect is possibly in these cases of a synergetic or competitive nature acting on the microvilli serving as taste receptors.

The increase of the response to sucrose was evident when alcohol was added. The cat is, however, not very well suited for such studies, as it has

very few fibres sensitive to sucrose (Pfaffmann, 1941). This part of the investigation should therefore be repeated on an animal which shows a good electrophysiological response to sucrose, like the dog (Andersson *et al.*, 1950).

The effect of alcohol on the response to quinine was most striking. This observation was evident in all animals. It is known from earlier studies that the cat displays a great gustatory sensitivity to quinine (Beidler, 1962) which makes it well suited for such studies. It may therefore be suspected that the observations depend on a competitive interaction on the taste receptors. Similar peripheral interactions triggered by other substances have been described and discussed earlier, e.g. the effect of Gymnema extract on the response to sugar (Andersson *et al.*, 1950 and Beidler, 1962) or between salt and sugar (Andersen, Funakoshi and Zotterman, 1962). Rubin, Griffin and Fischer (1962) reported a similar interaction between quinine sulfate and 6-*n*-propyl-thiouracil in man. From the human point of view it is interesting that the two taste qualities which were most affected often appear as constituents of alcoholic beverages.

This work has been supported by grants from "Svenska Maltadrycksforskningsinstitutet".

REFERENCES

ANDERSEN, H. T., FUNAKOSHI, M. and ZOTTERMANN, Y. 1962. Electrophysiologica. investigation of the gustatory effect of various biological sugars. *Acta physiol scand.* **56,** 362–375.

ANDERSSON, B., LANDGREN, S., OLSSON, L. and ZOTTERMAN, Y. 1950. The sweet taste fibres of the dog. *Acta physiol. scand.* **21,** 105–119.

BEIDLER, L. M. 1953. Properties of chemoreceptors of tongue of rat. *J. Neurophysiol.* **16,** 595–607.

BEIDLER, L. M. 1962. Mechanism of taste receptor stimulation. *Progress in Biophysics and biophysical chemistry.* **12,** 109–151.

DETHIER, V. G. and CHADWICK, L. E. 1947. Rejection thresholds of the blowfly for a series of aliphatic alcohols. *J. gen. Physiol.* **30,** 247–253.

DIAMANT, H., FUNAKOSHI, M., STRÖM, L. and ZOTTERMAN, Y. 1963. Electrophysiological studies on human taste nerves. *Olfaction and Taste.* Editor Y. ZOTTERMAN, Pergamon Press, Oxford. 193–203.

DIAMANT, H., OAKLEY, B., STRÖM, L., WELLS, C. and ZOTTERMAN, Y. 1965. A comparison of neural and psychophysical responses to taste stimuli in man. *Acta physiol. scand.* **64,** 67–74.

DODT, E. 1954. Schmerzimpulse bei Temperaturreizen. *Acta physiol. scand.* **31,** 83–96.

GRÜTZNER, P. 1894. Ueber die chemische Reizung sensibler Nerven. *Tübingen Pflügers Arch. ges. Physiol.* **58,** 101.

HALLENBERG, B. A. 1914. Beiträge zur Kenntnis der alkoholhaltigen Getränke. 1. Untersuchungen über die Geschmacks- und Geruchsschwelle einiger einatomiger Alkohole. *Skand. Arch. Physiol.* **31,** 75–80.

HELLEKANT, G. 1965a. Electrophysiological investigation of the gustatory effect of

ethyl alcohol. I: The summated response of the chorda tympani in the cat, dog and rat. *Acta physiol. scand.* 64, 392–397.

HELLEKANT, G. 1965b. Electrophysiological investigation of the gustatory effect of ethyl alcohol. II: A single fibres analysis in the cat. *Acta physiol. scand.* 64, 398–404.

HELLENKANT, G. 1965c. The effect of ethyl alcohol on non-gustatory receptors of the tongue of the cat. *Acta physiol. scand.* 65, 243–250.

HELLEKANT, G. 1965d. Studies of the effect of ethyl alcohol on the receptors of the tongue. Thesis Stockholm.

HENSEL, H. and ZOTTERMAN, Y. 1951. The response of mechanoreceptors to thermal stimulation. *J. Physiol.* (Lond.) **115,** 16–24.

MYERS, A. K. 1962. Alcohol choice in wistar and G-4 rats as a function of environmental temperature and alcohol concentration. *J. comp. physiol. Psychol.* **55,** 606–609.

PFAFFMANN, C. 1941. Gustatory afferent impulses. *J. cell. comp. Physiol.* **17:2,** 243–258.

RAETHER, M. 1905. Ueber die Einwirkung verschiedener einwertiger Alkohole auf sensible Nerven und Nervenendigungen. Druck von Gebr. Tönnes, Düsseldorf, 1–38.

RENQVIST, Y. 1920. Der Schwellenwert des Geschmackreizes bei einigen homologen und isomeren Verbindungen. *Skand. Arch. Physiol.* **40,** 117–124.

RUBIN, T. R., GRIFFIN, E. and FISCHER, R. 1962. A physico-chemical treatment of taste thresholds. *Nature,* **195,** 362–364.

ZOTTERMAN, Y. 1935. Action potentials in the glossopharyngeal nerve and in the chorda tympani. *Skand. Arch. Physiol.* **72,** 73–77.

GUSTATORY IMPULSES—THEIR NUMBER AND TEMPORAL PATTERNS

MASAMICHI ICHIOKA

Department of Physiology, School of Dentistry,
Tokyo Medical & Dental University

IN THE field of physiology of sensation a number of experiments have been done mainly to record the electrical activity of the peripheral and central nervous systems. There have been performed, on the contrary, only a few investigations in this field based upon the *reizphysiologische* point of view, which, however, together with the recordings of the discharges of impulses, may contribute to a fuller understanding of sensation. (Only a short report communicated by Köpchen and Seller[7] has to do something with this problem.) The purpose of the present paper is to describe experiments on the responsiveness of man, dog and rat to gustatory informations evoked by electrical stimulation of gustatory nerve or natural stimulation of the tongue, giving particular attention to the combined action of the temporal patterns of stimuli and their number with the hope of obtaining some clue to an understanding of the central processing mechanisms of the sensory codes.

EFFECTS OF THE STIMULATION OF THE LINGUAL NERVE UNDER CUTTING AND WITHOUT CUTTING THE MANDIBULAR NERVE ON THE SECRETION OF SUBMAXILLARY SALIVA IN THE DOG[10]

The submaxillary gland and the lingual nerve on one side of the face of dogs, of either sex, weighing between 8 and 16 kg, placed supine under Flaxedil and artificial respiration, following Nembutal-anaesthesia, were exposed after the procedures used by Cyon[2] (Fig. 1) and the nerve was stimulated at S indicated in the figure by a train of 10, 30, 50, 70 and 90 stimuli, each of them being a rectangular pulse of about 0·5 msec duration and of r.r. (repetition rate) of 2, 5, 10, 40, 80 and 200 per sec. The amount of saliva, secreted by reflex from the gland in 30 sec after the cessation of stimulation, was measured by a micropipette of 1×10^{-3} cc graduation,

inserted into the duct of the gland. Supplemental fluids (Dextran) were given as required. Special care was taken to keep the body temperature of animals constant.

One example of the results obtained is shown in Fig. 2, where the quantity of secreted saliva (Q cc) is plotted against both the stimulating r.r. (F per sec) and the number of stimuli (M), each in logarithmic scale. Two things can been seen in the figure: In the first place, the amount of secreted saliva (in logarithmic scale) is nearly directly proportional to the number of stimuli (in the same scale), especially in cases of $F=5$ and 10. Secondly,

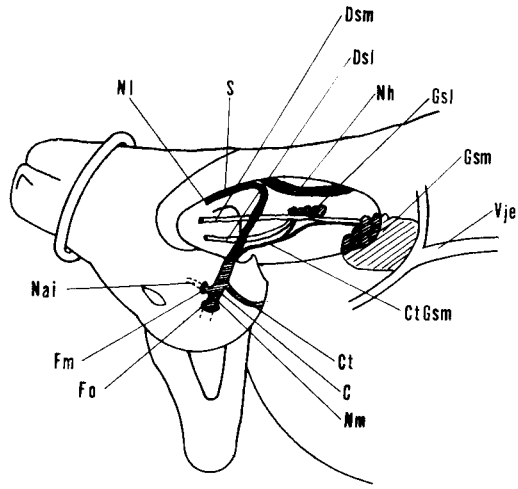


FIG. 1. Semi-schematic representation of the lingual nerve (Nl) and the submaxillary gland (Gsm) in the dog. Dsm: duct of the submaxillary gland, Ct: chorda tympani, Nm: mandibular nerve, CtGsm: submaxillary gland innervating branch of the chorda tympani, Nh: hypoglossal nerve, Vje: external jugular nerve, S and C: in text.

the saliva was most secreted at a stimulus r.r. of 10 per sec, independent of the number of stimuli. It became evident, as a result of all other experiments performed, that the stimulating r.r. of 2–20 per sec are the most powerful ones for the submaxillary secretion, no matter how many stimuli were delivered.

The above results were obtained from the experiments on the reflex due to the lingual nerve stimulation, i.e. on the reflex evoked by stimulating fibres of all the sensory modalities from the tongue (named Exp. Aa, for brevity). In order to eliminate the sensory fibres other than the taste ones, as usually believed, the mandibular nerve was cut at C in Fig. 1. Then, the stimulation of the lingual nerve at S produces the salivary reflex pro-

voked only by the gustatory afferents (named Exp. Ag). One of the results is illustrated in Fig. 3, which shows that the amount of secreted saliva after cutting the mandibular nerve (thin line group) was decreased by a factor of one to three or one to ten or in most cases even much less (in

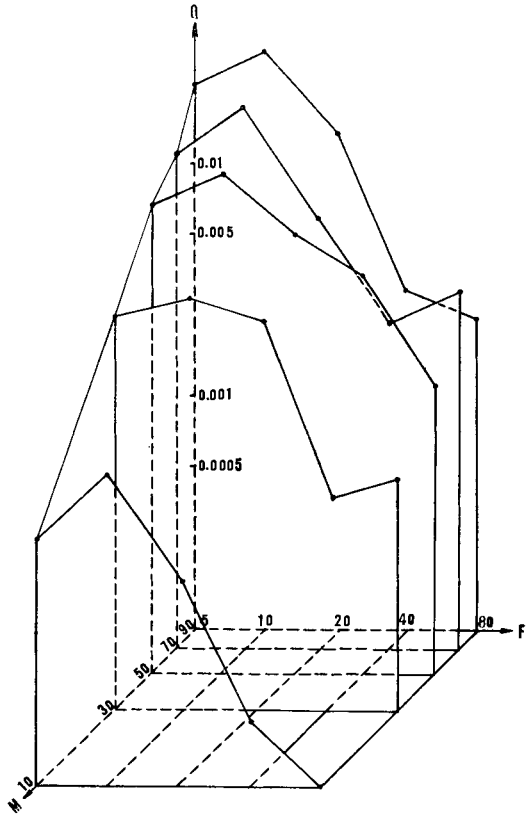


FIG. 2. Relationship between quantity of saliva secreted (Q cc), stimulating r.r. (F per sec) and number of stimuli (M), each one in a logarithmic scale: x-axis, F per sec; y-axis, M; z-axis, Q cc.

about half of all cases no secretion was observed) under all experimental conditions. According to the experiments previously performed by other investigators[3, 4, 11] on the effects of stimulating the *efferent* nerve fibres to the submaxillary gland (named Exp. E), the most effective r.r. of stimulation lay from 10 to 30 per sec. On the other hand, the most effective r.r. was about 2–20 per sec (Exp. Aa) in the present study, where it was intended to investigate input–output relations, stimulating the *afferent* nerve fibres from the tongue and observing effects produced by reflex at

the salivary gland. The experimental conditions of the present study are different from those of the previous ones in some respect, especially in that in the latter no attention was paid to the number of stimuli, which presumably play fairly important roles in the reflex action. The direct comparison between Exp. E and Exp. Aa as well as Exp. Ag, therefore,

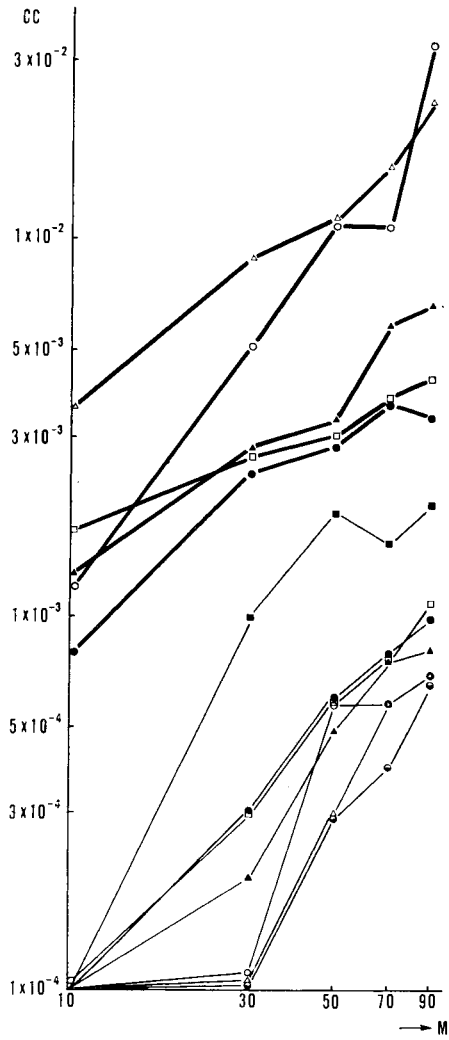


FIG. 3. Quantity of secreted saliva before (thick line group) and after (thin line group) cutting the mandibular nerve. △—△ : 5 per sec, ○—○ : 10 per sec, ▲—▲ : 20 per sec, □—□ : 40 per sec, ●—● : 80 per sec, ■—■ : 2 per sec, ◒—◒ : 200 per sec.

seems to be more or less unreasonable. But the slight, yet distinct, decrease of the optimal r.r. in Exp. Aa in comparison with Exp. E leads us to the presumption that the sensory information reinforces itself by a few to several-fold in the central nervous system. The mechanisms underlying this central processing of sensory codes are interesting and important, but are difficult to clarify at present. The decrease of the amount of the secreted saliva in Exp. Ag as compared with Exp. Aa depends, of course, on the diminution of the number of the afferent fibres. But does the reduction ratio of the quantity of saliva correspond to that of the afferent fibres stimulated in the lingual nerve cases in both? That is also an important problem, the solution of which may throw some light on the input–output relations in the central nervous system.

ELECTRICAL RESPONSES OF THE SUBMAXILLARY GLAND CELLS TO THE GUSTATORY STIMULATION OF THE TONGUE IN THE RAT[6]

Lundberg[8] classified the response types of the submaxillary gland cells of the cat into three groups according to patterns of their responses to the stimulation of the efferent nerves innervating the gland. The investigations carried out by us[6] on the rat have revealed, on the contrary, that

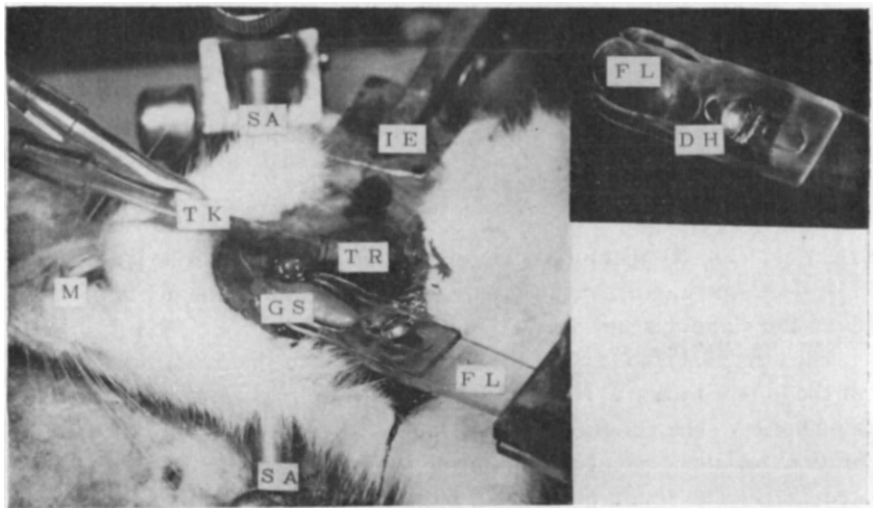


FIG. 4. Experimental arrangement for intracellular recording of the electrical activity of the submaxillary gland in the rat. M: mouth, TK: tracheal cannula, SA: stereotaxic apparatus, GS: submaxillary gland, FL: spoon, DH: half ring.

the electrical responses of the salivary gland cells to the gustatory stimulation recorded by microelectrodes are not so simple as those elicited by means of the efferent nerve stimulation.

The submaxillary gland of the rats (150–250 g), on their back under artificial respiration, was gently freed from surrounding tissues. The gland was fixed by a kind of little spoon and a part of the tunic of the gland was stripped. Both procedures made the microelectrode recording possible

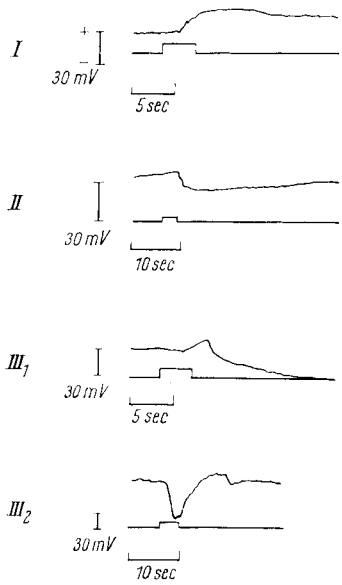


FIG. 5. Three types of action potentials recorded at the submaxillary gland cells in the rat.

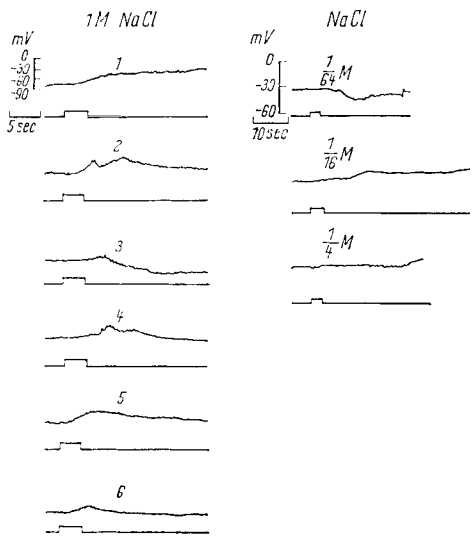


FIG. 6. Responses of the action potentials (type III).

and easy (Fig. 4). The glass microelectrode, filled with 3 M KCl and of 15–30 MΩ resistance, was inserted in most cases up to the depth of 560 μ from the glandular surface.

The electrical responses of the glandular cells to the natural stimulation of the tongue by NaCl, HCl and acetic acid solutions revealed a considerable variety. The variety of their responses in connection with the considerations of the present paper can be summarized as follows (Figs. 5, 6 and 7). (1) The action potentials of the glandular cells investigated were divided into three types according to their direction: Types I and II responded only with the de- and hyper-polarization throughout the whole period of the activity, respectively. All the other forms of the responses,

i.e. such action potentials as those which showed both hyper- and depolarization during the activity, were brought together into type III. (2) Some glandular cells did not respond to repetitive stimulation by a sapid solution even one of the same concentration with action potentials of definite

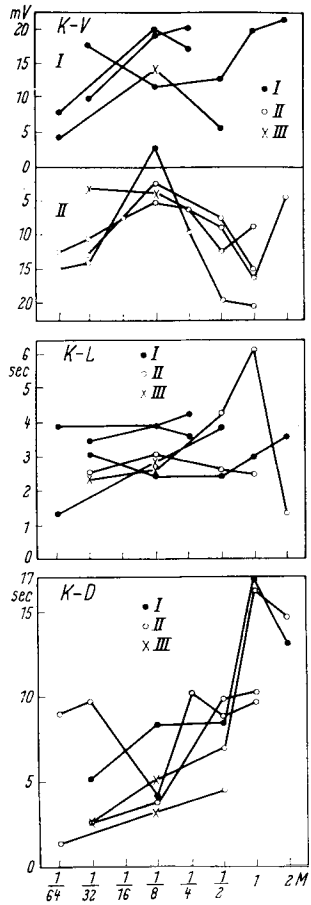


FIG. 7. Effects of NaCl-concentration on the maximal amplitude (K–V curve), on the latency (K–L curve) and on the half decay time (K–D curve). I, II and III mean action potential types.

direction. (3) No relations were observed between the characteristics of the action potentials of the cells, such as maximal amplitude, latency and half decay time, and the stimulus intensity.

Beidler[1] observed that the integrated response recorded from the chorda tympani in the rat increased with the NaCl-concentration reaching the

plateau at 0·5 M (Fig. 8a). He[1] also found that in the case of stimulation with 0·1 M NaCl the impulse frequency attained the steady state value about 2 sec (Fig. 8b). Our investigations, however, as described above,

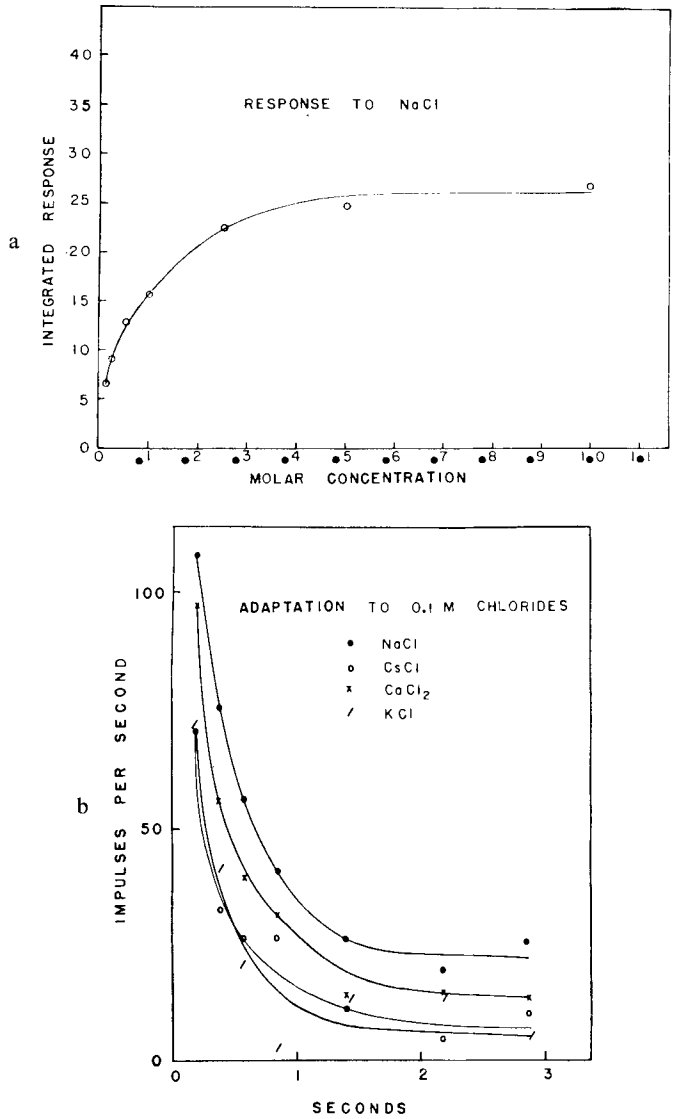


FIG. 8. a. Integrated response recorded on chorda tympani nerve in response to various concentrations of NaCl solution possed over tongue of rat (from Beidler[1]).

b. Adaptation to various 0·1 M chloride solutions possed over tongue of rat (from Beidler[1]).

demonstrated no relationship between the stimulus intensity and the amplitude of the action potentials. Moreover, no correspondence was observed between the form and duration of the electrical activity of the single units of the gland in our investigation and that of the integrated responses led off the peripheral nerve fibres in the rat by Beidler.[1] These discrepancies between the neural activity of the peripheral afferent nerve and the effector events produced by reflex activity mean that there exist certain unknown processes which correlate afferent codes with efferent ones in the central nervous system.

TASTE SENSATION ELECTRICALLY ELICITED[5, 9]

Anodally stimulating the tip of the tongue by means of a decatron stimulator through a glass electrode of about 1 mm end opening diameter by a train of rectangular pulses of varying intensity (V), duration (D), interval (I) and number (M), the taste sensation electrically elicited in man was investigated. Keeping constant two variables out of four, the relations between the other two were examined at the threshold sensation level. Two of the results deserve to be described here in relation to the present theme.

Figure 9[9] shows the relationship between the number of stimuli (M) in an arithmetic scale and the stimulus interval (I msec) in a logarithmic one under constant intensity (V) ($V=5$ volts (a), $V=12$ volts (b)) and constant duration (D) (1 : 0·6 msec, 2 : 1 msec, 3 : 1·6 msec, 4 : 2 msec, in both cases (a) and (b)). As is clearly seen on the figure, the stimulus interval increases in a geometrical progression, while the number of stimuli grows arithmetically, the gradient of the curves ranging in its value from three to more than ten. This means that a three to more than ten times increase in the stimulus interval can be overtaken by only one impulse, so far as the attainment of the threshold electrical taste sensation is concerned.

The next experiment[5] gives the relation between the intensity (V) and interval (I) of stimuli under conditions of a given stimulus duration (D) and number (M) (Fig. 10). The figure denotes that V and I can be correlated approximately as follows:

$$V = k_1\left(1-\exp\left(-\frac{I}{k_2}\right)\right).$$

As is pointed out by the equation, V approaches asymptotically to a definite value, when I becomes longer (in the case of $M=31$, V shows even a tendency to decline, as is distinctly seen in the figure). This means that each

stimulus interval, longer than a definite one, has an equal effect on the initiation of the electrically elicited taste sensation; in other words, a train of stimuli of longer interval produces the same effect, the threshold sensation, no matter how long the interval may be. This means that the afferent code provoked by each stimulus might be processed in the central nervous system.

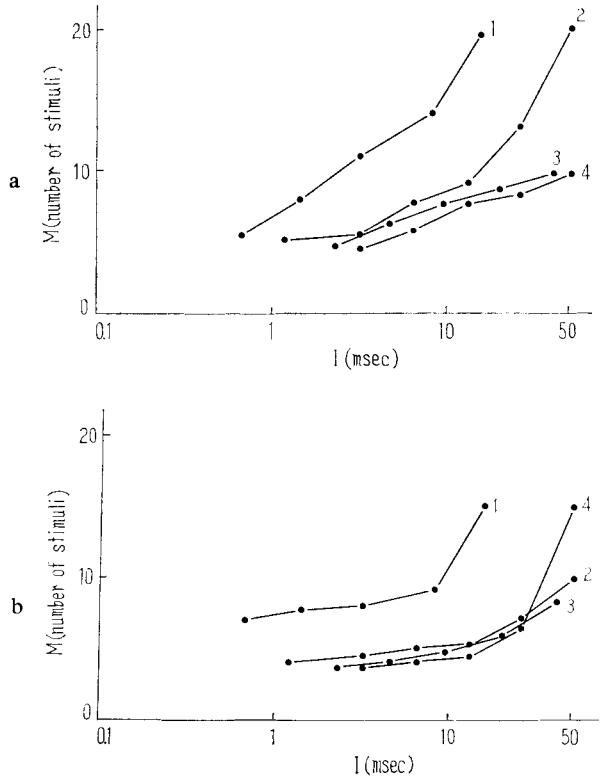


FIG. 9. Relation between stimulus interval (I in msec, in logarithmic scale) and number of stimuli (M, in arithmetic scale). Stimulus intensity, 5 volts (a) and 12 volts (b). 1, 2, 3 and 4 indicate the duration of each stimulus of 0·6, 1, 1·6 and 4·2 msec, respectively.

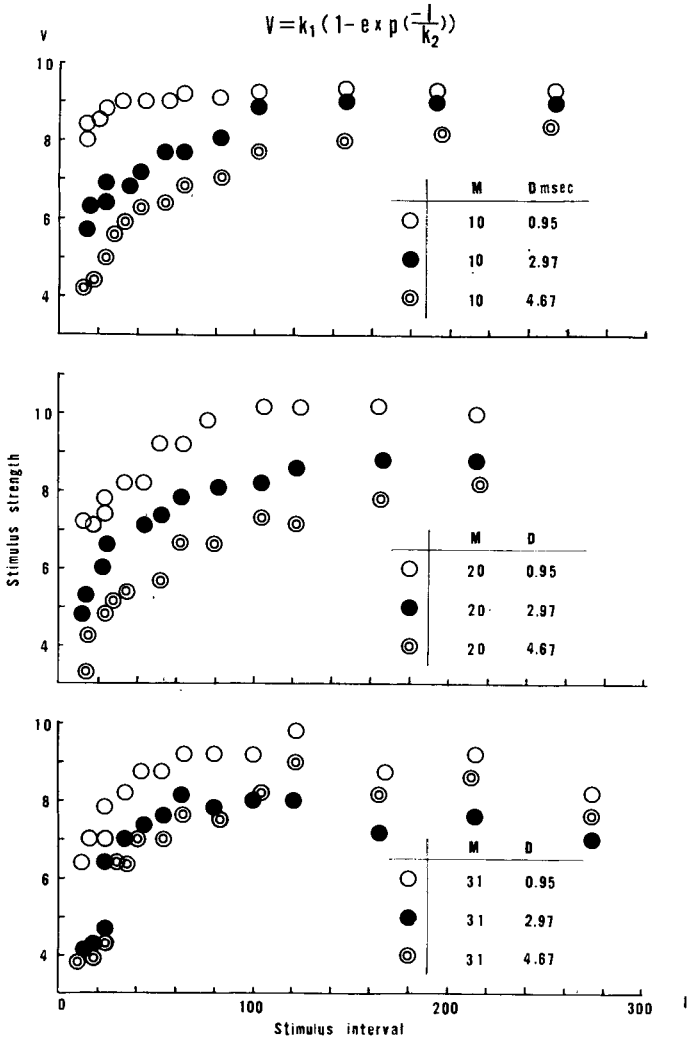


FIG. 10. Relation between stimulus intensity (in volts) and stimulus interval (in msec).

REFERENCES

1. BEIDLER, L. M. Properties of chemoreceptors of tongue of rat. *J. Neurophysiol.* **16,** 595–607, 1953.
2. CYON, E. *Methodik der physiologischen Experimente und Vivisektionen*, 264–274. Rickersche Buchhandlung, Giessen, 1876.
3. DIAMANT, H., ENFERS, B. and HOLMSTEDT, B. Salivary secretion in man elicited by means of stimulation of the chorda tympani. *Acta physiol. scand.* **45,** 293–299, 1959.
4. FUNAKOSHI, M. Neurophysiological studies on the mechanism of the secretion of saliva. *J. Physiol. Soc. Japan*, **19,** 553–561, 1957 (in Japanese).
5. ICHIOKA, M., OHBA, A. and SHIMIZU, H. Versuche über den elektrischen Geschmack. *Zschr. f. Biol.* **113,** 461–471, 1963.
6. ICHIOKA, M., SHIMIZU, H. and SHIMIZU, T. Elektrische Reaktion einzelner Submandibulardrüsenzellen auf salzigen und sauren Geschmaksreiz bei der Ratte. *Zschr. f. Biol.* **115,** 1–8, 1965.
7. KÖPCHEN, H. P. and SELLER, H. Wirkungen des Impulsmusters und Adaptationserscheinungen bei der zentralen Verarbeitung pressoreceptorischer Afferenzen. *Pflügers Arch. f. ges. Physiol.* **283,** R. 57, 1965.
8. LUNDBERG, A. The electrophysiology of the submaxillary gland of the cat. *Acta physiol. scand.* **35,** 1–25, 1955.
9. OHBA, A. An investigation on the mechanism of the initiation of the electric taste in main. *Koku-byo-Zasshi* **30,** 329–343, 1963 (in Japanese).
10. SHIMIZU, H., SAKAMOTO, M., KOMAMURA, T. and MATSUMOTO, K. Secretion of the submaxillary gland in reponse to stimulation of the lingual nerve in dog (in preparation).
11. WAKABAYASHI, T., SATO, Y. and OHSUMI, T. Temporal patterns of nerve stimulation and activity of effector. *J. Physiol. Soc. Japan*, **27,** 138 P, 1965 (in Japanese).

THE AFFERENT INNERVATION OF THE TONGUE OF THE SHEEP

A. IGGO and B. F. LEEK

Department of Veterinary Physiology,
University of Edinburgh

AT THE first International Symposium on Olfaction and Taste, the gustatory receptors in the tongue of the ruminant were described by Kitchell (1963). His results confirmed the general view that the receptors were similar to those found in other mammals. The concentration of different kinds of receptors varied — "salt" responses were most common in the chorda tympani, supplying the anterior two-thirds of the tongue, whereas "bitter", "acid" and "sweet" receptors were more common in the glossopharyngeal nerve, supplying the posterior one-third of the tongue. In the experiments to be described in the present paper, anaesthetised adult sheep have been used and single afferent fibres have been studied in either the chorda tympani/lingual nerve or the glossopharyngeal nerve. Single unit methods were used, because they allow the properties of individual receptors to be examined, avoid the confusion that can arise from whole nerve integrator methods and prevent the contamination of gustatory unit responses by mechano- and thermoreceptor activity. The latter are more likely to prove troublesome with the lingual and glossopharyngeal nerves. On the other hand, the single unit methods are particularly tedious when applied to the mixed nerves, i.e. lingual and glossopharyngeal, because the gustatory afferent fibres are thin and their action potential tends to be masked by the large mechanoreceptor activity. The single unit methods, in particular, make it possible to measure the conduction velocities of the individual afferent fibres and thereby to assess their diameters.

Several features of the afferent innervation of the tongue of the sheep were examined in the present work. These were (1) the receptor properties and diameters of gustatory afferent fibres in the lingual nerves (and chorda tympani) and the glossopharyngeal nerve, and (2) the sensitivity, receptive fields and axon diameters of thermoreceptors and mechanoreceptors.

The afferent nerves were exposed on the left side by a lateral approach.

The left mandible was removed from behind the incisor teeth. To prevent access of fluid from the buccal cavity to the exposed nerves, and vice versa, the dento-alveolar mucosal margins, exposed by removal of the lower jaw, were sutured. The nerves were covered with liquid paraffin B.P. which filled a pool formed by tying the skin margins to a metal ring. The methods for dissection of single units and for measuring conduction velocities were as described for the vagus nerve of the cat (Iggo, 1958). Particular trouble was caused by the constant flow of saliva from the parotid gland, by tissue fluid and, possibly, lymph. The fluid was removed by aspiration.

GUSTATORY AFFERENT UNITS

A total of forty-five single units were examined together with a much larger number of multi-unit preparations. The single units could be classified into the five basic types which are familiar from the earlier work of Zotterman and his colleagues and of Pfaffmann (1941). In agreement with Kitchell no positive response to distilled water (39°C) could be found. Instead, any background activity was reduced or abolished when water was made to flow over the tongue. To avoid interference from thermoreceptors, distilled water at 39°C was used to wash the tongue. However, even this precaution was not always satisfactory since there were some "warm" receptors that were excited by fluid at this temperature. A sequence of test solutions such as water at 39°C, 0·5 M NaCl at 39°C, water at 20°C was sufficient to distinguish this type of unit. All the test solutions were made up in distilled water.

"SALT" UNITS

The afferent fibres were present in the chorda tympani and glossopharyngeal nerve. They were excited by NaCl solutions. The threshold concentrations were less than 0·1 M and at this concentration only a weak, irregular discharge of impulses was elicited. A strong solution, e.g. 0·5 M NaCl gave a high frequency discharge that lasted several seconds until the solution was washed off the tongue. Only three examples of this type of afferent unit were examined as single units. (Fig. 4a).

"ACID" UNITS

Afferent units with a heightened sensitivity to acid solutions (the standard test solution was 0·2 M acetic acid, pH 2·5) were found in both the chorda tympani and the glossopharyngeal nerves (Fig. 1). These units

were not excited by the other test solutions (0·5 M NaCl, 0·5 M sucrose, 0·01 M quinine hydrochloride). The threshold pH was not established for any of the six units isolated.

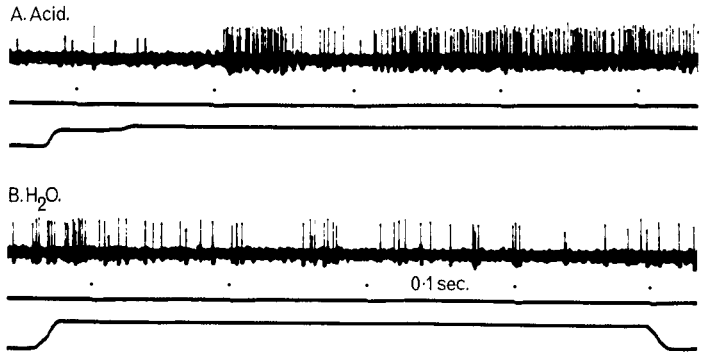


FIG. 1. The afferent discharge recorded from a thin nerve strand dissected from the left lingual nerve when a 0·2 M acetic acid solution (39°C) was allowed to flow over the rostral part of the tongue (A). This discharge, which persisted for several seconds after stopping the flow of the acid, could be reduced, or even abolished, by allowing distilled water (39°C) to flow over the tongue (B). An afferent discharge was not excited by salt, sweet or bitter test solutions. The afferent fibre was therefore innervating an "acid" receptor. Upper tracing—electrical record of afferent discharge, middle tracing—0·1 sec time marker, lower tracing—an upward movement of the trace indicates application of fluid.

"SALT/ACID" UNITS

The majority (23/45) of the single units examined were in this category. Both 0·5 M NaCl and 0·2 M acetic acid provoked a discharge of impulses (Fig. 2). The relative effectiveness of the solutions varied from a ratio of 1:1, salt:acid to ratios that were estimated at 1:4 to 4:1. When nerve strands containing many taste afferent units were recorded from, the largest discharge of impulses was obtained with the 0·5 M NaCl and the 0·2 M acetic acid. This was the case for both the chorda tympani and the glossopharyngeal nerves, in agreement with Kitchell's (1963) published records for the goat glossopharyngeal whole nerve preparation. Subdivision of these thick nerve strands in our experiments yielded single unit preparations, or strands in which the activity of single units could be distinguished. The units were most often of the "salt/acid" kind but occasionally, either specific "salt" or "acid" units were found.

These three categories of units were the most common "taste" units in the chorda tympani.

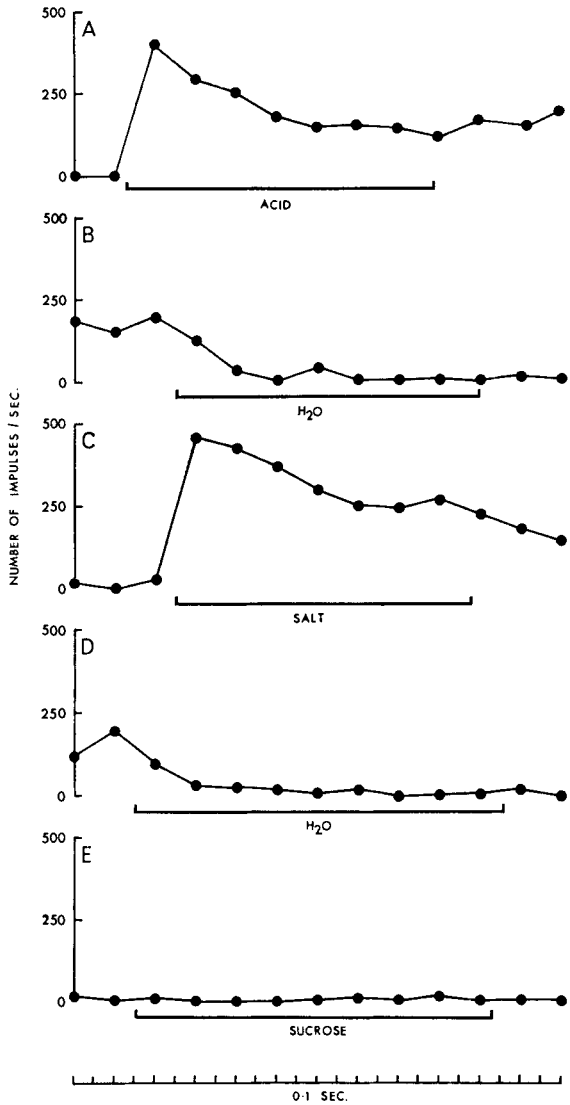


FIG. 2. The afferent discharge recorded from a thin nerve strand dissected from the left lingual nerve when 0·2 M acetic acid (A) and 0·5 M sodium chloride (C) solutions were applied to the rostral part of the tongue. The discharges, which persisted for several seconds after the flow of fluid had stopped, were much reduced when distilled water flowed over the tongue (B and D). An afferent discharge was not excited by 0·5 M sucrose (E) or 0·01 M quinine hydrochloride solutions. The gustatory receptor innervated by an afferent fibre in this strand was, therefore, excited by both salt and acid stimuli. The records A–E are sequential.

The glossopharyngeal nerve, in addition to numerous "salt" and "acid" and "salt/acid" units, also contained afferent fibres strongly excited by the other two test solutions, 0·5 M sucrose and 0·01 M quinine hydrochloride.

"SWEET" UNITS

The 0·5 M sucrose solution, in general, did not excite units in the lingual nerve/chorda tympani, in agreement with Kitchell's results. Conversely

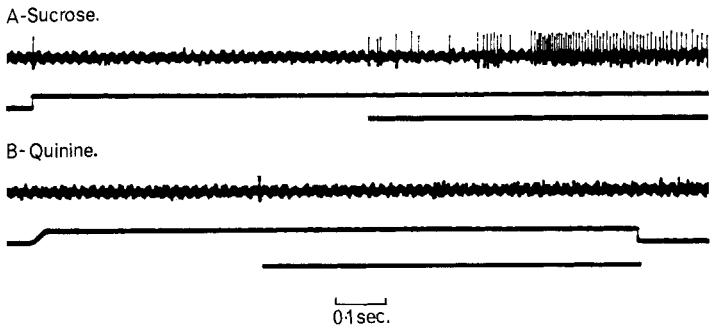


FIG. 3. The afferent discharge (upper tracing) in a single fibre dissected from the left glossopharyngeal nerve when 0·5 M sucrose (A) test solutions were applied to a circumvallate papilla (indicated by the raised part of the middle tracing) which was then moved mechanically with fine glass probe (indicated by the solid line of the lower tracing). Quinine hydrochloride (0·01 M) test solutions combined with movement of the papilla did not excite the unit (B). Sodium chloride and acid solution were also ineffective. The single fibre from which this record was made was innervating a gustatory receptor excited specifically by "sweet" stimuli. Since movement of the papilla was necessary before the sucrose solution excited a response, it seems likely that the receptor occupied a position deep in the groove of the papilla.

eight "sweet" units were isolated from the glossopharyngeal nerve. They were highly specific to sucrose and, to a lesser extent, fructose and were not excited by any of the other test solutions (Fig. 3).

"BITTER" UNITS

The 0·01 M quinine hydrochloride solution was ineffective in exciting afferent fibres in the lingual nerve or chorda tympani, except for one "salt" fibre in which there was a weak discharge. The quinine solution was effective on the back of the tongue, exciting fibres in the glossopharyngeal nerve. Only five single units were isolated which gave a response specifically to quinine but gave no response to the other test solutions. In several multi-

fibre preparations, however, quinine elicited a very vigorous and prolonged discharge and some of this activity could be distinguished as probably arising from quite specific units. The failure of quinine solution to excite any of the "salt", "acid", "salt/acid" or "sweet" single units also suggests that "bitter" units had a highly selective sensitivity.

RECEPTIVE FIELDS

The "taste" units in the lingual nerve and chorda tympani were restricted to the rostral part of the tongue (Fig. 7), as would be expected from the innervation of the tongue (see Appelberg, 1958). The afferent units were excited by allowing the test solutions to flow over the dorsal surface of the tongue and it was not necessary to supplement the flow with mechanical stimulation. Because of this ready excitability, the "taste" unit responses

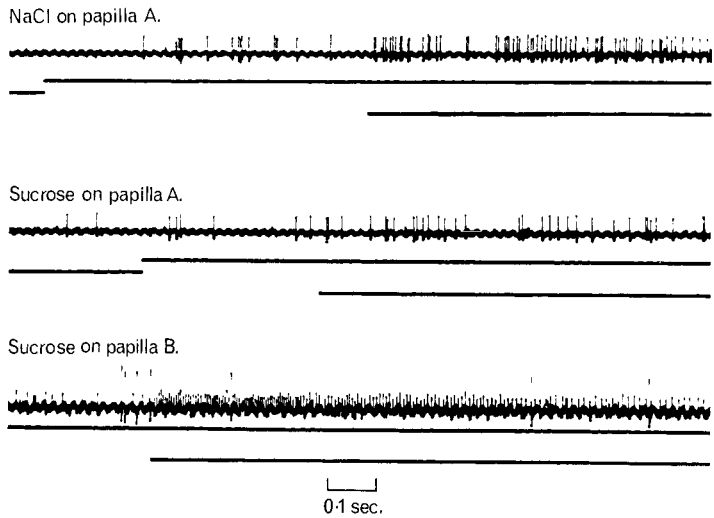


FIG. 4. The discharge of impulses in two taste units recorded from a thin nerve strand dissected from the left glossopharyngeal nerve. When 0·5 M sodium chloride solution was applied to papilla A there was a low frequency discharge which increased markedly when the circumvallate papilla was moved with a fine glass probe (lower signal trace). 0·5 M sucrose solution applied to the same papilla caused only a slight increase in the afferent discharge. This might be due to residual sodium chloride solution. When 0·5 M sucrose solution was applied to papilla B there was a very low frequency discharge in the second unit. This was markedly increased by moving the papilla. Sodium chloride solution did not excite this latter unit. Acid and bitter solutions did not excite either unit. Thus papilla A contains a specific "salt" receptor and papilla B a "sweet" receptor (signal markers as in Fig. 3). The amplification of the afferent discharge was increased two-fold in the last record.

were not found to be associated with any particular macroscopic structures in the tongue.

In contrast, the "taste" units in the glossopharyngeal nerve were associated with the conspicuous circumvallate papillae in the posterior third of the tongue (Fig. 7). Appelberg (1958) and Kitchell (1963) have previously reported that, in order to obtain excitation of the glossopharyngeal "taste" fibres, it is necessary to combine mechanical and "taste" stimuli. In Kitchell's records the combined response is evident as a large deflection (presumably due to the excitation of the large mechanoreceptor fibres) followed by a more slowly rising and falling discharge of variable amplitude and duration according to the kind of solution flowing over the tongue. In the present experiments it was found that a steady, slow flow of a test fluid over the tongue did not excite, or had only a very weak excitatory action (Fig. 4). When this flow was combined with brushing over the circumvallate papillae, there was, with the appropriate test solution, a vigorous discharge that ceased a few seconds after the mechanical stimulation stopped. Brushing alone did not elicit a discharge. An equally effective method was to direct a jet of the appropriate test solution directly at a circumvallate papilla. When this was done there was a vigorous discharge of impulses. In strands containing only "taste" units, this latter method was preferred, since it avoided the risk of injury to the mucosa caused by repeated stroking or rubbing. The glossopharyngeal responses, when tested with the jet stream of fluid, were always associated with the circumvallate papillae, and even, on occasion, with a single papilla. An even more defined method consisted of moving a circumvallate papilla with a fine, blunt-ended glass probe at the same time as test solutions were flowed onto the tongue (Fig. 4). In this way it was possible to decide exactly which papillae were being excited and thus innervated by a particular afferent nerve fibre.

DIAMETER OF THE "TASTE" AFFERENT FIBRES

The chorda tympani in the sheep contains many axons ranging in diameter between 2 and 8μ (Kitchell, 1963) as well as numerous non-myelinated fibres. In the present experiments the conduction velocities of 45 "taste" fibres were measured (Fig. 5), using the collision method (Iggo, 1958). Twenty-seven units which had been classed according to the sensitivity of the receptors were measured. The range of conduction velocities was 5 to 30 m/sec which corresponds to diameters of 1 to 5μ using the factor of 6 for conversion (Hursh, 1939). The sample is too small for very useful comparisons to be made for the different kinds of taste units but

it is noteworthy that all the higher conduction velocities, i.e. thicker fibres, were either "acid" or "salt/acid" units (Fig. 5A), and that these were the types most readily isolated. There were only five "sweet" units (5 to 15 m/sec), two "salt" units (8 and 11 m/sec) and two "bitter" units (5 and 16 m/sec). If these conduction velocities were characteristic of the "sweet", "salt" and "bitter" units, then their relative scarcity in our

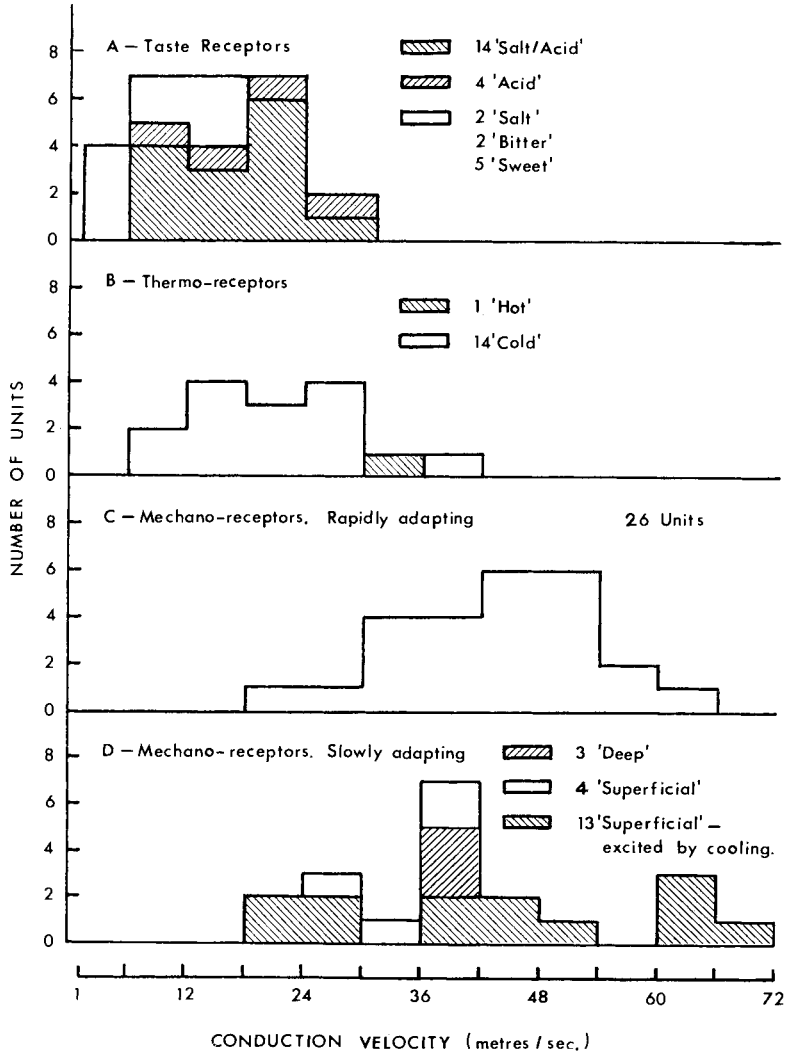


FIG. 5. Histograms to show the conduction velocities of afferent fibres dissected from the lingual/chorda tympani and glossopharyngeal nerves. Further details are given in the text.

sample, could be attributed to their small size. This would increase the difficulty of isolating them as single units, even when they were present in thick nerve strands before subdivision. In Kitchell's paper both quinine and sugar evoked responses comparable with those obtained with acid solutions, so that we expected to have no difficulty in finding the appropriate single units. The large response with salt, with the whole nerve technique, although due in part to the excitation of "salt/acid" units, may also be due to the excitation of "salt" units.

None of the "taste" units were found to be supplied by non-myelinated axons, but the conditions for dissection of the nerve trunks were difficult and the absence of non-myelinated units from our results may be due to this factor. These directly measured conduction velocities are in agreement with previous estimates of the size of the "taste" afferent fibres (Zotterman, 1935) which were based on action potentials spike amplitude.

No unexpected results have emerged in this work, which confirms the existence, in the sheep, of "taste" afferent units which can be grouped into classes on the basis of their relative sensitivity in salt, acid, sweet and bitter solutions. There is some slender evidence that the "acid" and "acid/salt" units had the largest afferent fibres. In some experiments, fluid obtained from the rumen was tested. It had an excitatory effect on "salt", "acid/salt" and "acid" units but did not bring up any additional activity in other categories of units.

THERMORECEPTORS

Numerous "cold" fibres were found in the lingual nerve, chorda tympani and glossopharyngeal nerve. The units were apparently unaffected by the chemical composition of fluids flowing over the tongue and were not excited by mechanical stimulation. They were in all respects, therefore, similar to the "cold" fibres described by Zotterman and his colleagues in the dog and cat. There was an increase in the rate of discharge when the tongue was cooled, and a steady discharge at constant temperatures.

The conduction velocities of fifteen thermoreceptors with afferent fibres in the lingual nerve and chorda tympani were measured and ranged from 7 to 40 m/sec (Fig. 5B). The majority were between 12 and 30 m/sec corresponding to diameters of 2–5 μ. Previous estimates of fibre size, based on spike amplitude comparisons (Zotterman, 1935), also indicated that the thermal afferent fibres in the cat were thin and myelinated, (A δ fibres).

The existence of "warm" fibres in the tongue has been disputed (Dodt and Zotterman, 1952; Boman, 1958). The present experiments confirm the

view that they are much less common than the "cold" fibres. Only one unit was found but its response was strikingly different from the "cold" fibres. It was excited by a rise in temperature of the surface of the tongue. The conduction velocity of this afferent fibre in the lingual nerve was 31 m/sec.

The afferent fibres supplying the "taste" receptors and the thermoreceptors thus have a similar range of diameters, with the majority falling between 2 and 5μ. For this reason, electrical stimulation of any of the afferent nerves supplying the tongue will fail to discriminate between "taste" and thermal afferent fibres, except that there were, in our sample, relatively more "taste" fibres below 12 m/sec, i.e. 2 μ diameter.

MECHANORECEPTORS

The presence of thick, myelinated afferent fibres in the lingual and glossopharyngeal nerves is a familiar and usually unwelcome fact for electrophysiologists studying "taste" by recording from the primary afferent fibres. This is because the high sensitivity to mechanical stimulation and the large action potentials may cause masking of the response evoked in taste afferent fibres by the flow of solutions over the tongue. This interference is a particular source of difficulty when the integrator methods are used.

In the present investigation, incidental observations were made on the mechanoreceptors. Three categories were recognised:

(a) rapidly-adapting mechanoreceptors in the mucosa; when carefully controlled mechanical stimuli were used there was a brief burst of impulses as the probe pressed on the skin and a further discharge when the probe was removed,

(b) slowly-adapting mechanoreceptors in the mucosa, from which a steady discharge of impulses could be evoked by steady pressure on the tongue (Fig. 6). The dynamic phase of the response was much less conspicuous than with the cutaneous touch afferent units (slowly-adapting, Type II). The mechanical sensitivity of these endings was strongly influenced by the dryness of the surface of the tongue, and, unless the tongue was kept moistened, the response to mechanical stimulation declined,

(c) slowly-adapting receptors with a high mechanical threshold. Some of these afferent units were excited by stretching the tongue and were not excited by direct mechanical stimulation unless there was evident distortion of the surface of the tongue, particularly the ventral surface.

The conduction velocities of a sample of these mechanoreceptors were

measured. The velocities ranged from 18 to 78 m/sec (Fig. 5 C and D), corresponding to diameters of 3 to 12 μ. The majority of the rapidly adapting units fall between 30–54 m/sec, in the middle range of velocities. The superficial slowly-adapting units covered the full range of velocities with no obvious preferred diameter, whereas the third type of unit, the more deeply situated, slowly-adapting units, had fibres in the range 36–41 m/sec.

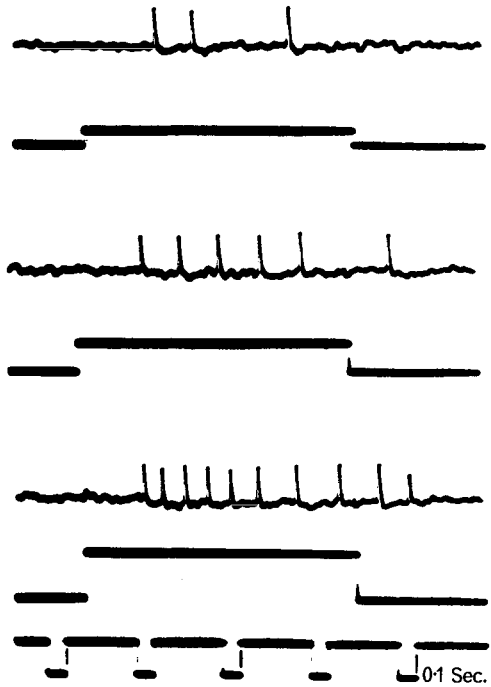


FIG. 6. The response of a slowly-adapting mechanoreceptor when a fungiform papilla was stimulated mechanically by an electrically driven probe with progressively greater displacements (indicated by lower tracing). These slowly-adapting receptors in the fungiform papilla also were excited when they were cooled. The response was abolished by excising the fungiform papilla.

THERMAL SENSITIVITY OF THE MECHANORECEPTORS

Only the superficial, slowly-adapting mechanoreceptors displayed any sensitivity to the temperature of fluids flowing over the tongue. If the discharge of a mechanoreceptor, identified by its sensitivity to touch or pressure on the tongue, increased or appeared when the tongue surface was cooled by the flow of water at 15°C, it was usually a slowly-adapting unit. The rapidly-adapting mechanoreceptors were not excited and the

slowly-adapting "deep" units were generally unaffected. This pattern of response corresponds very well with the thermal responses of mechanoreceptors in the cat's tongue, reported by Hensel and Zotterman (1951), who found that only a small proportion of the mechanoreceptors were excited by low temperatures, and that those that were, gave a brief discharge, lasting several seconds, during slow cooling of the tongue. It is not evident from their paper whether they distinguished the rapidly-adapting units from the slowly-adapting units. This difference in rate of adaption seems to underlie the response to sudden cooling, both in the tongue as well as the cutaneous mechanoreceptors. The present results distinguish the lingual mechanoreceptors on the basis of the functional properties of the receptors rather than on the diameter of the fibres. Hensel and Zotterman (1951), suggested, on the basis of spike heights, that the pressure receptors sensitive only to mechanical stimulation had larger axons (12–15 μ), than those sensitive to both thermal and mechanical stimuli which had thinner axons (8–10 μ). The present work has established, by direct measurement of conduction velocities, that the thermally insensitive units have diameters ranging from 4 to 11 μ, and that the slowly-adapting, temperature-sensitive mechanoreceptors range in diameter from 3 to 13 μ.

RECEPTIVE FIELDS

The receptive fields of the afferent fibres in the lingual nerve and chorda tympani were examined in greater detail than the glossopharyngeal because of the greater accessibility of the rostral end of the tongue. The distribution of receptive fields is shown in Fig. 7, which summarizes the results of 6 experiments. There is a very obvious crowding of the receptive fields to the tip and the lateral border of the dorsal surface of the tongue. Very few units were found on the under-surface and those were deep, slowly-adapting receptors. On the dorsum there appears to be a complete intermingling of the different kinds of receptors. The "taste" receptors were not usually localized with sufficient precision to be entered on the diagram. They were all on the dorsal surface.

In some of the later experiments the exact site of mechanoreceptors was established by punctate stimulation with fine probes. The rapidly-adapting units sometimes had very restricted receptive fields, i.e. spot-like, but other units appeared to end in an area as large as 1–2 cm^2. The endings were in the mucosa and the response of an afferent fibre to "natural" stimulation could be abolished by excising a small piece of the mucosa. The slowly-adapting units with spot-like receptive fields were most often

associated with the fungiform papillae. In a sample of seven units the discharge in each afferent fibre could be elicited from only one fungiform papilla and when the papilla was excised the response was abolished. The results do not allow us to say whether each fungiform papilla was innervated by only one slowly-adapting afferent unit. This association of a

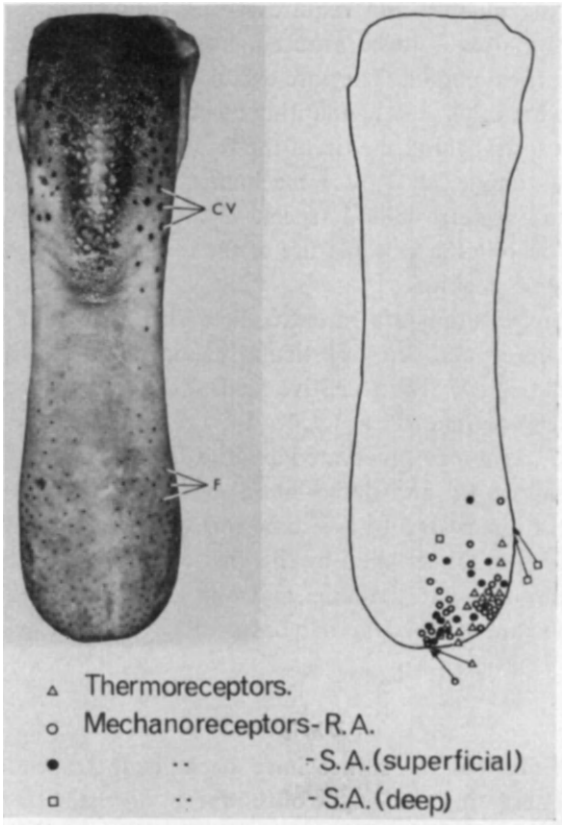


FIG. 7. The dorso lateral surface of the sheep's tongue contains fungiform papillae (F) which are particularly numerous on the tip and circumvallate papillae (C.V.) which are restricted to the caudal region. The rostral two-thirds of the tongue is innervated by the lingual nerve and the receptive fields of thermoreceptors and mechanoreceptors whose afferent fibres were dissected from the left nerve are shown on the right-hand diagram. Apart from a few slowly-adapting mechanoreceptors situated deep in the tongue musculature about 5 cm behind the tip, the remaining receptors were concentrated on the dorsum near the tip and lateral borders. Most of the superficial slowly-adapting (S.A.) mechanoreceptors were located in fungiform papillae. The rapidly-adapting (R.A.) mechanoreceptors and the thermoreceptors were not found to be associated with any particular macroscopic structures.

slowly-adapting mechanoreceptor with a distinctive structural element is similar to the cutaneous "touch corpuscle" in hairy skin (Iggo, 1963), except that the fungiform papilla is a more complex structure, and may contain taste buds as well as rapidly-adapting mechanoreceptors.

There are other slowly-adapting mechanoreceptors but these differ in their response characteristics from those that end in the fungiform papillae. Some are in the mucosa and require strong stimulation if a persistent discharge of impulses is to be aroused, but the receptive field does not include a fungiform papilla. They are excited by cooling the surface of the tongue. There are other slowly-adapting mechanoreceptors situated in our sample, about 5 cm behind the tip of the tongue. These responded only to stretching the tongue and gross mechanical distortion of the tongue particularly on its ventro-lateral aspect. We assumed, therefore, that they were situated deep in the musculature of the tongue and might have a rôle in sensing tongue position.

The thermoreceptors had a receptive field similar to that of the superficial mechanoreceptors, being particularly evident on the tip and dorso-lateral borders (Fig. 7). The receptive field of each unit was probably less than one square centimetre.

The "taste" receptors innervated by the lingual nerve likewise were concentrated at the tip and dorso-lateral borders of the tongue, but the size of the field innervated by one unit was not measurable. Conversely, the "taste" receptors innervated by the glossopharyngeal nerve were confined to the circumvallate papillae and one afferent "taste" fibre might innervate either one or two papillae only.

SUMMARY

Electrophysiological recordings have been made from single afferent nerve axons innervating the tongue of the sheep, dissected from the chorda tympani/lingual and glossopharyngeal nerves. This method allowed the independent investigation of the properties of gustatory, thermoreceptor and mechanoreceptor units. Although gustatory responses were found in small fibres (conduction velocity—5–30 m/sec) in both nerves, "taste" units in the chorda tympani/lingual nerve innervating the rostral region of the tongue were sensitive only to "salt and acid", "salt" and "acid" stimuli. "Taste" units in the glossopharyngeal nerve, on the other hand, were sensitive to "salt and acid", "salt", "acid", "sweet" or "bitter" stimuli, when test solutions were applied to a circumvallate papilla at the same time as it was being moved mechanically. Thermoreceptor responses

were obtained from all regions of the tongue and, with one exception, these units were excited by a fall of temperature. These afferent fibres were small, having conduction velocities ranging from 6 to 42 m/sec. Four types of mechanoreceptors were found: (a) a rapidly-adapting type with a diffuse receptive field and large afferent fibres (conduction velocities—11–66 m/sec); (b) a slowly-adapting type with a diffuse, superficial receptive field (conduction velocities — 24–42 m/sec); (c) a slowly-adapting type with a punctate receptive field restricted to the fungiform papillae and excited by cooling (conduction velocities — 18–72 m/sec) and (d) a deeply situated slowly-adapting type stimulated by stretching the tongue or by gross distortion particularly of its ventro-lateral surface (conduction velocities — 36–42 m/sec).

ACKNOWLEDGEMENT

We wish to thank the Agricultural Research Council for financial assistance.

REFERENCES

APPELBERG, B. 1958. Species differences in taste qualities mediated through the glossopharyngeal nerve. *Acta physiol. scand.* **44,** 129–137.

BOMAN, K. K. A. 1958. Elektrophysiologische Untersuchungen über die thermoreceptoren der Gesichtshaut. *Acta physiol. scand.* **44,** Suppl. 149, 1–79.

COHEN, M. J. S., HAGIWARA, S. and ZOTTERMAN, Y. 1955. The response spectrum of taste fibres in the cat: A single fibre analysis. *Acta physiol. scand.* **33,** 316–332.

DODT, E. and ZOTTERMAN, Y. 1952. Mode of action of warm receptors. *Acta physiol. scand.* **26,** 345–357.

HENSEL, H. and ZOTTERMAN, Y. 1951. The response of mechanoreceptors to thermal stimulation. *J. Physiol.* **115,** 16–24.

HURSH, J. B. 1939. Conduction velocity and diameter of nerve fibres. *Amer. J. Physiol.* **127,** 131–139.

IGGO, A. 1958. The electrophysiological identification of single nerve fibres, with particular reference to the slowest conducting vagal afferent fibres in the cat. *J. Physiol.* **142,** 110–126.

IGGO, A. 1963. New specific sensory structures in hairy skin. *Acta neuroveg.* **24,** 175–180.

KITCHELL, R. L. 1963. Comparative anatomical and physiological studies of gustatory mechanisms. *Olfaction and Taste.* Vol. 1, Ed. ZOTTERMAN, Pergamon Press, London.

PFAFFMANN, C. 1941. Gustatory afferent impulses. *J. cell. comp. Physiol.* **17,** 243–258.

ZOTTERMAN, Y. 1935. Action potentials in the glossopharyngeal nerve and in the chorda tympani. *Skand. Arch. Physiol.* **72,** 73–77.

ANION INFLUENCES ON TASTE RECEPTOR RESPONSE

LLOYD M. BEIDLER

Department of Biological Science, Florida State University
Tallahassee, Florida

INTRODUCTION

It has been shown that the cation has an excitatory effect on taste receptors and that the anion effect is of an inhibitory character (Beidler, 1954). It is the purpose of this paper to further examine the influences of anions on taste receptor responses with particular attention to both the inhibitory effect on the so-called "water response" and the response to weak acids.

Before considering the experimental data, it is advisable to discuss those properties of membranes that are important to a better understanding of taste receptor stimulation. This is done with the realization that little is known concerning the structure of the membrane of the taste receptor microvilli. It is thus necessary to consider membranes of other types of cells and then generalize to the specific case of taste receptors.

GROSS STRUCTURE OF TYPICAL CELL MEMBRANE

The rapid penetration of lipid-soluble molecules into cells suggests that cell membranes contain lipids which play an important role in controlling the passage of various molecules into the cell. Small aqueous-soluble molecules also penetrate rapidly and thus the concept of small holes in the lipid membrane was introduced. In 1925, Gorter and Grendel extracted the lipids from red cell membranes (red cell ghosts) and determined that there was enough lipid to twice cover the surface of the normal red cell, suggesting that the membrane was composed of a biomolecular lipid layer. Measurements of the electrical capacitance of the membranes of a large number of different types of cells showed that all were about 1 mfd./cm^2, again agreeing that a thin layer of low dielectric substance, such as a lipid,

separates the cell interior from its outside environment (Fricke, 1925; Cole, 1949).

Measurements of the surface tensions of cell membranes indicate that the outer layer of the membrane is not lipid but possibly protein. Optical polarization studies of the myelin membrane showed that the lipids were oriented radially and the protein molecules tangentially (Schmitt and Bear, 1939). These observations led Davson and Danielli (1943) to model the cell membrane as two layers of lipid molecules oriented radially to the

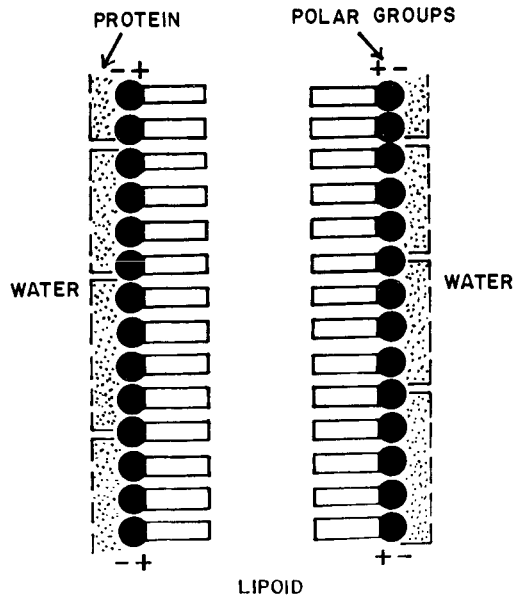


FIG. 1. Davson and Danielli (1943) model of structure of cell membrane.

membrane surfacewith the hydrophobic ends facing each other and the hydrophilics loosely bound to a small layer of protein (see Fig. 1). The gross composition of red cell membranes consists of 50% protein and 28% lipid on a dry weight basis (Williams, *et al.*, 1941). The lipid is 65% phospholipid and 24% cholesterol. The nerve myelin on the other hand, consists of 75% lipid and 25% protein, the lipid being 32% phospholipid and 20% cholesterol (Norton and Autilio, 1965). The amino acid composition of myelin protein is given in Table 1. Note the greater number of anionic groups than cationic. The detailed amino acid and lipid compositions of a given cell type may differ in various animal species (De Gier and Van Deenen, 1961).

TABLE 1. AMINO ACID COMPOSITION OF MYELIN PROTEIN

Polar		Apolar	
Cationic — 12%		Aliphatic — 40·79%	
Lysine	5·75	Glycine	10·87
Histidine	2·30	Alanine	9·65
Arginine	3·95	Valine	5·91
Anionic — 20·19%		Leucine	8·76
Aspartic	6·13	isoLeucine	4·52
Glutamic	7·14	Methionine	1·08
Tyrosine	3·14		
Cysteine	3·78	Cyclic — 10·21%	
		Phenylalanine	4·02
Hydroxyl — 16·81%		Tryptophan	5·08
		Proline	1·11
Threonine	6·41		
Serine	10·40		

From Vandenheuvel, 1965.

The cell's limiting membrane is but one of many; the membranes of cytoplasmic structures contribute great surface areas. Although these membranes may look similar, they have large functional differences. Similarly, the electron micrographs of plasma membranes of different types of cells or the same cell type in different species, all show the usual trilaminar structure, but the membranes' functions are quite diverse. Thus, the membrane skeletons may be the same but the particular lipid, protein, polysaccharide and enzyme molecules making up the membranes are quite different and lead to different functions. The plasma membrane need not be uniform in structure all around a given cell but may vary at different areas as indicated by histochemical studies. Projecting to the taste cell, the microvillus membrane may be quite different from the contiguous membrane at the side of the cell, which comes in very close contact with nerve endings.

The microvillus of a taste receptor contains a unit membrane 70–100 Å thick. Below the base of the microvilli, the limiting membranes of adjacent receptor cells fuse to form a trilaminar membrane of 150–180 Å thick. Slightly below this region the receptor cell membranes are once again separated by extracellular fluid. Thus, fusion of the receptor cells near the microvilli prevents taste solutions applied to the surface of the tongue from coming in contact with the extracellular space and bare nerve endings contained within the taste bud. The plasma membranes of the nerve axon

and the receptor cell are in close contact with numerous vesicles nearby (Gray and Watkins, 1965).

The membranes of some cells also contain polysaccharides, particularly sialic acid. The active groups of sialic acid, such as acetylneuraminic acid, contribute to the total surface charge of the red cell which determines its electrophoretic mobility as well as some of its antigenic properties.

CELL MEMBRANE ASYMMETRY

The most important function of a membrane is the maintenance of an asymmetry in function between the inside and outside of the cell. For example, a membrane may separate the high potassium ion concentration within the cell from the much lower potassium concentration outside. The Davson–Danielli model would indicate that the inside and outside surfaces of a membrane are symmetrical. However, divalent ions are quite toxic to nerve membrane when applied to their inside but not when applied to their outside. Tetrodotoxin exerts its effect only when applied externally and has no effect when injected internally (Narahashi, 1965). Thus, the membrane itself may have structures which give different functional properties to its inner and outer surfaces.

TASTE CELL DYNAMICS

The cell membrane is not necessarily a static structure. The microvilli of the taste cells come in contact with a wide variety of environmental conditions, many of which we believe to be quite injurious to their cell membranes. It is reasonable to expect that there might be some self-repair of the microvilli membranes. Evidence for membrane synthesis comes from the study of pinocytosis, phagocytosis and secretion. During phagocytosis and pinocytosis part of the plasma membrane of a cell is utilized to form a vesicle which then passes to the interior of the cell. In order to maintain a constant volume, the cell must resynthesize some of its membrane. The Golgi bodies of secretory cells form the membranes of vesicles which transport secretory granules to the exterior of the cell. Thus, new cell membranes are continually being formed in these cells and this membrane manufacture is accompanied by a sharp increase in the turnover rate of membrane phosphate, as is also true for phagocytosis and pinocytosis.

Not only should we consider that the taste cell membrane may be repaired over short periods of time, but it also has been shown that entire cells within the taste bud are replaced (Beidler, 1961; 1963; Beidler and Small-

man, 1965). Autoradiography, with tritiated thymidine, shows that the average life span of the rat taste cell is about ten days. The epithelial cells surrounding the taste buds undergo mitotic division and some of the daughter cells pass into the taste buds and move slowly toward the center. The life span of these cells is not uniform but is presumably determined by random injury to the cells while inside the taste bud. How the plasma membranes of the various cells within the taste bud differ one from another and whether the structure of the membrane of any given cell changes with age are not known. The membranes of other cells are known to change their properties of permeability as the cells age (Hoffman, 1958).

The continuous reproduction of taste cells presents a convenient means of altering the taste sensitivity in response to changes in the internal or external environment of the animal. The genetic concepts (repressors) of the recent Nobel Laureates (Lwoff, A., Monod, J. and Jacob, F.) should be considered for application to the taste receptors since their function should reflect the chemical needs of the animal.

CELL ELECTRICAL POTENTIALS

There is a potential difference of approximately 10–150 mV across the plasma membrane of most cells as measured by one electrode placed inside and another electrode outside the cell. This difference of potential is related to the differences in ionic concentrations between the inside and outside of the cell. In nerve this is determined by the concentration differences of sodium, potassium and chloride ions as controlled by the permeabilities of the membrane and may be calculated by the Hodgkin–Huxley equation:

$$V = \frac{RT}{F} \ln \frac{P_{K}[K^{+}]_{i} + P_{Na}[Na^{+}]_{i} + P_{Cl}[Cl^{-}]_{o}}{P_{K}[K^{+}]_{o} + P_{Na}[Na^{+}]_{o} + P_{Cl}[Cl^{-}]_{i}},$$

where P_{K}, P_{Na}, P_{Cl} are permeability coefficients of K^{+}, Na^{+} and Cl^{-} respectively. Thus, we may assume that in the taste cell, as in other sensory cells, the source of energy for electrical stimulation of the associated nerve fiber is the ionic concentration gradient across the taste cell membrane. This is in agreement with microelectrode experiments on taste cells (Kimura and Beidler, 1961; Tateda and Beidler, 1964) which measured across the membrane a cell depolarization whose magnitude is dependent upon the chemical stimulus applied to the tongue surface.

A potential difference across the cell membrane is associated with any ion to which the membrane is permeable. This gives rise to the thought that salt or acid stimulation of the taste receptors might act by membrane

penetration which would directly depolarize the microvilli cell membrane and create a current sink whose influence would spread decrementally to the area of the associated nerve fiber. If this were true and followed the Hodgkin–Huxley equation, then as potassium chloride is applied to the surface of the tongue at various concentrations, there would be some point at which the external concentrations would be identical to the internal K^+ concentration and the potential difference across the membrane would fall to zero, assuming the contribution of other ions to be negligible due to their low permeability coefficients. Higher or lower concentrations would stimulate or inhibit taste responses. This has never been observed when the taste cell was investigated with microelectrodes. On the contrary, as the concentration of potassium chloride is varied on the surface of the tongue, it stimulates the taste cell, as all other electrolytes do, by increasing the response of the taste cell as the concentration is increased. Thus, it is probable that the membrane of the taste cell microvillus is impermeable to such electrolytes whereas the contiguous membrane of the cell near the nerve endings may be quite different and act like the surface of any other excitable cell.

The approximate resting potential of rat and hamster taste cells was measured with microelectrodes to be about 10–50 mV, being negative on the inside with respect to the outside. These potential differences can be changed by certain drugs or agents such as cocaine and iron chloride, both of which decrease these potential differences and even reverse their polarities. Experiments (Tateda and Beidler, 1964) utilizing these chemicals show that any stimulus applied to the surface of the tongue causes the taste cell potential, whether initially positive or negative with respect to the outside, to return to some equilibrium level as shown in Fig. 2. This indicates that chemical excitation of taste cells results in an effective increase in the permeability of the cell membrane at some point distant from the microvilli, which results in an electrical depolarization great enough to excite the nearby nerve endings. Thus, information concerning microvilli stimulation must be transmitted within a few milliseconds to another area of the membrane 15–30 μ away. Such rapid transmission is expected to occur along the membrane itself rather than through the cytoplasm. The exact nature of such transmission is not known but it is conceivable that it could include a transfer of charge or a molecular re-orientation along the well-ordered structure of the cell membrane, particularly that of the ordered phospholipid, or protein and water molecules.

Great emphasis is usually given to the potential differences across the cell membrane as measured by microelectrodes placed within and without

the cell since these potentials play a great role in nerve excitation. When one considers the properties of the membrane of a living cell, there are other potentials that may be of great importance in the ultimate regulation of their function.

Most cell surfaces have a net negative charge since there are more anionic than cationic groups in the cell membrane, as shown in Table 1 for myelin protein. The density of these excess anionic groups in the membrane gives rise to a surface potential. Such potentials have been described by Debye–Hückel and application of their theory to cell surfaces shows that they

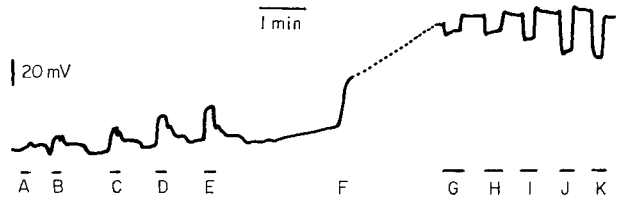


FIG. 2. Change in receptor potential of rat in response to various concentrations of NaCl (*A* and *G*, 0·05 M; *B* and *H*, 0·1 M; *C* and *I*, 0·25 M; *D* and *J*, 0·5 M; *E* and *K*, 1·0 M) before and after the application of cocaine at *F*. Each bar on the bottom of the record shows the duration of stimulus application. Note that before cocaine the changes are projected upward and after cocaine they are projected downward, both tending to approach some equilibrium value at high concentrations. (Tateda and Beidler, 1964.)

should be surrounded by a diffuse layer of counterions. The counterion concentration in this diffuse boundary layer declines away from the cell and the potential drops exponentially. How fast the potential declines away from the surface of the cell is related to the ionic strength of the medium surrounding the cell as is shown in the equation:

$$d = \frac{3{\cdot}05 \text{ Å}}{\sqrt{\text{ionic strength}}} \tag{1}$$

where ionic strength is equal to $^1/_2$ the sum of the ionic concentrations, each multiplied by the square of the ionic valence, and d is the distance away from the cell surface to the center of gravity of the diffuse layer. Thus, the extent of the surface potential surrounding the taste microvillus membrane is dependent upon the ionic strength of the solution flowing over the tongue surface. The magnitude of this potential may be approximated if the double layer is considered as a parallel-plate condenser or:

$$V = \frac{4\pi d\sigma}{D} \tag{2}$$

where σ is the charge density of the surface and D is the dielectric constant of the medium where the diffuse layer exists.

The charge density may be estimated if the excess anionic over cationic sites per unit surface of the microvillus are known. If the charge is due to the membrane protein and if the microvillus membrane is similar to that of myelin, then each amino acid residue is contained in an area of 32·6 Å^2

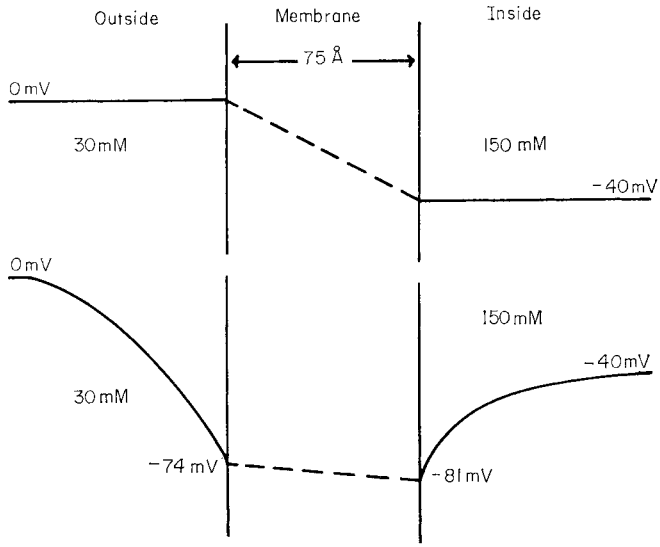


FIG. 3. Effect of fixed charges on membrane potential gradient. Top Curve: Potential gradient of 40 mV across the membrane due to resting potential of cell. Bottom Curve: Potential gradient of 7 mV across the membrane due to same resting potential above together with the surface potentials produced by fixed charges.
(The assumption of a linear gradient within the membrane is not reasonable, but is used for simplicity and is consistent with similar assumptions made by most researchers.)

(Vandenheuvel, 1965). Since 8·2% of the total residues are excess anionic sites, then each such anionic site is contained in every 400 Å^2 of membrane surface of a charge density of $2{\cdot}5 \times 10^{13}$ charges/cm^2. The dielectric constant of pure water is 80 although the dielectric constant near the phosphate group of the phospholipid may be as low as four. Equation (2) assumes there is no binding of electrolytes to the anionic sites, thus the charge density of the surface remains constant as the electrolyte concentration changes. However, this assumption is erroneous since as the electrolyte concentration increases more cations are bound to the anionic sites and the net surface charge decreases.

Hodgkin and Chandler (1965) considered the role of fixed charges in the axon membrane in order to account for the voltage across the membrane when the nerve fibers were perfused internally with electrolytes. They assumed a charge density of $1{\cdot}4\times10^{13}$ electronic charges/cm^2 which corresponds to a linear separation of 27 Å between the charges.

Although these surface charges do not contribute to the voltage as measured by microelectrodes inserted into and outside the cell, they do result in a considerable change in the voltage gradient across the membrane and thus may affect the membrane's internal structure where the taste stimuli are presumably adsorbed (see Fig. 3). It is interesting to note that the average field strength across a 100 Å membrane that has a difference of potential of 50 mV is 5×10^4 V/cm. Much larger field strengths exist at local places within the membrane. (Goldman, 1964, calculated field strengths of $4{\cdot}0\times10^8$ to $4{\cdot}6\times10^7$ V/cm for the dipole of phosphatidyl choline in the membrane.) Surface potentials would change the membrane's field strength to a degree (see Fig. 3) and create changes in local molecular orientations within the membrane.

FINE STRUCTURE OF THE MEMBRANE

There is seen a similar backbone structure in all membrane studied. However, we know that the function, which is dependent upon the fine structure within the membrane, does change from one membrane to another. Little information is available concerning the fine structure of any membrane. Perhaps myelin is the most studied membrane and consideration of it may give us some insight into the membrane of the taste microvillus. A model of the structure of myelin has recently been presented by Vandenheuvel (1965). The myelin protein is assumed to be similar to the soft keratin in human epidermis. The amino acid composition is shown in Table 1, wherein is noted the high excess anionic charge (8·2%) in the collection of amino acids. The average amino acid residue is contained in an area of 32·6 Å^2. This corresponds to proteins whose side chains are 3·33 Å apart and where the various protein chains are oriented parallel to one another with a separation of 9·8 Å. The protein chains are presumed to be held together by ionic and dipole interactions or hydrogen bonding of adjacent chains with water molecule networks separating them. Similar forces are involved in binding the protein to the lipid beneath. Any disturbance of this equilibrium by foreign ions or molecules presented to the membrane should alter considerably the equilibrium. It is this effect that we will discuss more fully in the next section.

The hydrated protein layers are in close contact with the polar groups of the phospholipids. The thickness of the bimolecular lipid layer is 51 Å as measured between the phosphate groups of the two layers of phospholipid molecules. London and Van der Walls interactions bind the lipid layer of molecules together.

SITE BINDING OF TASTE STIMULI

In 1954 a theory of taste stimulation was proposed which assumed that the cations adsorb to the microvillus membrane and elicit ultimately the neural activity along the taste nerve (Beidler, 1954). Anion binding was shown to be inhibitory rather than excitatory and nonionic stimuli were later assumed to be adsorbed to the membrane by way of hydrogen bonds. This site theory of taste receptor stimulation proved useful in directing attention to quantitative aspects of taste data and in designing new experiments. The simplicity of the hyperbolic equation relating the magnitude of taste response to the concentration of the stimulus enhanced the attractiveness of the theory (see Fig. 4). This equation has been applied, usually but not always with success, by many researchers to taste data derived from many different experiments on various species of animals. Other equations could presumably be used to describe taste data but those pre-

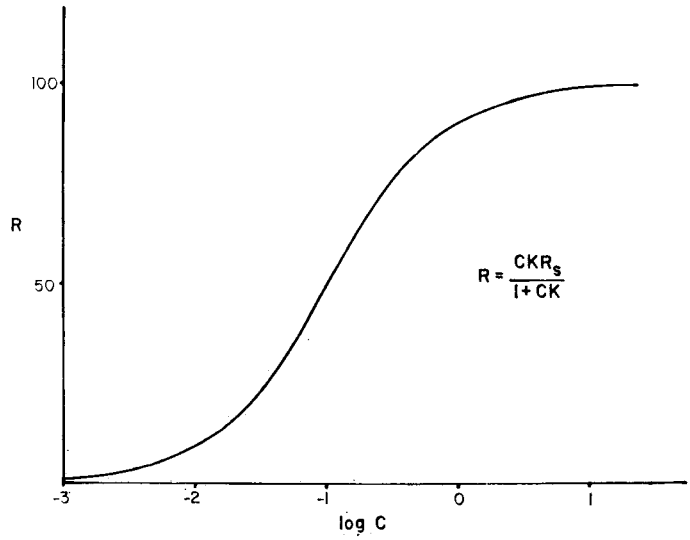


FIG. 4. The magnitude of taste response as a function of log concentration as determined by the taste equation. Notice the almost linear relation through the middle concentration range.

sented to date lack a simple physicochemical basis and include ill-defined constants. The simplicity of the quantitative description which makes a theory attractive also tends to limit its usefulness when it is applied to all the complex data obtained with numerous chemical stimuli. Thus, equations were derived to describe competitive inhibition and multiple site adsorption (Beidler, 1961).

The mathematical theory of taste receptor stimulation was based on the mass action law as applied to the taste stimulus binding to the receptor sites to form complexes. The steady level of the neural activity of the taste nerve was used as a measure of the magnitude of response. The response as seen in single nerve fiber activity varies considerably from one second to the next. If, however, a number of single taste nerve fibers' activities are added together by an electronic summator, and if the stimuli are not applied too rapidly in order to avoid adaptive effects, then the steady level of the neural response to chemical stimuli flowed over the tongue surface is a very stable magnitude and can be used with confidence as a measure of the average taste receptor response. In applying the taste equation:

$$\frac{C}{R} = \frac{C}{R_s} + \frac{1}{KR_s} \tag{3}$$

a decision must be reached as to whether the reaction is at a steady state or at equilibrium. In biological systems it is often difficult to discriminate between the two. Equilibrium states but not steady states would result in a magnitude of response to a given stimulus independent of whether it is approached from a lower to a higher stimulus concentration or from a higher to a lower stimulus concentration. Experiments with various stimuli on the rat taste receptors indicate that the taste response is more likely in an equilibrium rather than a steady state; the K in the taste equation refers to an equilibrium constant. This constant is a function of the individual constants that describe the stimulus binding at each receptor site. It also is a function of those constants determined by recording from single nerve fibers, since each fiber may have a different sensitivity.

Many additional taste data are now available such that the site theory should be re-evaluated and extended. Perhaps it is best to consider taste receptor stimulation in light of present information available concerning membranes of a variety of cells. The model of a typical membrane shown in Fig. 5 would indicate that there are many charged amino acid side chains as well as phosphate groups of the phospholipids that could bind ions. The question is, to what do the taste stimuli bind? If they are bound to the

amino acid of a protein, then the binding should change considerably with pH as more or fewer of these groups become available. Figure 6 shows that the binding of hydrogen ions to the taste receptor does not occur until low pH's are reached and that the pK of the reaction is about 1·8 (Furchgott and Ponder, 1941, measured an isoelectric point of 1·7 for the surface of human red cells). Comparing this pK with that of the amino acids found in membrane protein shows that the majority of the amino acid groups

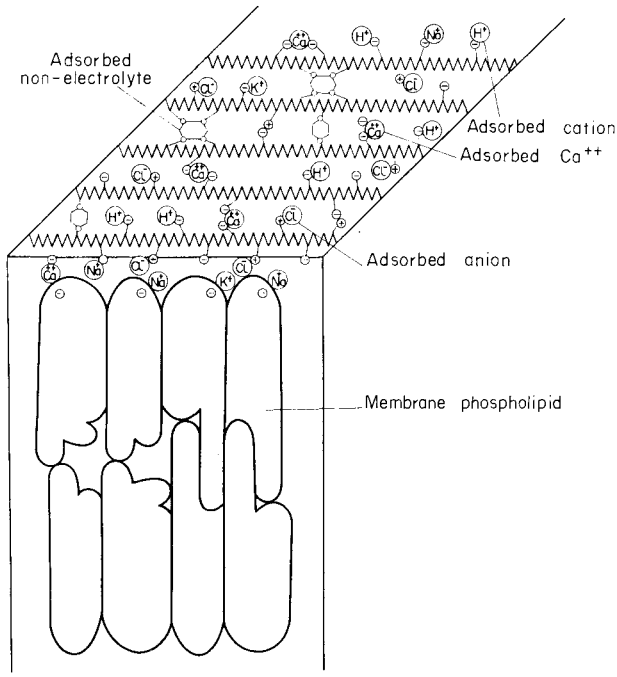


FIG. 5. Schematic description of lipid-protein layer of cell membrane showing electrolyte and non-electrolyte bindings.

are not involved in the binding that leads to a taste response. Even aspartic and glutamic acid have pK's (about 4·7) that are much higher than those found for taste receptor stimulation. There is the possibility that the pK's would be shifted to lower values if the aspartic and glutamic groups were contained in a fixed structure such as the membrane, which also has a net surface charge. It can be reasonably assumed that most of the cations of a taste stimulus are actually bound to the phosphate groups of the phospholipids, although it is realized that at the same time a few

of the amino acid groups may bind alkali cations and may bind hydrogen ions but such binding has little effect on the total taste response.

It might be helpful at this point to describe what is meant by ion-site binding and to classify other types of ion interactions that occur at the cell membrane. Four types of such interactions have been suggested (Lifson 1957):

(a) Interactions between the small ions and the electrostatic field of the membrane polyelectrolytes,

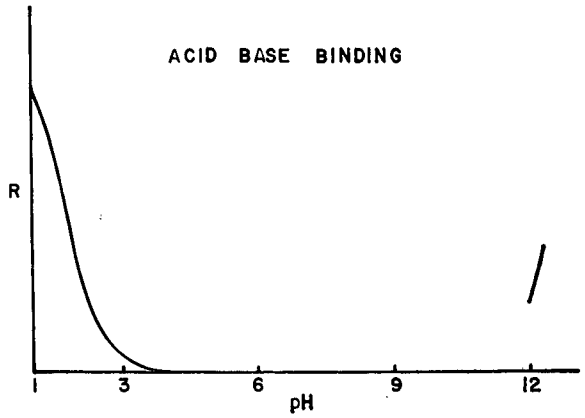


FIG. 6. The magnitude of summated response of rat to various concentrations of HCl and NaOH shows a pK about 1·8 for H^+ binding.

(b) Electrostatic interactions which follow the Debye–Hückel theory as outlined earlier,

(c) Interaction of one ion with a number of reactive groups such as occurs in chelation,

(d) Association between a small ion and a single reactive group (bonding of Na^+ to phosphate of phospholipid or COO^- group of amino acid side chain, for example.) This is what is referred to in this paper as site binding and has been shown important in binding of ions to polyelectrolytes (Harris and Rice, 1954; Rice, 1956) and to binding of Na^+, K^+ and Li^+ to polyphosphates in particular (Strauss, Woodside and Wineman, 1957; Strauss and Ross, 1959).

DIFFERENCES IN RECEPTOR MEMBRANES

When a large number of different salts are utilized in an experiment to stimulate the taste receptors of a given animal, various single nerve fibres may differ in their relative response to the same series of salts. Thus, most of the rat single taste fibres respond much better to NaCl than to KCl at the same concentration, but there is always a certain number of fibers that respond in an opposite manner. In another species, the cat, the population shifts such that most of the single fibers now respond to K^+ much better than to Na^+, but some may be similar to those found in the rat. Thus, we are presented with the fact that some of the binding sites might respond better to sodium than to potassium whereas other sites behave differently. Since the only character of monovalent ions that can account for a difference in binding is the total hydrated size of the ion, differences in binding must be accounted for by differences in effective degree of hydration around the ion. Ling (1962) and Eisenman, Rudin and Casby (1957) have shown that if the site possesses a high electrostatic field strength then the sodium ion is essentially stripped of some of its hydrated water so that the Na^+ ion can move closer to the anionic membrane site than can K^+. The more hydrated cations shed their water of hydration before the less hydrated cations so that only a limited number of rank orders of cation effectiveness exist. The electrostatic field strength of a carboxyl group, for example, may change with its environment so that its cation selectivity may also change. This concept would explain many taste data obtained from a large number of species. Another way of describing this effect is that the sodium ion penetrates part of the atmosphere surrounding the phosphate group.

Most chemical groups bind potassium in preference to sodium. Polyelectrolytes containing numerous phosphate groups do prefer sodium, however (Strauss and Ross, 1959). The ability of taste receptors to respond well to sodium over potassium has been correlated in many species to the $\frac{Na^+}{K^+}$ ratio of the content of the red cells of the same species. (The correlation between taste responses and red cell content breaks down in those animals such as sheep and cows where the individuals of a given species may have red cells with either high Na or high K. A single gene determines the K content of each individual sheep (Hoffman, 1962).) A causal relation is difficult to conceive. However, the Na^+ and K^+ contents of red cells have been related to the amount of ATP^{ase}enzyme activity found in the red cell membrane (Tosteson, Moulton and Blaustein, 1960). An ATP^{ase} takes part in the active transport of the Na^+ across the

membrane. Whether an ATP^{ase} is involved in cation taste receptor stimulation is not known, but available data merely suggest that phosphate groups may be involved in the cation binding and that the magnitude of taste response is proportional to the number of cations so bound.

Because of the nature of the hydrated ion, sodium and lithium ions act similarly, as do potassium, rubidium and cesium. Furthermore, these five ions presumably act on the same sites (the three latter also bind to other sites) but with a magnitude of binding dependent upon the electrostatic field strength of the anionic membrane site. The action of the divalent ions is somewhat different. For example, one can completely adapt taste receptors to calcium without affecting seriously the subsequent sodium response. A divalent ion can bridge the gap between two anionic sites in the same membrane as shown in Fig. 5. Other polyvalent ions, such as copper and iron, may be bound tightly (chelated) to certain groups within the membrane so that these sites become unavailable for monovalent ions. It should be possible to make such sites again available for monovalent ions if a strong chelating agent is added to the tongue surface to attract such polyvalent ions away from the membrane temporarily. Ammonium ions react similarly, but not identically, to other monovalent ions. In all different species studied, ammonium chloride is a good taste stimulus whereas the relative binding efficiency of sodium and potassium is species dependent. Larger organic ions, such as choline, may also interact with the membrane to elicit a taste response.

STIMULATION WITH NONELECTROLYTES

Little attention has been given by current taste theories to stimulation of the receptors with nonelectrolytes, although such molecules contribute greatly to our daily taste sensations. It has been shown that taste responses to sugars can be described by the same type of equation that has been successfully applied to salt and acid stimulation (Beidler, 1961).

It is also known that molecules with widely different structures, such as urea, alcohols, glycosides, sugars and steroids, bind to proteins (Klotz, 1953). Similarly, a large group of molecules bind to lipids. Hydrogen bonding is expected to play a large role in the binding of nonelectrolytes to the protein layer of the cell membrane. The organization of the protein side chains into the rather rigid structure of the membrane presents many specific sites for binding of nonelectrolytes. Since hydrogen bonding is involved, the steric configuration of the stimulating molecules becomes important. Such binding would result in a local re-orientation in the mem-

brane and thus initiate a taste response in a manner not too dissimilar to that of electrolytes.

Exact knowledge concerning the intimate details of the forces active in the binding of a given taste molecule should not be expected without long study. Pharmacologists have studied specific drug reactions intensively without being able to completely describe their interactions. An example of a diligent search is given by Wilson (1957) who studied the specificity in cholinesterase reactions.

ANIONS AND THE "WATER RESPONSE"

It has been shown that anions have an inhibitory effect on the action of the cations. The ability of an organic anion to bind with protein (albumin) increases with chain length as well as with the size of the anion (Klotz, 1953). Sodium propionate produces a smaller magnitude of taste response than does sodium formate, whose anion chain length is smaller, due to the stronger binding of the inhibitory propionate over the formate anion. Since cation binding to taste receptor membranes is excitatory and anion inhibitory, the net magnitude of the taste response may depend upon the relative number of cationic to anionic membrane sites available to the taste stimulus as well as their relative binding constants for a given stimulus. Certain taste receptors may differ in their membrane fine structures so that the ratio of anionic to cationic sites available for taste stimuli may also vary. The net response to stimulation would then depend upon the relative effectiveness of the stimulus cation in relation to that of the anion. If the rat taste receptor membrane contains a predominance of anionic sites, then the cations of most stimuli presented to the tongue dominate and the anions play a lesser role. Since stimulation depends upon the net number of positively charged sites, there will be little spontaneous activity when the tongue is covered with water and the anionic membrane groups are more numerous. Even this small amount of spontaneous activity can be decreased if a stimulus is chosen where the cation has a poor stimulating effect, such as potassium, and the anion a good inhibiting effect, such as benzoate (see Fig. 7). Low concentrations of potassium benzoate will decrease spontaneous activity since the relative number of free cationic membrane sites will decrease as the strongly bound benzoate ion will fill the cationic sites. At higher concentrations, when many of the cationic membrane sites are filled, the potassium will bind to the more numerous anionic sites and taste receptor stimulation will occur.

Such effects due to the balance between anionic and cationic membrane

sites are much more dramatic in the frog, cat and rabbit which exhibit large water responses (Zotterman, 1956). In these animals it might be hypothesized that the cationic membrane sites are more numerous than the anionic and thus, the membrane is spontaneously active. When a salt is applied to the tongue, the anions will be bound to the membrane in greater number and efficiency than the cations, and the magnitude of the spontaneous activity will decrease. As most of the cationic membrane sites

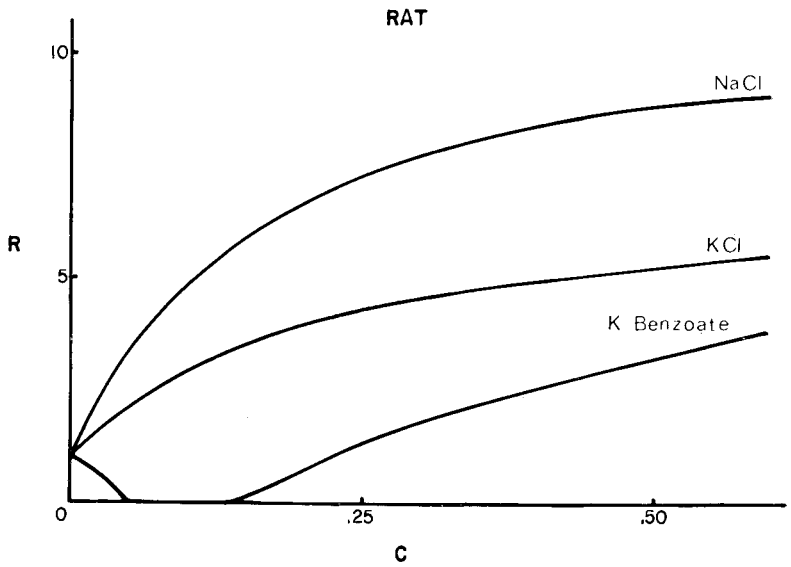


FIG. 7. Expected magnitude of taste response of rat as a function of concentration of three different electrolytes.

are filled, the stimulus cation binds to the anionic membrane sites and a taste response occurs. The ability to decrease the spontaneous activity in such preparations depends upon the relative effectiveness of the anion-cation of the stimulus. Since a benzoate ion will be adsorbed more strongly than a chloride ion, one would expect sodium benzoate to be much more effective than sodium chloride in depressing the rabbit spontaneous activity. Experiments conducted in our laboratory on the water response of the rabbit indeed indicate that it is possible to predict the effectiveness of a stimulus in depressing spontaneous activity by a knowledge of the inhibitory effect of the anions and the stimulatory effect of the cations. Those animals that respond better to K^+ than Na^+ may show very little inhibition with KCl since the chloride ions bind to the cationic membrane groups in a very ineffective manner, whereas the K^+ is effective and will produce

a taste response. If now potassium benzoate is used instead of KCl then the benzoate ion will bind to the cationic membrane groups, decreasing their number relative to the free anionic membrane groups and thus decreasing the spontaneous activity. At higher concentrations the K^+ begins to bind and the taste receptors respond well. Sodium chloride, on the other hand, contains both a poor stimulating cation and anion so that a higher concentration must be used in order to inhibit the spontaneous activity of

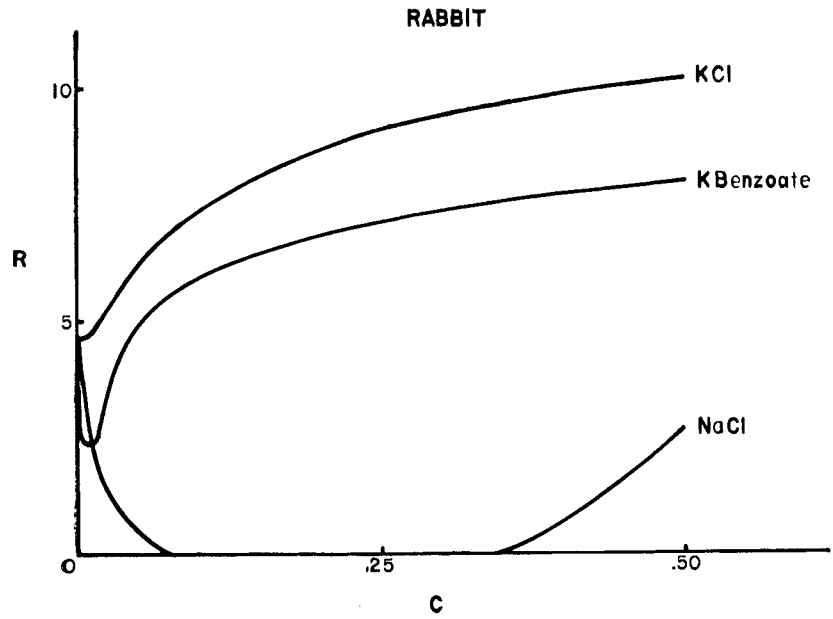


FIG. 8. Expected magnitude of taste response of rabbit as a function of concentration of three different electrolytes. Compare with Fig. 7.

a "water fiber" and an increase in response to Na^+ will not be obtained except at very high concentrations. (see Fig. 8).

According to the above hypothesis, the term "water response" may be misleading since actually the receptor is not responding to water but is merely spontaneously active due to the relative number of anionic-cationic groups in the membrane. It is the relative effectiveness of these two opposed binding groups that determines the response of the receptor to the stimulus. Thus, there is no fundamental difference between "salt" and "water" fibers but only a difference in degree. Again, this model emphasizes the differences in membrane characteristics from one cell to another in the same species of animal, or one group to another in different species of animal.

ACID BINDING

Most proteins, myosin being an exception, do not bind alkali cations appreciably but do bind hydrogen ions in large quantities. As the pH of the taste solution is decreased, numerous H^+ attach to the taste receptor membrane and its surface charge increases. This increased positive charge associated with the membrane hinders the addition of more H^+ ions. (Although many H^+ ions may be adsorbed to membrane sites that are not effective in eliciting a taste response, these bound H^+ ions also increase the charge of the membrane and thus hinder additional H^+ ions from binding to taste receptor sites.) Anions are adsorbed at other sites independently of the H^+ ions and are restricted only by their affinity for the sites and the total membrane charge. Thus, the magnitude of the taste response should be quite dependent upon the anion of the dissociated acid. In addition, the pK of the "taste titration" curve shown in Fig. 6 is much lower than one would expect if H^+ ions were bound to similar sites contained in a small soluble protein or free amino acid which is free of the effect of the positive charge associated with the larger fibrous proteins of the receptor membrane.

Twenty different acids were chosen for study of the effect of the anion on the acid response, and the concentration of each necessary to elicit a magnitude of response equal to that of 5 mM hydrochloric acid was electrophysiologically determined using summated methods of recording with rats. Table 2 shows the wide range of molar concentrations that elicit the same magnitude of response with these various acids. Notice that it is necessary to use 150 mM concentration of butyric acid to elicit the same magnitude of response as that elicited by 5·0 mM hydrochloric acid. That this magnitude of taste response is not dependent upon the hydrogen ion concentration alone is shown by the fact that the millimolar hydrogen ion concentration varied from 0·73 for glutaric acid to 7·7 for dichloroacetic acid. A similar effect has been known in the science of taste for many years.

Sour taste has been associated with acid substances and, therefore, it was expected that the intensity of acid taste would be related to the hydrogen ion concentration and that acid solutions of equal pH would be equally sour in taste. However, Richards (1898) found that hydrochloric acid is more sour than an equal molar concentration of acetic acid; however, acetic acid is more sour than hydrochloric acid of the same pH. This result was confirmed by Kahlenberg (1898). Liljestrand (1922), Kendrick (1931) and Beatty and Cragg (1935) noted the ability of weak organic acids to stimulate taste receptors at a lower hydrogen ion concentration than strong inorganic acids. They explained this behavior by assuming

TABLE 2. RESPONSE EQUAL TO RESPONSE TO 5 mM HCl

Acid	Acid mM	$[H^+]$ mM	pH	$[H_3A]$ mM	$[H_2A]$ mM	[HA] mM
Sulfuric	2·2	4·20	2·38	0	0	0·2
Oxalic	3·3	3·50	2·46	0	0·2	2·7
Hydrochloric	5·0	5·00	2·30	0	0	0
Citric	5·5	1·97	2·71	3·71	1·61	1·16
Tartaric	5·9	2·38	2·62	0	3·85	1·69
Nitric	5·9	5·90	2·23	0	0	0
Maleic	6·4	4·82	2·32	0	1·59	4·79
Dichloroacetic	9·0	7·70	2·11	0	0	1·3
Succinic	10·0	0·824	3·08	0	9·2	0·74
Malic	10·0	1·91	2·72	0	8·2	1·68
Monochloroacetic	10·4	3·13	2·50	0	0	7·27
Glutaric	11·0	0·73	3·14	0	10·3	0·67
Formic	11·6	1·32	2·88	0	0	10·3
Adipic	14·0	0·74	3·13	0	13·3	0·68
Glycolic	15·0	1·45	2·84	0	0	13·5
Lactic	15·6	1·41	2·85	0	0	14·2
Mandelic	25·0	3·11	2·51	0	0	21·8
Acetic	64·0	1·06	3·00	0	0	63·0
Propionic	130·0	1·32	2·88	0	0	129·0
Butyric	150·0	1·44	2·84	0	0	149·0

that the weak acids maintained a rather constant pH by further dissociation when the acid reacted with saliva. However, a flow chamber was used in those electrophysiological experiments from which the data in Table 2 were determined and thus any contribution due to saliva was eliminated.

Why do various weak acids behave differently in stimulating the taste receptor? We have already considered the effect of the surface charge on the membrane. A formal mathematical treatment was given by Gilbert and Rideal (1943) and applied effectively to protein binding of H^+. Is this the dominant factor in taste stimulation with acids or do other effects also contribute? Let us consider other possibilities.

If it is primarily the hydrogen ion concentration that determines the magnitude of taste response, one may safely assume that the concentration of the anion is also important since it has already been indicated that the anion concentration has an inhibitory effect on the salt response. If these were the only factors involved in weak acid stimulation, one would expect that at equal molar hydrogen ion concentrations of hydrochloric acid and acetic acid, the acetic acid response would be of much smaller magnitude since both acids possess the same concentration of the excitatory H^+ cation and the acetic acid possesses an anion that is quite inhibitory.

However, just the opposite is true; acetic acid is more effective than HCl at equal pH. Therefore, the anion inhibitory factor does not contribute greatly to the acid response. We must also consider the effect of the undissociated molecule.

Taylor *et al.* (1930) measured the concentration of various acids that elicited the same intensity of sourness as did 0·002 N oxalic acid. He postulated that the undissociated molecule penetrated the taste receptor membrane and then dissociated to form H^+ ions. The same intensity of sourness for various acids was assumed to be reached at equal H^+ concentrations within the cytoplasm. However, his theory could not account for the sourness of HCl and other strong acids since they are completely dissociated. He therefore had to assume both the H^+ and anions could also penetrate, thus severely limiting the acceptance of the theory.

The lipid solubility of weak acids can be correlated to their taste effectiveness. Possibly the acids penetrate and react with the lipid layers of the receptor membranes. Thus, sourness should increase with the chain length of the acid in a homologous series or the effectiveness of the H^+ stimulation should be: butyric > propionic > acetic > formic. This is exactly what is found to be true experimentally. On the other hand, the anion adsorption also follows the same sequence predicted by the theory of Gilbert and Rideal (1943) when anion influences described by Klotz (1953) are considered.

In order to resolve experimentally the difference between the effects of the undissociated acid molecule and its dissociated anion, buffered acids were used as taste stimuli. The taste response to 0·1 M acetic acid was compared with the response to 0·1 M acetic acid plus 0·01 M sodium acetate. Although the pH was increased from 2·88 to 3·76, the neural response was only slightly lower in magnitude than that obtained to 0·1 M acetic acid alone. Thus, although the hydrogen ion concentration decreased by a factor of seven or more, the response only declined slightly. The various concentrations of ionic and nonionic species of both solutions are compared in Table 3. Note that there is only a one percent change in the concentration of the undissociated molecule and therefore it contributes little to the enhancement of the response. The acetate anion was increased by a factor of about eight. It has been previously shown with sodium organic salts that organic anions bound to taste sites tend to inhibit slightly the salt response and a similar mechanism probably occurs with acid stimulation. However, the greater effect of anion binding decreasing the positive membrane surface charge, as predicted by Gilbert and Rideal (1943), dominates and increases the total response rather than decreases it. The

TABLE 3. EFFECT OF BUFFERS

	0·1 M HAc	0·01 M NaAc+0·1 MHAc
pH	2·88	3·76
$[H^+]$	1·32 mM	0·175 mM
[HAc]	99·0 mM	100 mM
$[Ac^-]$	1·32 mM	10·175 mM
$[Na^+]$	0	10·0 mM
Ionic strength	1·32	10·175

added response due to 10 mM sodium ions is inconsequential compared to the magnitude of response to 0·1 M acetic acid alone. One concludes that the observed increase in magnitude of response to the buffer solution over that expected for acetic acid of pH 3·76 can only be explained by the increased H^+ binding to the lesser charged membranes caused by simultaneous anion binding.

The effects of buffers on the sourness of acids were already predicted in 1922 by the experimental results of Liljestrand (1922). He showed that a buffer mixture of acetic acid and sodium acetate gave a sour threshold at pH 5·6 whereas the threshold to acetic acid alone was pH 3·9. Additionally, an acetate buffer mixture of pH 3·9 tasted much more sour than did acetic acid at 3·9.

Notice that the increased ionic strength of the buffered solution, as shown in Table 3, will tend to collapse the diffuse double layer surrounding the microvilli membranes. When the distance of the center of gravity of the layer, as calculated by Equation (1), becomes less than the distance that separates the anionic membrane sites which contribute to the taste response, then the membrane protein acts like a small soluble protein and the sites act without electrostatic interactions.

The magnitude of the taste response to acids may be calculated from Equation (3) if it is assumed that the H^+ cations are the stimuli and no electrostatic interactions are present.

$$\frac{[H^+]}{R} = \frac{[H^+]}{R_s} + \frac{1}{K_i R_s},$$

where K_i is the association constant of the reaction of the binding of H^+ to equivalent anionic sites of a soluble protein. Solving for the functional response, $\frac{R}{R_s - R}$, (which is related to the fractional numbers of sites

filled):

$$\frac{R}{R_s - R} = [H^+]K_i,$$

$$\log \frac{R}{R_s - R} = \log [H^+] + \log K_i,$$

$$\log \frac{R}{R_s - R} = -\text{pH} - \text{p}K_i.$$

This equation does not adequately describe the taste response to weak acids since the electrostatic repulsion of H^+ ions at low pH was not assumed. If the surface charge of the membrane is considered, then a relation similar to that obtained by Gilbert and Rideal (1943), (see Alexander *et al.* 1963), and adapted by Tanford (1954, 1961) for the binding of H^+ to protein is obtained. Thus, the dissociation of H^+ from a maximum of n binding sites is given by:

$$\log \frac{r}{n - r} = \text{pH} - \text{p}K_{\text{int}} + 0{\cdot}868Zw,$$

where r is the number of dissociated sites at a given pH, K_{int} is the intrinsic dissociation constant, Z is the net charge of the protein at the given pH, and w is an electrostatic factor. Notice that the factor $0{\cdot}868Zw$ describes the effect of the positive surface charge of the protein on H^+ ion binding. The charge Z is not only dependent upon the number of H^+ ions bound, but also upon the number of anions bound at the same pH. Thus, the affinity of the anions for the cationic membrane sites greatly determines the nature of the acid titration curve or the sourness of various acids at different pH's.

SUMMARY

The factors involved in the binding of ions to a model membrane were applied to taste phenomena. Many anionic sites in the protein and phospholipid may bind H^+ and a few bind alkali cations. The binding of some of these anionic membrane sites elicits a taste response, whereas the other sites only contribute indirectly; for example, in determining the net charge density of the receptor membrane. The extent and specificity of binding is greatly influenced by the electrostatic field strength of the individual anionic membrane sites, the relative distribution of which varies from receptor to receptor, with species, and with the electrical field strength across the membranes (see Goldman, 1964).

The binding of anions to certain cationic membrane sites decreases the magnitude of the taste response elicited by the cations. In addition to this

general effect, anion binding to any area of the membrane decreases the net positive surface charge due to cation binding and thus may enhance further cation binding at low pH's where the contribution of the bound H^+ ions becomes great.

The ionic strength of the taste solution, as determined by the concentration of both cations and anions, regulates the distance the diffuse layer of counterions extends away from the membrane. This in turn helps to determine the electrical field strength across the membrane and thus the orientation of charged groups and dipoles within.

The relative number of anionic and cationic membrane sites, as well as the concentration and effectiveness of each kind of cation and anion in the taste solution, determines not only the magnitude of taste response but also its direction, whether inhibitory or excitatory.

ACKNOWLEDGMENTS

The author's work was supported in part by National Science Foundation Grant No. G-14334.

REFERENCES

ALEXANDER, P. HUDSON, R. F. and EARLAND, C. 1963. *Wool: Its Chemistry and Physics*, Franklin Publishing Co., New Jersey.

BEATTY, R. and CRAGG, L. 1935. The sourness of acids. *J. Amer. Chem. Soc.* **57**, 2347–2351.

BEIDLER, L. M. 1954. A theory of taste stimulation. *J. Gen. Physiol.* **38**(2), 133–139.

BEIDLER, L. M. 1961. Taste receptor stimulation. In: *Progress in Biophysics and Biophysical Chemistry*, Vol. 12, ed. BUTLER, J. A. V., HUXLEY, H. E. and ZIRKLE, R. E., Pergamon Press, New York, pp. 107–151.

BEIDLER, L. M. 1963. Dynamics of taste cells. In: *Olfaction and Taste*, ed. Y. ZOTTERMAN, Pergamon Press, London, pp. 133–148.

BEIDLER, L. M. and SMALLMAN, R. 1965. Renewal of cells within taste buds. *J. Cell Biol.* **27**(2), 263–272.

COLE, K. S. 1949. Some physical aspects of bioelectric phenomena. *Proc. Nat. Acad. Sci. U.S.A.* **35**, 558–566.

DAVSON, H. and DANIELLI, J. F. 1943. *The Permeability of Natural Membranes*, Cambridge University Press, London.

DE GIER, J. and VAN DEENEN, L. L. 1961. Some lipid characteristics of red cell membranes of various animal species. *Biochim. Biophys. Acta* **49**, 286–296.

EISENMAN, G., RUDIN, D. O. and CASBY, J. V. 1957. Glass electrode for measuring sodium ion. *Science*, **126**, 831–834.

FRICKE, HUGO 1925. The electric capacity of suspensions with special reference to blood. *J. Gen. Physiol.* **9**, 137–152.

FURCHGOTT, R. F. and PONDER, E. 1941. Electrophoretic studies on human red blood cells. *J. Gen. Physiol.* **24**, 447–457.

GILBERT, G. A. and RIDEAL, E. K. 1943. The combination of fibrous proteins with acids. *Proc. Roy. Soc.* **182A**, 335–346.

GOLDMAN, D. E. 1964. A molecular structural basis for the excitation properties of axons. *Biophys. J.* **4**(3), 167–188.

GORTER, E. and GRENDEL, F. 1925. On bimolecular layers of lipoids on the chromocytes of the blood. *J. Exp. Med.* **41**, 439–443.

GRAY, E. C. and WATKINS, K. C. 1965. Electron microscopy of taste buds of the rat. *Zeit. für Zellforschung* **66**, 583–595.

HARRIS, F. E. and RICE, S. A. 1954. A chain model for polyelectrolytes. I and II. *J. Phys. Chem.* **58**, 725–739.

HODGKIN, A. L. and CHANDLER, W. K. 1965. Effects of changes in ionic strength on inactivation and threshold in perfused nerve fibers of *Loligo*. *J. Gen. Physiol.* **48**(5), 27–33.

HOFFMAN, J. F. 1958. On the relationship of certain erythrocyte characteristics to their physiological age. *J. Cell Comp. Physiol.* **51**, 415–423.

HOFFMAN, J. F. 1962. Cation transport and structure of the red-cell plasma membrane. *Circulation*, **26**, 1201–1213.

KAHLENBERG, L. 1898. The action of solutions on the sense of taste. *Bull. Univ. Wisc. Sci. Serv.* **2**, 1–31.

KENDRICK, F. 1931. The sour taste of acids. *Trans. Roy. Soc. Canada*, Ser. 3, **25**, 227.

KIMURA, K. and BEIDLER, L. M. 1961. Microelectrode study of taste receptors of rat and hamster. *J. Cell. Comp. Physiol.* **58**(2), 131–140.

KLOTZ, I. M. 1953. Protein interactions. In: *The Proteins*, Vol. 1, part A, ed. H. NEURATH and K. BAILEY, Academic Press, New York, pp. 727–806.

LILJESTRAND, G. 1922. Über den Schwellenwert des sauren Geschmacks. *Arch. Neerl. de Physiol.* **7**, 523–537.

LIFSON, S. 1957. Potentiometric titration, association phenomena, and interaction of neighboring groups in polyelectrolytes. *J. Chem. Phys.* **26**(4), 727–734.

LING, G. N. 1962. *A Physical Theory of the Living State*, Blaisdell Publishing Co., New York.

NARAHASHI, T. 1965. Potential, structure, and excitability of giant axon membrane. *J. Gen. Physiol.* **48**, 19–25.

NORTON, W. T. and AUTILIO, L. A. 1965. The chemical composition of bovine CNS myelin. *Ann. N. Y. Acad. Sci.* **122**(1), 77–85.

RICE, S. A. 1956. On the bjerrum relation and the formation of ion parts. *Amer. Chem. Soc. J.* **78**, 5247–5252.

RICHARDS, T. 1898. The relation of the taste of acids to their degree of dissociation. *Amer. Chem. J.* **20**, 121–126.

SCHMITT, F. O. and BEAR, R. S. 1939. The ultrastructure of the nerve axon sheath. *Biol. Rev.* **14**, 27–50.

STRAUSS, U. P., WOODSIDE, D. and WINEMAN, P. 1957. Counterion binding by polyelectrolytes. I. Exploratory electrophoresis, solubility and viscosity studies of the interaction between polyphosphates and several univalent cations. *J. Phys. Chem.* **61**, 1353–1356.

STRAUSS, U. P. and ROSS, P. D. 1959. Counterion binding by polyelectrolytes. II. The determination of the binding of univalent cations by long-chain polyphosphates from conductivity and electrophoresis data. *Amer. Chem. Soc. J.* **81**, 5292–5295.

STRAUSS, U. P. and ROSS, P. D. 1959. Counterion binding by polyelectrolytes. IV. Membrane equilibrium studies of the binding of univalent cations by long-chain polyphosphates. *Amer. Chem. Soc. J.* **81**, 5299–5302.

TANFORD, C. and WAGNER, M. L. 1954. Hydrogen ion equilibria of lysozyme. *Amer. Chem. Soc. J.* **76**, 3331–3336.

TANFORD, C. 1961. *Physical Chemistry of Macromolecules*, John Wiley & Sons, Inc. New York.

TATEDA, H. and BEIDLER, L. M. 1964. The receptor potential of the taste cell of the rat. *J. Gen. Physiol.* **47**(3), 479–486.

TAYLOR, N., FARTHING, F. and BERMAN, R. 1930. Quantitative measurements on the acid taste and their bearing on the nature of the nerve receptors. *Protoplasma* **10**, 84–97.

TOSTESON, D. C., MOULTON, R. H. and BLAUSTEIN, M. 1960. An enzymatic basis for the difference in active cation transport in two genetic types of sheep. *Fed. Proc.* **19**, 128.

VANDENHEUVEL, F. A. 1965. Structural studies of biological membranes: the structure of myelin. *Ann. N. Y. Acad. Sci.* **122**(1), 57–76.

WILLIAM, H. H., ERICKSON, B. N. and MACY, I. G. 1941. Chemical structure of the red blood cell. *Quart. Rev. Biol.* **16**, 80–89.

WILSON, I. B. 1957. Specificity in cholinesterase reaction. In: *Molecular Structure and Biological Specificity*, ed. L. PAULING and H. A. ITANO, American Institute of Biological Sciences, Washington, D. C., pp. 174–185.

ZOTTERMAN, Y. 1956. Species differences in water taste. *Acta Physiol. Scand.* **37**, 60–70.

ALTERED TASTE RESPONSES FROM CROSS-REGENERATED TASTE NERVES IN THE RAT*

BRUCE OAKLEY

Department of Zoology, University of California
Los Angeles, California

INTRODUCTION

When a mammalian taste nerve is cut the denervated taste buds degenerate and when the nerve grows back the taste buds re-form (von Vintschgau, 1880; Whiteside, 1927; Guth, 1958; Oakley and Benjamin, 1966). The present experiment attempted to answer the following question. Is it the taste nerve that determines the reactivity of taste receptor cells to different chemicals? That is, does the taste nerve not only induce the formation of the taste bud but also dictate to what degree the taste receptor cells will respond to different chemicals?

The answer was sought through electrophysiological recording from cross-regenerated taste nerves. Normally the chorda tympani nerve innervates the anterior two-thirds of the rat tongue and the IXth (glossopharyngeal) nerve the posterior one-third (Fig. 1A). By cross-union it is possible to cause the IXth nerve to regenerate to the front and, in other rats, to cause the chorda to regenerate to the back of the tongue.

A number of barriers had to be surmounted before such recording experiments from cross-regenerated taste nerves could be carried out and meaningfully interpreted. First, it was necessary to record quantifiable taste responses from the normal IXth nerve. There are no previous reports of attempts to quantify taste responses from the whole glossopharyngeal nerve in mammals. Second, it was necessary to demonstrate that the chorda

* This work was carried out during the author's tenure as a U.S. National Academy of Sciences-National Research Council Senior Fellow in Physiological Psychology. Professor Y. Zotterman provided the laboratory facilities for this research and Mrs. J. Davis assisted in analysis of the data. The data analysis was supported in part by grants from the National Science Foundation and the National Institutes of Health to Professor T. H. Bullock.

tympani and IXth nerves were reliably different in some taste response characteristics for at least some chemicals. By examining the neural responses to these chemicals it was assumed that it would be possible to determine whether the cross-regenerated taste nerve retained its response characteristics, or changed in some fashion, e.g. responding like the nerve

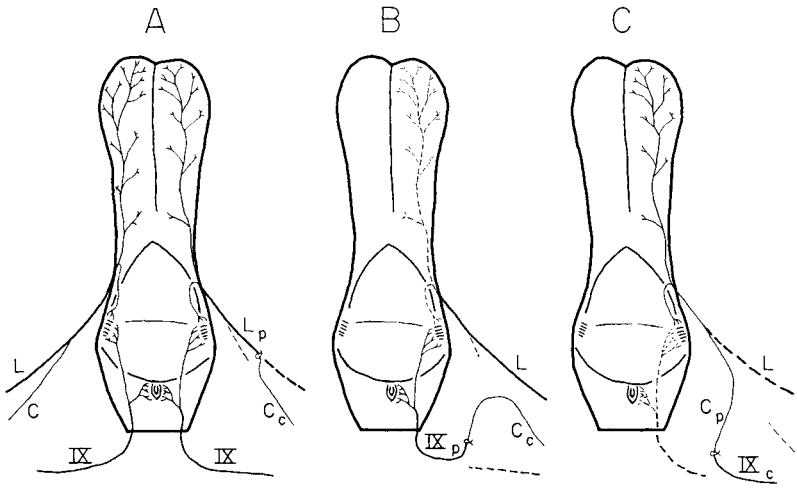


FIG. 1. Schematic drawing of taste nerve crosses and normal taste nerve innervation of the rat tongue. The normal innervation is shown on the left side of the tongue in Fig. 1A. The glossopharyngeal nerve (IXth) supplies taste buds in the circumvallate and foliate papillae. The chorda tympani nerve (C) joins the lingual nerve (L) before entering the tongue and distributes to the anterior two-thirds of the tongue including a small contribution to the foliate papillae. On the right side in Fig. 1A the chorda has been sutured to the lingual nerve and allowed to grow to the tongue ($C_e - L_p$). In Fig. 1B the central part of the chorda (C_c) is sutured to the peripheral part of the IXth nerve (IX_p); $C_c - IX_p$. In Fig. 1C the central part of the IXth nerve (IX_c) is sutured to the peripheral part of the chorda (C_p); $IX_c - C_p$. In this instance the lingual has been cut. In Fig. 1B and 1C normal innervation is present on the left side but not shown. Dashed lines represent either degenerated nerves or nerves with frustrated regeneration. Non-taste fibers are not shown within the tongue.

which normally innervates that region of the tongue. Third, it was necessary to study regeneration, *per se*, in order to determine whether taste nerves that had been cut and allowed to regenerate would change in their reactivity to different taste stimuli. Fourth, techniques had to be developed or bringing together and suturing the IXth nerve with the chorda tympani nerve.

The absence of data on the variability of summator records from taste nerves (between different rats, within the same rat, within the same nerve

at different times, etc.) handicapped the present experiment. Initial recordings from normal rats suggested that there were individual differences between rats, but little variability between left and right chorda tympani nerves of the same rat. Thus, in so far as possible each rat served as its own control.

METHODS

This report is based upon results from 14 female Sprague-Dawley albino rats having either regenerated or cross-regenerated taste nerves.

Operative Procedure

Four kinds of unilateral end-to-end taste nerve anastomoses were performed: C_c-C_p, C_c-L_p, C_c-IX_p, and IX_c-C_p.

1. C_c-C_p $N = 2$. The chorda tympani nerve was cut and the central portion of the chorda rejoined to the peripheral portion.

2. C_c-L_p $N = 2$. The chorda was cut and the lingual nerve cut central to the branching out of the chorda. Then, the central portion of the chorda was joined to the peripheral portion of the lingual (Fig. 1A).

3. C_c-IX_p $N = 4$. The central portion of the chorda was sutured to the peripheral part of the IXth nerve. In order to accomplish this the IXth nerve was sectioned at its entrance into the posterior lacerated foramen and directed anteriorly against the medial surface of the tympanic bulla. The chorda, sectioned at the point of union with the lingual nerve, was diverted in a medial direction against the anterior surface of the tympanic bulla, and joined to the IXth. It was not necessary to cut any muscles to achieve this anastomosis (Fig. 1B).

4. IX_c-C_p $N = 6$. The central part of the IXth nerve (the pharyngeal branch was cut away) was joined to the peripheral part of the chorda tympani. In order to join the two nerves it was helpful to pull the chorda out of the tympanic bulla for some distance before cutting. Usually the pterygoid muscles were partially cut. The lingual branch of the IXth nerve was sectioned anterior to the hyoid bone and pulled under the digastric muscle to join the chorda at the bulla. The lingual nerve was cut in the first two animals (Fig. 1C).

All operations were performed under semi-sterile conditions. Each rat received an intramuscular injection of streptomycin and penicillin.

Because of the small size of the chorda (about 100 μ in diameter) it was necessary to make miniature sutures for joining it to other nerves. Monofilament nylon thread (20 μ in diameter) from a nylon stocking was

glued to a fine tungsten needle with epoxy resin cement. Heating for 24 hr at 80°C ensured a firm junction between needle and thread. The tungsten needle (about 1·0 mm long, 40 μ base diameter tapering to 1–5 μ at the tip) was made from tungsten wire electropolished in KNO_2 (Hubel, 1957). The needle was passed through the side of each nerve near the cut end and the tips of the two nerves drawn together in end-to-end anastomosis with a single suture. The operations to join the chorda and the IXth nerve usually required about 4 hours. Some of the initial animals were prepared with small strands of silk thread, but these sutures were difficult to fabricate properly and had less strength per unit diameter than the monofilament nylon.

Recording

After an average of 15 weeks (range 12–19 weeks) the rats were reoperated under pentobarbital anesthesia to expose the regenerated nerve for electrophysiological recording. The trachea was cannulated. The operations to expose the C_c-IX_p and IX_c-C_p crosses for recording usually required about 5–6 hr. Flaxedil and artificial respiration were often combined with pentobarbital to prevent reflex gasping when stimulating the back of the tongue. Whenever possible records of spike activity were obtained not only from the regenerated nerve but also from the normal chorda tympani and IXth nerves on the other side of the rat. In all cases the nerves were cut, the sheath stripped back, and the bare, whole nerve laid on a pair of 120 μ diameter nichrome wire electrodes separated by about 2 mm. Impulses recorded with these electrodes were fed push–pull into an RC amplifier whose single sided output led to a monitoring audio system and oscilloscope, and into an electronic summator. The summator output was recorded by a Grass polygraph inkwriter. The summator, whose circuit has been described previously (Diamant *et al.*, 1965), produced a running average of neural activity by rectifying and smoothing the spike output of much of the nerve. The rise time constant was 0·075 sec and the fall time constant 0·22 sec. A thermocouple connected to one polygraph channel continuously monitored the temperature of the stimulus solutions at the tongue.

The taste solutions were applied through a gravity flow system from a funnel-stopcock arrangement above the animal's head. They flowed through a small glass tube onto the surface of the tongue. Usually, no tongue chamber was used. To stimulate the region of the circumvallate papilla the glass tube was inserted far into the throat. Often considerable rinsing was ne-

cessary before sizable taste responses could be obtained from the back of the tongue. Responses to cooling were very easy to elicit from the IXth nerve and as a result it was necessary to keep the solutions at a constant warm temperature of approximately 34°C. The following sequence of chemicals was given at least twice for each nerve tested: 0·3 M NH_4Cl, 0·3 M NaCl, 0·3 M KCl, Ringer, 0·3 M NH_4Cl, 1·0 M sucrose, 1·0 M fructose, 2·0 M glycerol, 0·3 M NH_4Cl, distilled water control, 0·05 M Na saccharin, 0·05 M quinine hydrochloride, 0·3 M NH_4Cl, HCl (pH 2·18), citric acid (pH 2·50), 0·3 M NH_4Cl, acetic acid (pH 2·55). Each of these chemicals was followed by a distilled water rinse. When the peak amplitude of the response to 0·3 M NH_4Cl varied by more than $\pm$ 15% from the previous application of 0·3 M NH_4Cl, the last four stimuli were repeated. 0·3 M NH_4Cl elicited good responses from both the normal chorda tympani and IXth nerves and for this reason it was used as the standard reference stimulus. All solutions were made up in distilled water. About 25 cc of stimulus and 50 cc of distilled water rinse were used.

In the analysis of the summator records the peak responses were measured and these values set relative to the peak response to 0·3 M NH_4Cl which was called 100.

RESULTS

In Fig. 2 the average values for the peak summated response are shown for different chemicals for the normal chorda and the normal IXth nerves. It is clear that some chemicals stimulate the two nerves to differing degrees. On a relative basis Ringer and NaCl are much better stimuli for the chorda while quinine and saccharin are more effective with the IXth nerve. (Although it may be obvious I should like to emphasize that it is inaccurate to say, for example, that quinine activates the IXth nerve more than the chorda. These measurements are relative to the response to 0·3 M NH_4Cl; they are not absolute values). These are the chemicals that are of interest here because their responses can indicate whether a cross-regenerated taste nerve is responding like a normal chorda or a normal IXth nerve. Thus, in the succeeding figures, responses to Ringer, NaCl, quinine and saccharin are presented. (The acetic acid response also discriminated between the two nerves, but the time course of the response was variable, and furthermore the normal IXth nerve value is an average of results from only two animals.) The response to KCl, relative to 0·3 M NH_4Cl, is about the same for the chorda tympani and IXth nerves. Yet, as was particularly clear during the actual recording, KCl was a good discriminator between the two nerves when compared with other salts. That is, in the normal chorda the re-

sponse to KCl was without exception smaller than that to Ringer or NaCl, while in the normal IXth, with one exception KCl produced a larger peak response than either NaCl or Ringer. The inclusion of responses to KCl in the succeeding figures makes the analysis of the data independent of the use of 0·3 M NH_4Cl as the reference chemical.

The variability in Fig. 2 is due primarily to individual differences among rats. (In Figs. 3 and 4 variability within the same rat can be seen and compared with individual differences.)

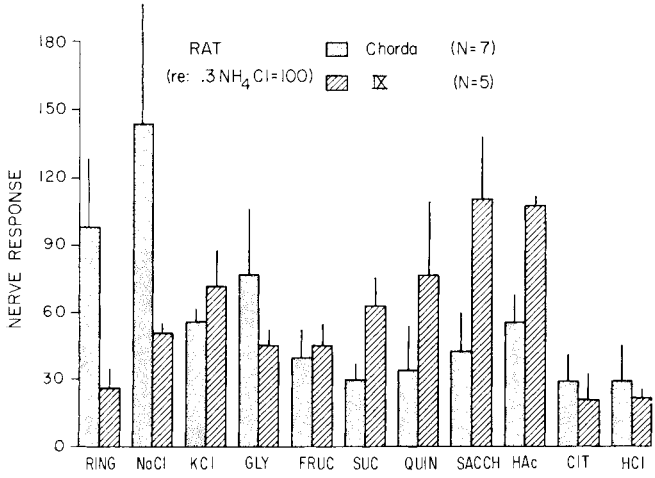


FIG. 2. Peak summated action potential discharge to various taste solutions for the normal chorda tympani and IXth nerves. For the chorda $N = 6$ for the acids. For the IXth nerve $N = 3$ for sucrose and citric acid, $N = 2$ for HAc and HCl. See methods for stimuli. In Figs. 2–7 responses are relative to the peak response to 0·3 M NH_4Cl. In Figs. 2–7 one standard deviation is plotted for each of the bars.

The effect of regeneration, *per se*, must be considered. If the chorda is simply cut and allowed to regenerate will this change the response? In Fig. 3 results are shown for the two rats with regenerated chordas ($C_c - C_p$). The black bars correspond to the response from the regenerated chorda and the dotted bars to the response from the normal chorda of the same rat. For each chemical the pair of bars is essentially the same height. Note that rat No. 22 gave a greater relative response to quinine and saccharin than rat No. 20. This figure indicates that regeneration, *per se*, had no effect upon the relative peak summated response to these chemicals.

It is possible that while simple regeneration might not effect the response, forcing a taste nerve to regenerate to the tongue through a different nerve

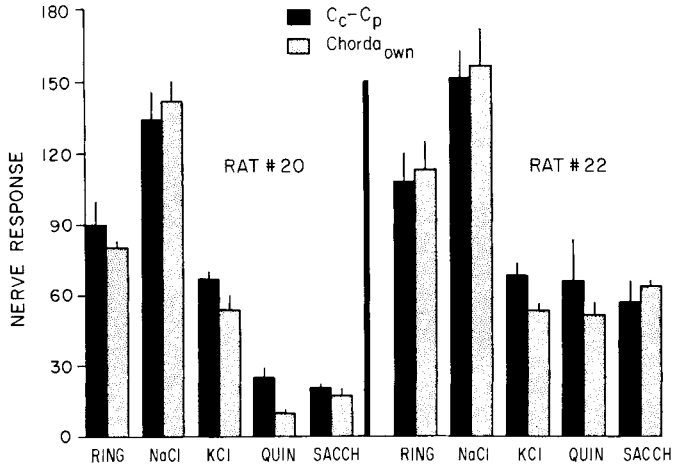


FIG. 3. Peak summated responses of the right regenerated chorda tympani nerve compared with responses from the left normal chorda tympani nerve of the same rat. Taste stimuli: Ringer, 0·3 M NaCl, 0·3 M KCl, 0·05 M quinine hydrochloride, 0·05 M Na saccharin. All responses are relative to 0·3 M NH_4Cl whose peak response is called 100. Data for two rats.

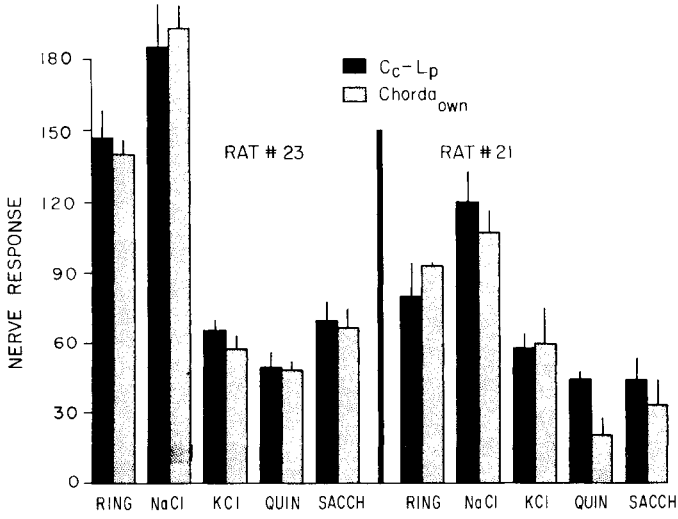


FIG. 4. Peak summated responses of the right chorda tympani which has regenerated through the peripheral stump of the lingual nerve to the anterior part of the tongue. These responses are compared with responses of the normal left chorda tympani to five different taste solutions. Data for two rats.

might alter the response. Figure 4 compares the response from the normal chorda with the response from the chorda which has regenerated down the old lingual stump ($C_c - L_p$; see Fig. 1A). This situation also differs from the simple regeneration of Fig. 3 since the normal non-taste nervous innervation of the tongue has also been disturbed, for gross observations at the time of recording indicated that the central part of the lingual had not

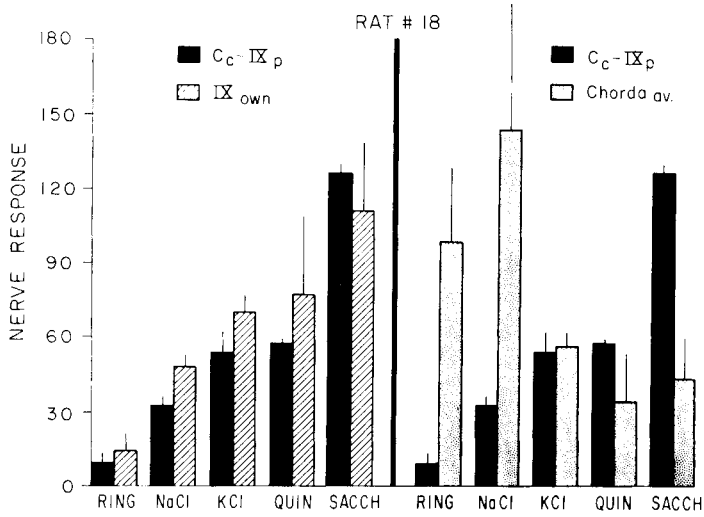


FIG. 5. Peak summated responses from the cross-regenerated chorda tympani nerve ($C_c - IX_p$) growing to the back of the tongue compared with average values for normal chorda tympani nerve responses (see Fig. 2) on the right of the figure, and with responses from the rat's own normal IXth nerve on the left of the figure. Rat No. 18. The black bars depicting the response of the cross-regenerated chorda are the same on the left and right sides of the figure.

regenerated into the tongue. It is clear from Fig. 4 that there is no difference in response for the $C_c - L_p$ compared with the rat's own normal chorda. This is true even in the presence of sizable individual differences in the relative magnitude of the responses to Ringer and NaCl.

The values shown in Figs. 3 and 4 for the four control rats (Nos. 20–23) are averages of at least two quantifiable records of the response to each chemical. Usually three measures were averaged. From Figs. 3 and 4 it may be concluded that regeneration of a taste nerve does not alter the relative peak summated responses to taste solutions. It also follows that the right and left chorda of the same rat give approximately identical relative responses, in contrast to chordas from different rats.

Diverting the chorda tympani nerve to the back of the tongue through

the IXth nerve (C_c—IX_p; see Fig. 1B) alters the taste responses that can be recorded from this new cross-regenerated chorda tympani nerve. In Fig. 5 responses from the cross-regenerated chorda of rat No. 18 are compared with group values for the normal chorda on the right side of the figure and with the rat's own normal IXth nerve responses on the left side of the figure. Responses from the cross-regenerated chorda have taken on the

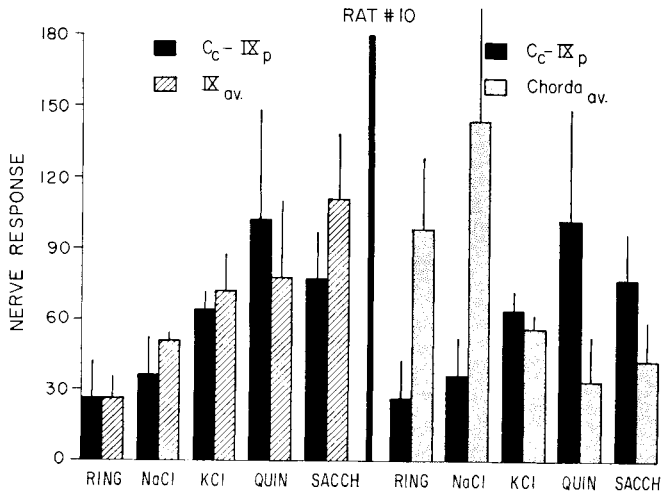


FIG. 6. Same plot as in Fig. 5 for rat No. 10. Average normal IXth and normal chorda values from Fig. 2.

characteristics of the normal IXth nerve and are no longer the same as responses from the normal chorda. Note that the relative response to KCl in the cross-regenerated chorda is much greater than the response to NaCl or Ringer—a condition that has never been observed in the normal chorda. The same results may be seen in Fig. 6 for rat No. 10. Responses from the cross-regenerated chorda resemble those of the normal IXth. Again KCl is a better stimulus than either NaCl or Ringer. (One would not expect the KCl response, itself, to change since it is of about the same relative magnitude for the normal chorda and normal IXth. See Fig. 2.) Two additional rats with cross-regenerated chordas (C_c—IX_p) gave the same results as those presented in Figs. 5 and 6.

The logical parallel of the above experiment is, of course, to make the IXth nerve grow through the chorda to the front of the tongue (IX_c—C_p; see Fig. 1C). In Fig. 7 the responses of the cross-regenerated IXth nerve of rat No. 15 are compared with average taste responses from the normal

IXth and normal chorda tympani nerves. The relative responses from the cross-regenerated IXth nerve bear a considerably greater resemblance to responses from the normal chorda than to responses from the normal IXth nerve. The values in Figs. 5–7 for the cross-regenerated taste nerves are averages of at least four measures for each chemical with two exceptions; two measures for No. 18 for quinine and saccharine and three for No. 15 for quinine.

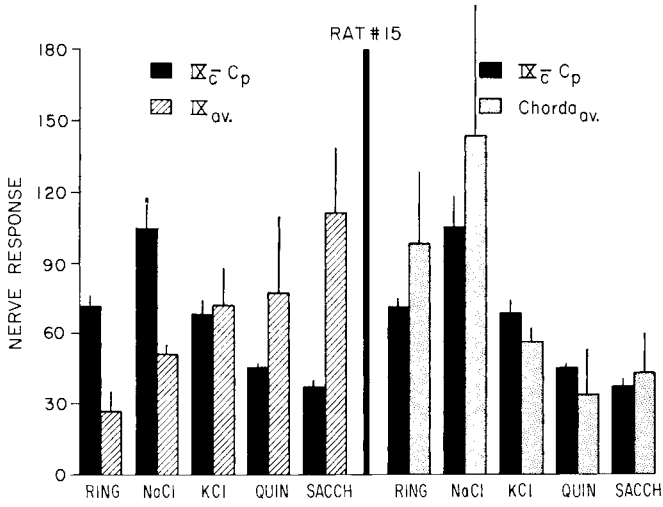


FIG. 7. Peak summated responses from the cross-regenerated IXth nerve ($IX_c - C_p$) growing to the front of the tongue compared with average values for the normal IXth on the left and average values for normal chorda on the right of the figure. Rat No. 15.

The necessity for summarization of data makes it expedient to select one characteristic of the response such as the peak magnitude, but it should be pointed out that the responses of the cross-regenerated nerves changed not only in relative peak magnitude but also in overall form. The original summator records of the cross-regenerated IXth nerve ($IX_c - C_p$) of rat No. 15 may be seen in the center column of Fig. 8. The left column shows responses from a normal chorda and the right column responses from a normal IXth nerve. Scanning each row it is clear that the cross-regenerated IXth nerve has changed its electrophysiological characteristics. Its summated responses now look like those of the normal chorda, not the normal IXth nerve. This is true for both peak amplitude and waveform.

The $IX_c - C_p$ union in rat No. 1 failed. The union in rat No. 7 was suc-

cessful, but the author mistook other degenerated tissue for the experimental nerves and did not discover the union until the animal had been sacrificed. Electrophysiological records were obtained from the other three

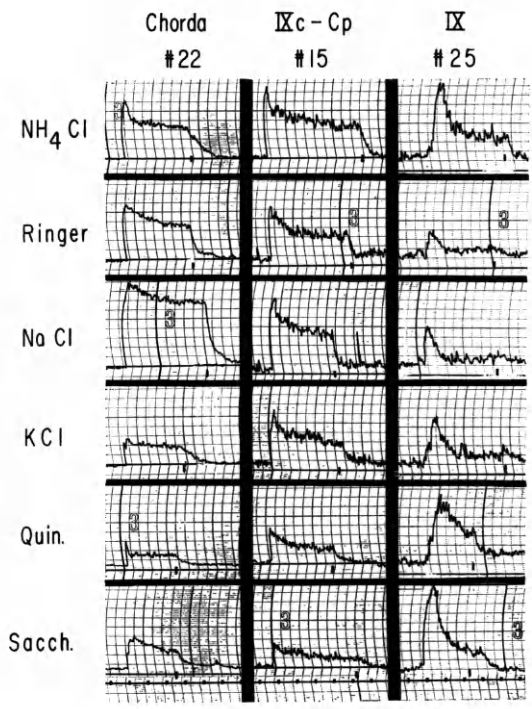


FIG. 8. Original summated records of action potential activity from three nerves: the normal chorda tympani nerve of rat No. 22 in the left column, the cross-regenerated IXth nerve ($IX_c - C_p$) of rat No. 15 in the center column, and the normal IXth nerve of rat No. 25 in the right column. Each row represents the response of these three nerves to a specific taste stimulus. The small vertical dash beneath each record is a marker which gives a crude indication of the onset of the distilled water rinse. Time base in 10 sec intervals. The summated responses from the normal chorda of rat No. 22 and the normal IXth of rat No. 25 (a normal control rat) were selected for presentation in this figure because the peak values closely approximate the average peak values for the normal chorda and IXth nerves as presented in Fig. 2.

$IX_c - C_p$ preparations and these results confirm the findings presented for rat No. 15 in Figs. 7 and 8.

A discussion of the general issues raised by the present experiment will not be included here.

SUMMARY

Regenerated taste nerves will re-form taste buds. The chorda tympani nerve normally innervates taste buds on the anterior two-thirds of the rat tongue, and the IXth nerve the posterior one-third. It was possible to join the chorda tympani and IXth nerves using microsutures, such that the chorda tympani grew to the back of the tongue, and in other rats, the IXth nerve grew to the front of the tongue. The experiment compared summated neural activity from the following whole taste nerves: the normal IXth, the cross-regenerated IXth, the normal chorda and the cross-regenerated chorda.

CONCLUSIONS

1. It is possible to quantify taste activity from the normal IXth nerve in the rat. For some taste stimuli the relative magnitude of the summated response is different for the normal IXth and the normal chorda tympani nerves.

2. Regeneration of the chorda tympani nerve into the posterior portion of the tongue changes the relative magnitude and time course of the summated nerve responses so that they closely resemble those of the normal IXth nerve, and not the normal chorda.

3. In a parallel fashion regeneration of the IXth nerve into the anterior portion of the tongue causes the taste responses to closely resemble those of the normal chorda tympani nerve, and not of the normal IXth.

4. Regeneration, *per se*, does not alter these taste response characteristics.

5. The simplest plausible hypothesis which accounts for the above results is that the relative reactivity of taste receptor cells to different chemicals is determined by inherent properties of the tissue from which the cells are formed—the epithelium at the front differs from that at the back of the tongue.

6. When unsupported by additional assumption, the following two hypotheses can be rejected:

(a) that in the adult rat the relative responsiveness of taste receptor cells to different chemicals is determined by the taste fibers (chorda tympani and IXth nerves) which innervate them.

(b) that free taste nerve endings are normally directly stimulated by chemicals. (The response properties of cross-regenerated taste nerves should not change.)

REFERENCES

DIAMANT, H., B. OAKLEY, L. STRÖM, C. WELLS and Y. ZOTTERMAN. A comparison of neural and psychophysical responses to taste stimuli in man. *Acta Physiol. Scand.* **64,** 67–74, 1965.

GUTH, L. Taste buds on the cat's circumvallate papilla after reinnervation by glossopharyngeal, vagus and hypoglossal nerves. *Anat. Record,* **130,** 25–37, 1958.

HUBEL, D. H. Tungsten microelectrode for recording from single units. *Science,* **125,** 549–550, 1957.

OAKLEY, B. and R. M. BENJAMIN. Neural mechanisms of taste. *Physiol. Revs.* **46,** 173–211, 1966.

VON VINTSCHGAU, M. Beobachtungen über die Veränderungen der Schmeckbecher nach Durchschneidung des N. glossopharyngeus. *Pflüg. Arch. ges. Physiol.* **23,** 1–13, 1880.

WHITESIDE, B. Nerve overlap in the gustatory apparatus of the rat. *J. Comp. Neurol.* **44,** 363–377, 1927.

CHEMOTOPIC CODING FOR SUCROSE AND QUININE HYDROCHLORIDE IN THE NUCLEUS OF THE FASCICULUS SOLITARIUS*

BRUCE P. HALPERN

State University of New York, Upstate Medical Center
Syracuse, New York

IN several sensory systems, modification of non-intensive characteristics of the stimulus produces a change in the location of maximum neural response magnitude within central nervous system transfer nuclei. Such a

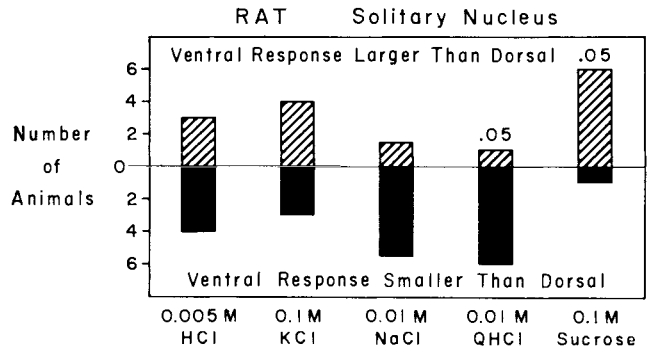


FIG. 1. Number of animals with an increased, or a decreased, relative response magnitude at the more ventral of two electrode depths along a single vertical puncture in the nucleus of the fasciculus solitarius (NFS) (at each depth, magnitude re: response to 0·1 M NaCl). Median separation: 50 microns. Based on seven rats. Anterior tongue region stimulated by each of the five chemicals shown, and by the 0·1 M NaCl reference standard.

change in maximum responsiveness when found in the auditory system is termed tonotopic coding. A somewhat similar type of organization exists in the gustatory system (Halpern, 1964; Halpern and Nelson, 1965;

* Supported by NIH Grant NB-04121. Present address: Dept. of Psychology and Division of Biology Cornell University, Ithaca, New York.

Halpern, 1965). The term chemotopic coding has been applied to this gustatory encoding pattern. In previous studies of this gustatory phenomenon, neural responses were recorded at two depths along single electrode tracks in the nucleus of the fasciculus solitarius (NFS) of the rat (Halpern, 1964; Halpern, 1965). It was found that responses to gustatory stimulation of the anterior region of the tongue with sucrose were larger at the more ventral recording sites. In contrast, responses to quinine hydrochloride (QHCl) were smaller at the more ventral sites. Both differences were significant at the 5% level (Fig. 1). Responses to NaCl also tended to

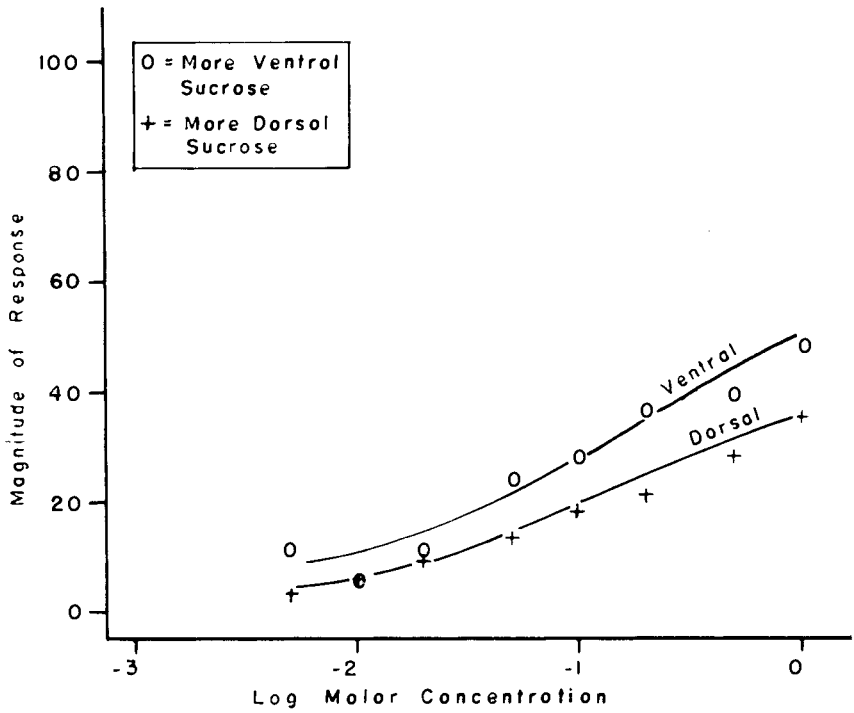


FIG. 2. Relative response magnitudes recorded at two depths along a single electrode track in NFS to sucrose solution stimulation of the anterior region of the tongue (response magnitude to 0·1 M NaCl at each depth = 100). Medians of three rats (not those included in Fig. 1). Separation between recording depths: 50 microns (median).

decrease ventrad, but the change in magnitude did not reach significance. For a wide range of sucrose concentrations, neural response magnitudes were larger at more ventral recording sites (Fig. 2). Thus, previous studies

demonstrated that responses to sucrose increased with greater depth within NFS, while responses to QHCl became smaller.

The present experiment was designed to examine the chemotopic organization of NFS in more detail.

METHODS

The results to be reported were obtained from 21 Wistar rats of both sexes. The general surgical, recording, stimulating, and histological procedures have been previously described (Halpern and Nelson, 1965). Procedures specific to the present experiment will be given in detail, while others will be outlined.

The animals were anesthetized, and the dorsal surface of the medulla oblongata exposed for exploration. A thermometer was placed between the ventral surface of the animal and a d.c. body warmer upon which the animal rested. The temperature was measured as soon as surgical anesthesia was reached, and then maintained, $\pm 2°C$, throughout the experiment.

The recording electrode was nickel–chrome wire, 25·4 microns in diameter. With the exception of the cross-sectional area at the tip, the electrode was completely insulated with enamel. Stiffening was produced with one to two coats of Epoxylite 6001 – M Electrode Insulator.* Electrode resistance was approximately 80 ohms.

The multiunit neural activity was led to a Grass P-5 a.c. preamplifier. Frequency response was set to be down 50% at 35 cps and 2 kc, down 10% at 100 cps and 650 cps. The amplified neural activity was led thru a pulse height gate (variable bottom clipper), and then into a summator. The summator output drove a critically damped, rectilinear Texas Rectiriter (Fig. 3). Stimuli were confined to the anterior region of the tongue by a plexiglas chamber. Stimuli were applied in a *W-ABC-,W-CBA-W-ABC-W* sequence†. When several concentrations of different chemicals were used, the lowest concentration of each solution was presented first. Each stimulus consisted of 25 ml of liquid which flowed over the anterior region of the tongue in approximately 8 sec. The flow chamber was allowed to remain empty for at least 12 sec after stimulus flow, and then a 45 ml distilled water wash began. Forty seconds elapsed between the completion of water wash and the onset of the next stimulus. All liquids were maintained at $24 \pm 1°C$. The conductivity or pH of solutions was checked regularly. Sugar solutions were prepared on the day of use from concentrated stock solu-

* The Epoxylite Corporation, South El Monte, California, U.S.A.

† *A*, *B* and *C* are three different stimuli. *W* is water, presented as a stimulus.

tions stored at 1°C. The sucrose, NaCl, and HCl were A.R. grade. The maltose was reagent grade. The QHCl was N.F., and the quinine sulphate (QSO_4) and caffeine were U.S.P. The fructose was C.P. (sp. rot. $-85{\cdot}6°$ in water $C = 2$. Paper CHR : Homogeneous). The sucrose octa-acetate (SOA) was $99^{+}\%$. The sodium dehydrocholate was General Biochemical No. 5434 F, and the saccharin was Matheson Coleman, and Bell SX5. The quinidine hydrochloride was K and K Laboratories No. 18110.

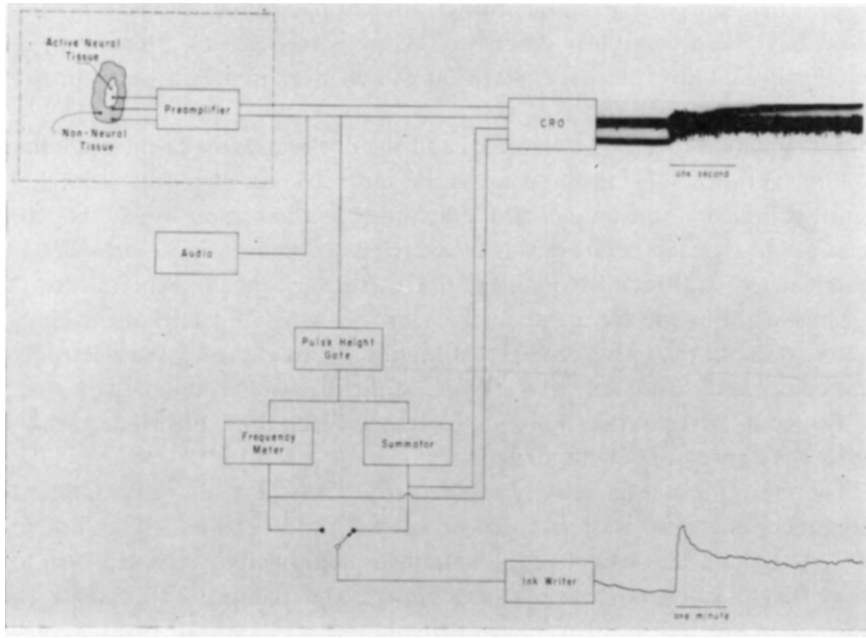


FIG. 3. Schematic representation of the recording setup. The active neural tissue was NFS. The non-neural tissue, which was contacted with silver electrodes, was muscle near the recording site. The spike-like trace at the CRO is a multiunit response to 0·1 M NaCl. The solid, slowly rising CRO trace is the summator output to the same stimulus. The summator had a 0·75 second rise time, a 5·5 second fall time. The Ink Writer was a critically damped 0—1 ma. Texas Recti-riter. The Frequency Meter was not used in the present experiment.

The recording procedure for each of these experiments may be divided into four successive phases.

Phase One: The recording electrode was moved ventrad thru NFS in steps of 50 to 100 microns until the ventral limit of the gustatory response zone was reached. The median distance from dorsal to ventral limit (total response area) was 370 microns. At each step, response magnitudes to

0·05 M and 0·1 M NaCl were measured. The recording electrode remained at the ventral limit of the response zone for at least 10 min.

Phase Two: The recording electrode was now moved dorsad, in steps of 20 to 30 microns, with 0·05 M NaCl stimulation at each step. This dorsal passage was continued until an obvious change in response magnitude was observed, or until a cumulative electrode movement of 100 microns was reached, whichever occurred first. The electrode remained at this depth for at least 5 min (this time delay was also used in subsequent *phases*). Then, the stimuli of the particular experiment were applied. Following presentation of these stimuli, dorsal electrode movement resumed. This dorsad sequence continued until the response to 0·05 M NaCl reached 10 to 20% of the largest 0·05 M NaCl response measured during *phase one* (the initial ventral passage of the electrode).

Phase Three: When this 10 to 20% magnitude was reached during the dorsal movement of the electrode, subsequent dorsal steps were of fixed size. At each of these steps, the full complement of stimuli was used. This fixed step procedure was continued until the magnitude of responses to 0·05 M NaCl decreased to 10% of the maximum magnitude of the response to this solution.

Phase Four: When this diminished response to NaCl was reached, the variable step dorsad sequence, *phase two*, was reinstituted. Dorsal movement was continued until the dorsal limit of the gustatory response zone was reached. At least 6 and often 10 hours were required to map a single electrode track. Therefore, data were collected from only one responsive electrode track in each animal.

RESULTS

A criterion was established for designating the response at a depth as unusually large (a "maximum"), using previous multiunit data recorded from the rat chorda tympani and from NFS sites. Chorda tympani response magnitudes to 0·1 M sucrose were less than 25 (0·05 M NaCl = 100), while responses to 0·02 M QHCl ranged from 19 to 25 (Halpern, 1959; Pfaffmann, 1962). Previous chemotopic studies of NFS found that the largest response magnitudes to 0·02 M QHCl and 0·1 M sucrose exceeded the usual chorda tympani magnitudes by at least one-third (Halpern, 1964; Halpern, 1965). Therefore, for the present experiment, the criterion was established that for a "maximum" to be noted, the median response at a

depth must be 42% or more of the largest response to 0·05 M NaCl recorded anywhere in that puncture.*

The above criterion segregated the 21 response patterns obtained into four categories (Table 1). Animals in the Q category had a maximum for QHCl but not for sucrose. In confirmation of previous results, the QHCl maxima were relatively dorsal (Fig. 4).†

TABLE 1. CHEMOTOPIC RESPONSE CATEGORIES IN THE NUCLEUS OF THE FASCICULUS SOLITARIUS (NFS)

		Q	QS	NP	S
Characteristics		Maximum[1] to 0·02 M QHCl, not to 0·1 M sucrose	Maxima to both 0·02 M QHCl and 0·1 M sucrose	No maxima	Maximum to 0·1 M sucrose, not to 0·02 M QHCl
Number of animals		8	8	3	2
Depth of largest response[2,3]	QHCl	39±9	42±13	40±12	64
	Sucrose	55±5	47±13	37±12	66
Largest response magnitude[3]	QHCl	71±9	68±13	32±4	39
	Sucrose	21±12	55±11	24±6	58

[1] Criterion for a maximum is magnitude ≥42% of largest response to 0·05 M NaCl in that puncture.

[2] Percentage of total response area depth (dorsal to ventral limit).

[3] Median, ± semi-interquartile range, across individual rat median [magnitude, (0·05 M NaCl = 100)].

A second category, QS, had maxima for both QHCl and sucrose. The QHCl maxima were in the dorsal half of the response area in all eight animals. Each sucrose maximum was ventral to its associated QHCl maximum (Fig. 5). The Q and QS categories contain 76% of the animals reported. An interesting relationship exists between the Q and QS maxima for QHCl, and single unit responses to QHCl. The median for all QHCl maxima in the present experiment was 69 (0·05 M NaCl = 100). Oakley reported that the "composite" response to 0·02 M QHCl of single units in NFS was 60 (re: 0·05 M NaCl) (Oakley, 1962).

* A median response at each depth to water, when presented as a stimulus, was subtracted from all other response magnitudes at that depth before any relative response magnitudes were determined.

† Dorsal 50% of response area in 6/8 animals. QHCl maximum dorsal to largest sucrose response in 7/8.

Thus the areas designated QHCl maxima in the present experiment may correspond to those in which a large proportion of second order neurons strongly fired by QHCl stimulation are concentrated. Since QHCl maxima

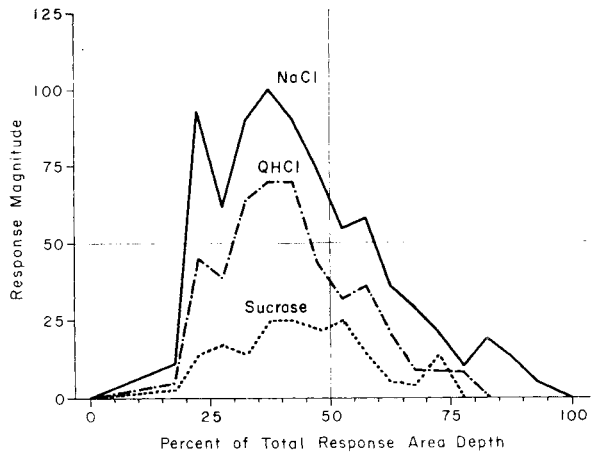


FIG. 4. Summated response magnitudes recorded in the NFS of one "Q" rat. In this and subsequent graphs (except 6A), response magnitudes are expressed as percentages of the maximum response of each rat to 0·05 M NaCl (0·05 M NaCl = 100). The distance from the beginning (dorsal limit) to the end (ventral limit) of the responsive area is converted into percentages. Responsive area depth (dorsal to ventral limit) was 520 microns in this animal. Solid lines will represent NaCl; broken lines, QHCl (quinine hydrochloride) and dotted lines, sucrose. Except when otherwise indicated, NaCl is 0·05 M; QHCl, 0·02 M; and sucrose, 0·1 M.

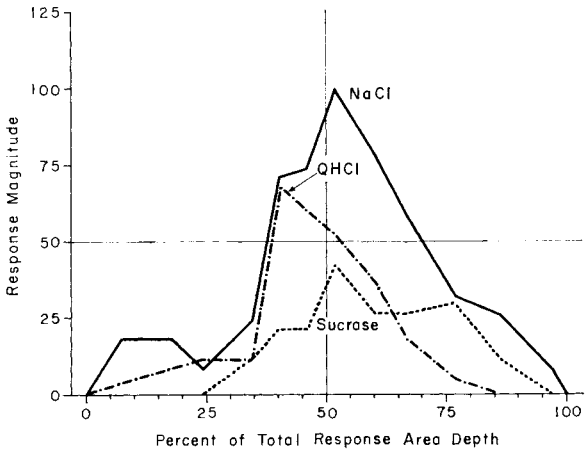


FIG. 5. Summated response magnitudes recorded in the NFS of one "QS" rat. The total response area depth was 301 microns (dorsal to ventral limit).

occurred in 76% of the animals in the present study, and were relatively dorsal in NFS, these areas should be sampled often in microelectrode exploration.

This correspondence between the magnitude of chemotopic maxima and single unit response magnitudes did not occur for 0·1 M sucrose, however. The "composite" single unit response magnitude to 0·1 M sucrose was 30, while the median size of sucrose maxima in the present experiment was 56, re: 0·05 M NaCl. Since sucrose maxima occurred in only 48% of the responsive animals, and were ventral, they may be infrequently found in microelectrode punctures, due to the sampling problems inherent in microelectrode techniques.

A third category, NP, did not have maxima for either sucrose or QHCl. The final category, S, had a maximum for sucrose but not for QHCl (Fig. 6A, B). This pattern, which was found in only two animals, is not very different from the QS pattern. For the 21 animals, the depth of the largest sucrose response was significantly ventral to the depth of the largest QHCl response ($p < .01$, Wilcoxon Signed-Rank Test).

It is not proposed that the above response categories represent, in different animals, several dissimilar homogeneous types of NFS. Instead, the converse of this is believed to exist. Only a single responsive electrode track was studied in each animal. Therefore, specialized sub-areas of NFS were probably sampled. A more complete exploration of a single NFS would presumably encounter most or all of the categories. Thus, the present data suggest that the anterior tongue zone of NFS is not a homogeneous structure. At least two classes of non-homogeneity exist. One is the dorsal-ventral separation between QHCl and sucrose maxima. The second is the several response patterns (categories) found in separate punctures. This latter effect may have a medial-lateral and/or rostral caudal orientation as well.

Previous studies showed that the gross dorsal-ventral difference in response magnitudes to sucrose occurred through a wide concentration range (Fig. 2). In the present experiment, the effects of concentration change on the chemotopic patterns were examined with sucrose, NaCl, and QHCl. Five animals were used, four going into the Q category (Fig. 7A, B, C) and one into the QS category (7D). The depth of the largest responses to QHCl and NaCl was not greatly affected by concentration. Similarly, peak magnitudes of responses to sucrose did not shift depth between 0·05 M and 0·1 M concentrations. However, responses to 0·5 M sucrose were considerably different from the lower concentrations. Thus, the chemotopic patterns and response maxima are not fortuitous products of a particular concentration.

The pattern change with 0·5 M sucrose may indicate that a non-electrolyte at such concentrations enters a different class of stimuli.

The maxima were located on the basis of responses to sucrose or QHCl. However, it seemed unlikely that the highly responsive areas should be

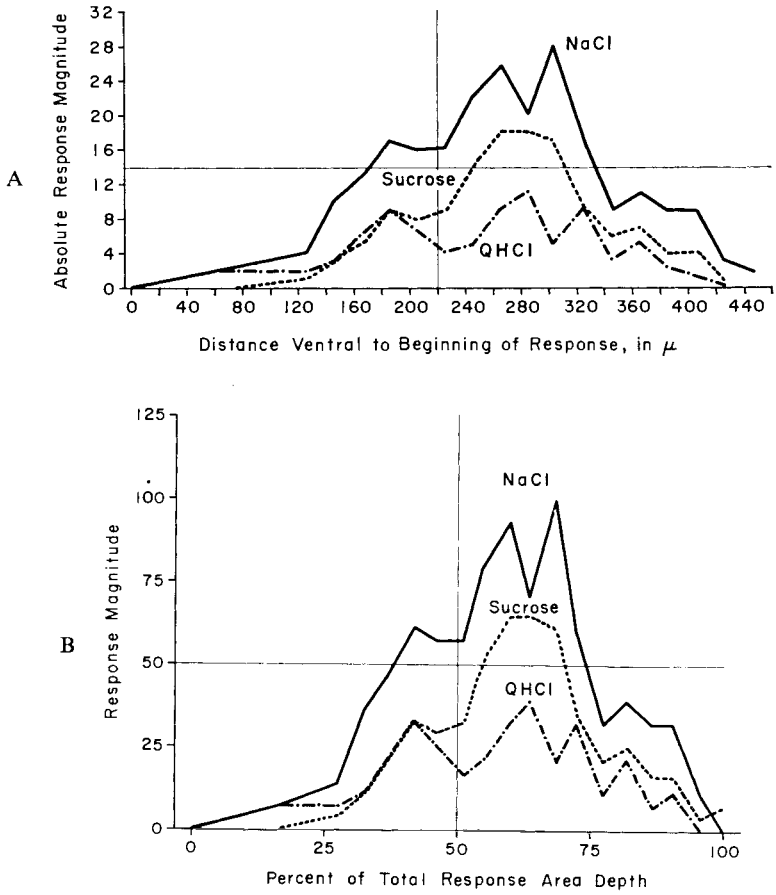


FIG. 6. Summated response magnitudes recorded in NFS of one "S" rat. *A*. Response magnitudes in absolute units. One unit is 2·5 mm pen deflection; maximum pen excursion is 50 units. Abscissa is measurements of electrode position, using a dial indicator marked in 10 micron steps. *B*. Data of *A*, converted as described for Fig. 4.

unique to those two chemicals. A number of other chemicals were therefore screened (Table 2). Of the compounds that are often described as "bitter", only the stereoisomer of QHCl, quinidine HCl, produced maxima. The depths of the quinidine HCl maxima were within ten percentage points of

the QHCl maxima. Saccharin produced very large maxima, also located within 10 percentage points of the depth of the corresponding QHCl maximum.

HCl produced maxima in three out of four cases. The magnitudes of the responses represented considerable increases over the usual responses to 0·001 M HCl. Chorda tympani response magnitudes to 0·001 M HCl ranged

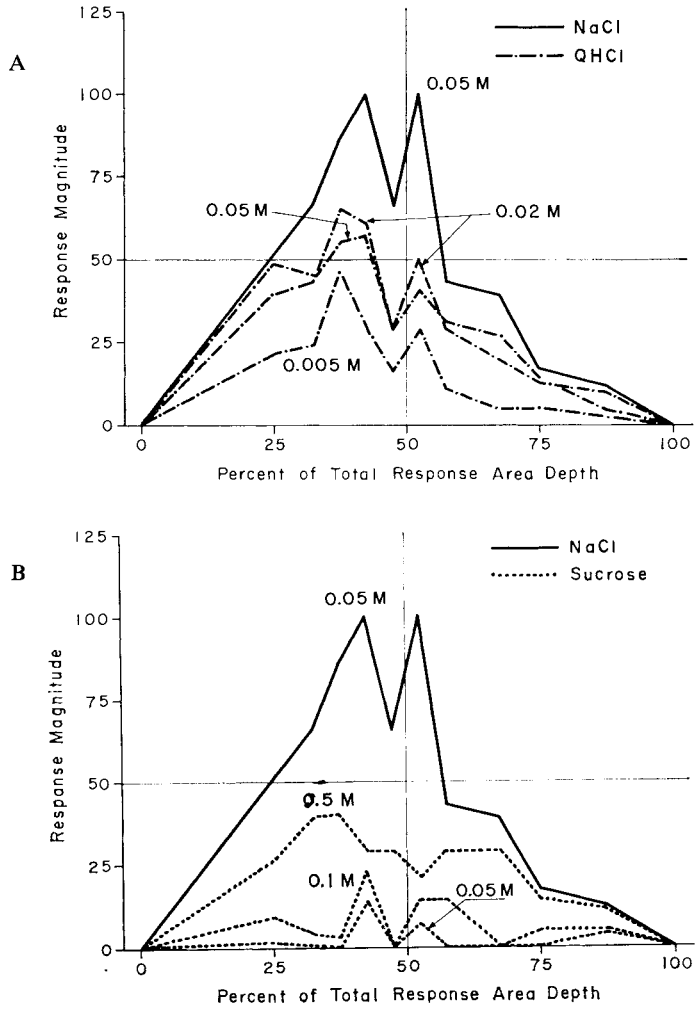
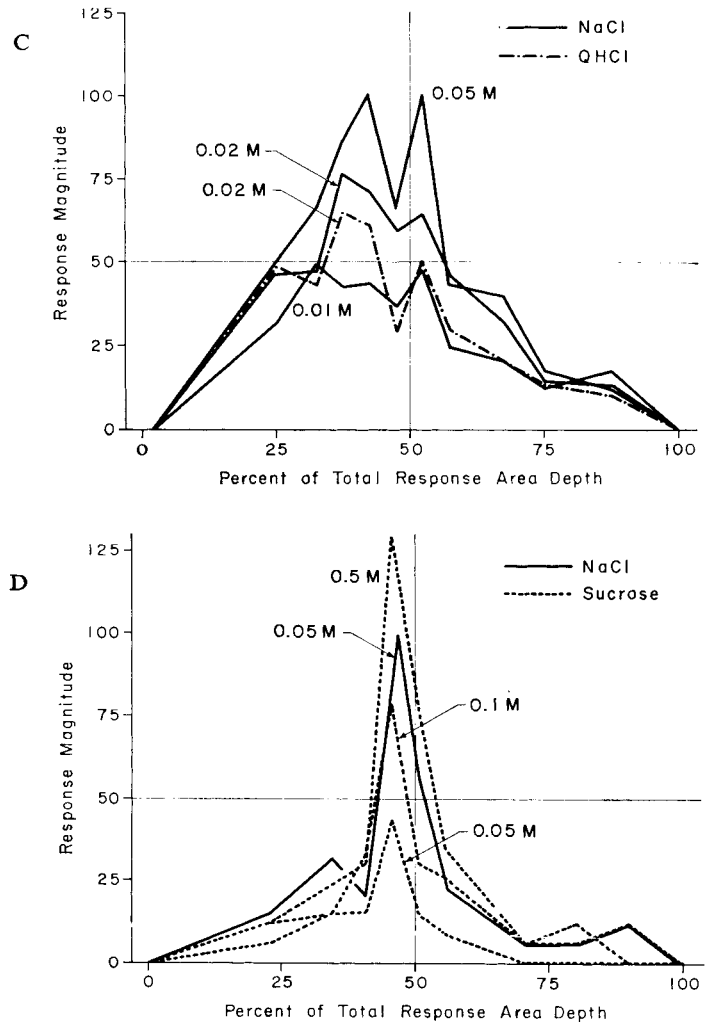


FIG. 7. Summated response magnitudes recorded in NFS. *A*, *B*, *C*. Median responses of four "Q" rats. *A*. Response magnitudes to 0·005 M, 0·02 M, and 0·05 M QHCl, and to 0·05 M NaCl. *B*. Response magnitudes to 0·05 M, 0·1 M, and 0·5 M

from 11 to 25 (0·05 M NaCl = 100) (Pfaffmann, 1962; Hardiman, 1964). This may be contrasted with the median response at NFS maxima of 61 (0·0005 and 0·001 M HCl). Hardiman's analysis of chorda tympani response functions suggested that QHCl and HCl would be related stimuli (Hardiman, 1964). This does not involve pH, however, since 0·02 M QHCl is pH 6·15, while 0·001 M HCl is pH 3.



sucrose, and to 0·05 M NaCl. *C*. Response magnitudes to 0·01 M, 0·02 M, and 0·05 M NaCl, and to 0·02 M QHCl. *D*. Response magnitudes of one "QS" rat to 0·05 M, 0·1 M, and 0·5 M sucrose, and to 0·05 M NaCl.

TABLE 2. LARGEST RESPONSE MAGNITUDES[1] OF Q AND QS RATS TESTED WITH STIMULI IN ADDITION TO QHCl, SUCROSE, AND NACl

Animal	QHCl[2] ·02 M	Quinidine HCl ·02 M	QSO_4[3] ·001 M	Caffeine ·01 M	Caffeine .1 M	SOA[4] ·001 M	Sodium Dehydrocholate ·02 M	Saccharin ·01 M	HCl ·0005 M	HCl ·001 M	Sucrose ·1 M	Fructose ·1 M	Glucose ·1 M	Maltose ·1 M
Q Animals														
R143U	63	—	17	4	—	0	—	92	—	—	25	—	—	—
R170U	60	—	0	—	0	—	—	110	—	—	10	10	—	—
R192U	56	56	—	—	—	7	4	—	—	—	21	11	—	—
QS Animals														
R141U	60	—	—	—	—	—	—	—	—	—	60	—	7	13
R179U	68	53	16	—	5	—	—	—	—	66	42	21	—	—
R195U	83	—	—	—	—	—	—	—	67	—	50	33	—	—
R196U	35	—	—	—	—	—	—	—	33	—	42	8	—	—
R200U	67	—	—	—	—	—	—	—	56	—	67	33	—	—

Dashes indicate that a particular stimulus was not used with that animal.

[1] Response magnitudes are given as percentages of the largest response to 0·05 M NaCl of each rat (i.e. ·05 M NaCl = 100).

[2] QHCl = quinine hydrochloride.

[3] QSO_4 = quinine sulphate.

[4] SOA = sucrose octa-acetate. Each number is the median of three responses, all three recorded at the same depth.

The anatomical locations of the response sites were studied in all animals reported. At the completion of each recording experiment, the electrode was moved ventral from the dorsal limit of the response zone, until large responses to NaCl were observed. When these large responses were encountered, an electrolytic lesion was made. In 20 of the animals, the marker lesion was located in the anterior tongue response zone of NFS. The brain of one rat (an S animal) was lost during histological processing.

DISCUSSION

The data of this experiment argue that, within NFS, the areas of maximum responsiveness to QHCl and sucrose are not coincident in depth. Rather, maximum responsiveness to sucrose is transposed ventrad, relative to QHCl. In addition, responses to HCl, saccharin, and quinidine HCl appear to reach maximum magnitude at depths similar to those for QHCl. The largest responses ("maxima") to these chemicals exceeded, by at least one-third, the magnitude of chorda tympani responses to them. These increases in magnitude suggest that those second order neurons which are highly responsive to peripheral gustatory stimulation by certain QHCl related compounds, or to sucrose, are concentrated at different depths in NFS. Finally, maxima to both QHCl and sucrose are found along some electrode tracks, but only QHCl maxima along others. This existence of double maxima along certain tracks and single maxima along other electrode tracks implies that some of the congregations of highly responsive units are not continuous throughout the anterior tongue zone of NFS. Further study is required to determine the molecular characteristics of the effective stimuli for the several maxima areas, as well as the spatial organization of these areas and their role in gustatory encoding.

REFERENCES

HALPERN, B. P. 1959. Gustatory responses in the medulla oblongata of the rat. Cited in: FROMMER, G. P., Gustatory afferent responses in the thalamus, in: KARE, M. R., and B. P. HALPERN, editors *Physiological and Behavioral Aspects of Taste* (University of Chicago Press, Chicago, 1961) 50.

HALPERN, B. P. 1964. Areal differences in gustatory responses recorded in the nucleus of the fasciculus solitarius. *Federation Proceedings*, **23**, 465 (Abstract).

HALPERN, B. P. and L. M. NELSON. 1965. Bulbar gustatory responses to anterior and to posterior tongue stimulation in the rat. *Amer. J. Phys.* **209**, 105.

HALPERN, B. P. 1965. Chemotopic organization in the bulbar gustatory relay of the rat. *Nature*, 208, 393.

HARDIMAN, C. W. 1964. Rat and hamster chemoreceptor responses to a large number of

compounds and the formulation of a generalized chemosensory equation. Ph.D. Thesis. The Florida State University, Tallahassee, Florida.

OAKLEY, B. 1962. Microelectrode analysis of second order gustatory neurons in the albino rat. Ph.D. Thesis. Brown University, Providence, Rhode Island.

PFAFFMANN, C. 1962. Sensory processes and their relation to behavior: Studies on the sense of taste as a model S-R system, in: S. KOCH, editor. *Psychology: A study of Science* (McGraw-Hill, New York) 380.

THALAMIC REPRESENTATION OF TASTE QUALITIES AND TEMPERATURE CHANGE IN THE CAT

NOBUSADA ISHIKO, MASAHIRO AMATSU and YOSHIHIKO SATO

Department of Physiology, Kumamoto University Medical School, Kumamoto, Japan

THALAMIC responses within or in the vicinity of the *nucleus ventralis posteromedialis* (VPM) were recorded from a cat, anesthetized with sodium amobarbital and/or chloralose, with a platinum-plated indium electrodes of 20–100 μ in tip diameter. To quantify the electrical activity from a small cluster of neurons around the tip of the electrode, an ink-writing recorder preceded by a "summator" or the current-averaging device was employed, while impulse discharges were monitored on an oscilloscope. In most of the cats, the whole surface of the tongue was stimulated with the aid of a lucit box (Ishiko and Amatsu, 1964). As taste stimuli, sodium chloride, deionized water, hydrochloric acid and quinine hydrochloride were employed, the latter two being dissolved in Ringer's solution. As thermal stimuli, warm or cold Ringer's solution was used.

INTEGRATION OF TASTE AND TEMPERATURE RESPONSES

Thalamic responses of 11 cats to taste and temperature stimuli were recorded from 112 clusters of neurons. Three kinds of responses were observed at various recording sites of different animals: (i) The facilitatory response (Fig. 1 A), which showed an increase in the spontaneous activity (middle trace in each record) in response to application of stimuli (bottom bar) accompanied by an upward deflection of the summed record (top trace), (ii) the inhibitory response (B), which indicated a decrease in the spontaneous activity associated with a depression of the summed record, and (iii) the off-response (C), observed after replacement of stimulating solution by Ringer.

All these three types of the responses, such as shown in Fig. 1, were recorded with every kind of taste stimuli as well as thermal stimuli. But, as

shown in Fig. 2, the frequency of appearance of the facilitatory (filled bars) or the inhibitory (empty bars) response type depends on the kind of stimuli applied. With a given stimulus, a characteristic distribution in the

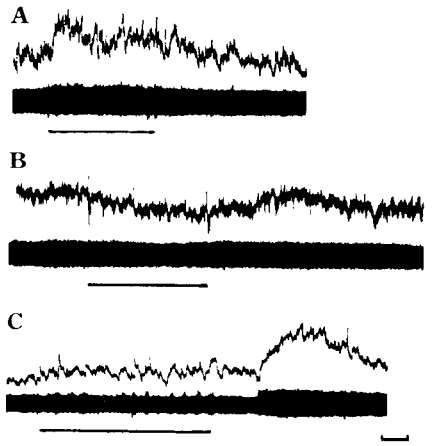


FIG. 1. Records indicating the facilitatory response to 7 mM (A), the inhibitory response to 14 mM HCl (B) and the off-response after replacement of 14 mM HCl to Ringer's solution (C), respectively. In each record, obtained from different cats, the top trace indicates the summed thalamic response, the middle the impulse discharge and the bottom bar the duration of stimuli. A bar at the bottom in the right-hand corner shows 10 sec.

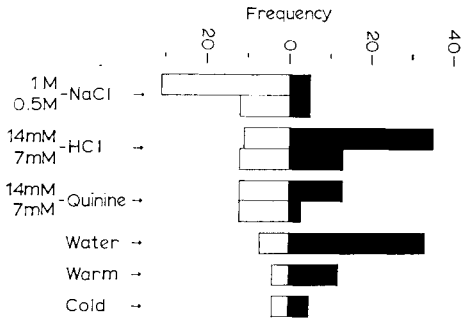


FIG. 2. Frequency distribution of the facilitatory (filled bars) and inhibitory (empty bars) responses of 112 neurons to taste and temperature stimuli.

frequency of appearance exists between the facilitatory response and inhibitory one, and reduction in the stimulus intensity results in a decrease of the frequency distribution in either one of the two response types.

In a statistical sense, such a distinct frequency distribution of the two response types for each stimulus could happen within the thalamic relay

nucleus for taste and temperature in every cat. To integrate the activity within the whole nucleus the response type distribution relative to that of the facilitatory response to 1 M NaCl was computed for each kind of stimulus, while the magnitude of all the summed responses to taste and thermal stimuli at every 5 or 10 sec interval during and after cessation of stimulus was totalized for each response type. Frequency distribution of each response type expressed by the relative value was then multiplied by the mean

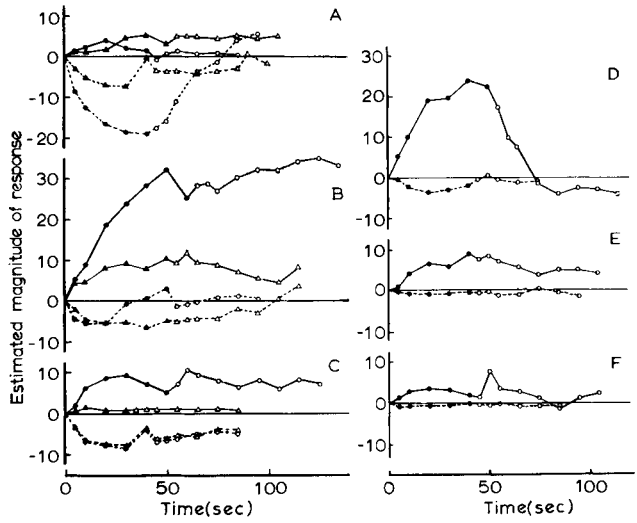


FIG. 3. Spatio-temporal pattern of NaCl (A), HCl (B), quinine (C), water (D), warm (E) and cold (F) Ringer's solution, the temperature range in the latter two being shown in Table 1. Solid and broken lines indicate the facilitatory and inhibitory responses, respectively, during (filled symbols) and after (empty ones) stimulus. In A–C, circles and triangles show the response to 1 M and 0·5 M for NaCl, 14 mM and 7 mM for both HCl and quinine.

magnitude of the response to the respective stimulus obtained at successive times; the time course of the response thus obtained is indicated in Fig. 3 A–F. The spatio-temporal pattern of the responses to 4 kinds of taste stimuli, as represented in Fig. 3 A–D, differs not only in the magnitude but also in the temporal pattern of both the inhibitory (dotted lines) and the facilitatory (solid ones) response with each other, e.g. the facilitatory response to HCl (B) and quinine (C) persists for more than 1 min even after cessation of the stimulus (hollow symbols), while the facilitatory response to water (D) and the inhibitory response to NaCl (A) returns to the resting level (zero line) within 20–30 sec after the rinse with Ringer's solution. In addition, the difference in stimulus strength appears to be

expressed in the thalamic neurons by a change in the integrated magnitude of the responses of either one of the two types; lowering of NaCl concentration from 1 M (circles) to 0·5 M (triangles) decreases the magnitude of the inhibitory response but not the facilitatory response magnitude, whereas the difference in the concentration of both HCl and quinine (7 mM and 14 mM) is represented only by variation of the facilitatory response magnitude. In Fig. 3 A–F, the inhibitory and the facilitatory responses to every kind of stimuli were drawn on the same time scale because both events should take place within the same region but at different neurons at the same time. Therefore, the amount of the activity at a given time can be expressed by the sum of both the inhibitory and facilitatory response magnitudes. The magnitudes of the responses 20 sec after the onset of stimuli calculated in this way are summarized in Table 1 together with the results obtained from the chorda tympani and the glossopharyngeal nerve.

TABLE 1. MAGNITUDE OF TASTE AND TEMPERATURE RESPONSES IN THE THALAMIC RELAY NUCLEUS AND THE GUSTATORY NERVES

	HCl (14mM)	Quinine (14 mM)	Water	NaCl (1 M)	Warm	Cold
Thalamic relay nucleus	116	78·1	110	100	36·5 (+10~17)	20·4 (−13~21)
Chorda tympani	222·2	142·5	121·2	100	60·5 (+13)	120·0 (−18)
Glossopharyngeus	241·5	110·5	20·1	100	55·0 (+13)	120·0 (−18)

All values were expressed relative to the magnitude of response to 1 M NaCl.

Values for the chorda tympani and glossopharyngeal nerve indicate the mean of 6 and 7 cats, respectively. Parentheses indicate the temperature (°C) of Ringer's solution higher (+) or lower (−) than that of the room.

MODALITY AND QUALITY SPECIFICITY OF THALAMIC NEURONS

Summed responses (top trace in each record) of a cat's thalamic neurons to 4 kinds of taste stimuli at different depths (Y) and at varying caudo-rostral positions (X) are shown in Fig. 4 together with those obtained simultaneously from the chorda tympani (middle trace). The figure illustrates clearly a specific responsiveness of neurons to only one kind of taste stimuli applied (bottom bar). Variation in the magnitude of the chorda

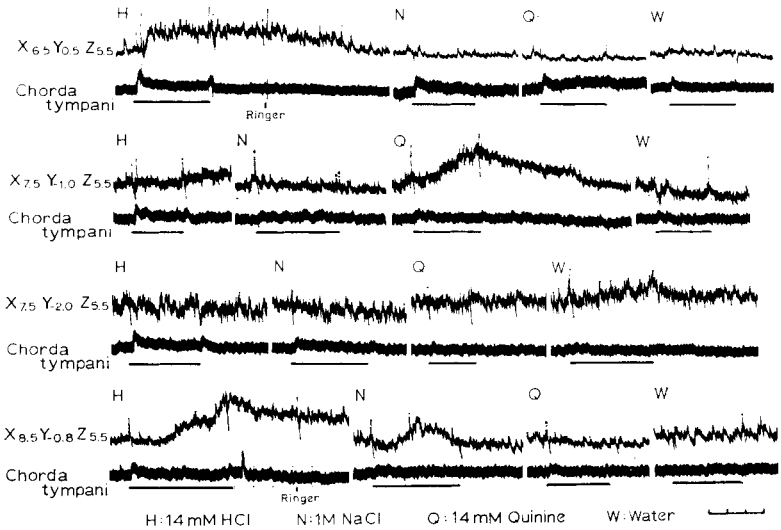


FIG. 4. Records indicating, from left to right, summed responses to successive application of 4 kinds of taste stimuli (bottom bar in each record) obtained from the thalamus (top trace) and from the chorda tympani (middle trace). Electrode position is expressed on Horsley-Clark coordinates. One division of the scale in the right-hand corner shows 10 sec.

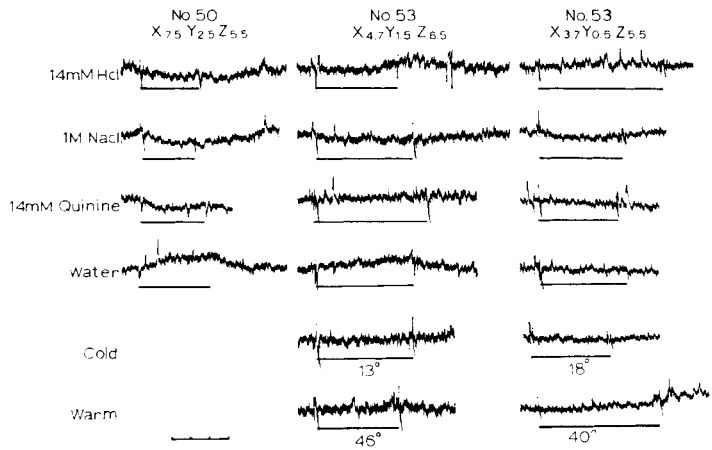


FIG. 5. Summed thalamic responses (upper trace in each record) to taste and thermal stimuli (lower bar), indicating different types of the response at each recording site (uppermost figures). Records should be read from top to bottom. 10 sec time scale for one division of the scale in the lower left-hand space.

tympani response does not necessarily indicate a change in the sensitivity of the tongue receptors to taste stimuli (Ishiko and Amatsu, 1964). In Fig. 5 three examples are shown, in which varying types of responses to more than three kinds of stimuli were recorded from the same recording site. Classification of 112 clusters of neurons with regard to their modality specificity to taste and/or temperature stimuli is summarized in the upper part of Table 2, and quality specificity of 103 taste-sensitive neurons is shown in the lower part. It appears that a great increase in the quality as well as modality specificity occurs in the cat thalamus, as compared with the results obtained from the rat medulla (Makous, Nords, Oakley and Pfaffmann, 1963) or those from the chorda tympani of cat (Sato, 1963) and of rat (Pfaffmann, 1955), though accurate comparison of these two sets of experimental results are difficult because multi-neuron responses were recorded in the present experiments.

TABLE 2. NUMBER OF THALAMIC NEURONS RESPONDING TO TASTE AND/OR TEMPERATURE STIMULI TO THE TONGUE

Stimuli	Number of neurons
Taste alone	72
Temperature alone	9
Taste and temperature	31
TOTAL	112
Number of taste-sensitive neurons	
Single taste quality	40
Two taste qualities	27
More than two taste qualities	36
TOTAL	103

All 112 recording sites, determined by the procedure similar to that reported by Appelberg and Landgren (1958), were plotted on the Horsley-Clark coordinates (Snider and Niemer, 1961) and shown in Fig. 6, in which filled circles indicate the sites at which responses to thermal and taste stimuli other than the inhibitory response to NaCl (empty ones) were recorded.

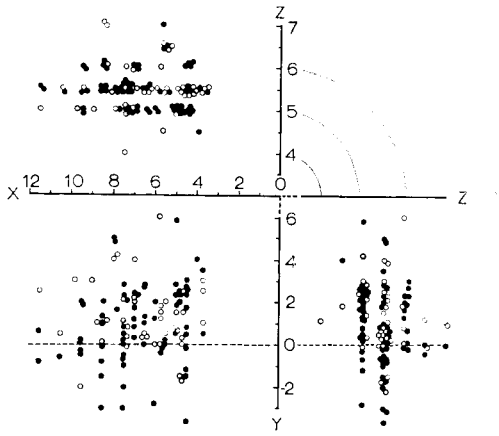


FIG. 6. Representation of 112 recording sites on caudo-rostral (X), medio-lateral (Z) and dorso-ventral (Y) axis of Horsley-Clark coordinates. Note no difference between the area where the inhibitory response to NaCl (o) and the responses to taste and/or temperature stimuli (●) were obtained.

ANALYSIS OF THE RESPONSE TO WATER AND TO NaCl

Multi-unit activity of individual cluster of neurons was recorded with the electrodes having tip diameters of less than 100 μ. The inhibitory response of neurons to varying concentrations of NaCl was examined with nine cats; in six cats the whole surface of the tongue was stimulated while in the remaining three stimulation was limited to the anterior two-thirds of the tongue with a flow chamber (Nagaki, Yamashita and Sato, 1964). Such a variation in the area of stimulation yielded a difference in the time course of the response to NaCl but not in the relationship between the maximum magnitude of the inhibitory response and the NaCl concentration (Table 3 and Fig. 7 A and B). To compare the temporal pattern of the response, the rate of inhibition (the time necessary to reach half of the maximum magnitude) and that of recovery (the time required to decay from the maximum to half of the maximum magnitude) is tabulated in Table 3. A quick depression followed by a rapid adaptation of the response to NaCl is shown in the case of the limited tongue stimulation, while in the other case a progressive increase in the recovery time is observed with increasing NaCl concentration.

The time course of the facilitatory response to water and of the inhibitory response to NaCl is plotted in Fig. 8; a decrease in purity of water or an increase in NaCl concentration resulted in a decrease of the response magnitude (left), while increase in NaCl concentration to more than 0.1 mM

brought about an increase in the inhibitory response in magnitude (right). The magnitude of response to water and NaCl of varying concentrations obtained in the thalamic neurons is shown by solid lines in Fig. 9, while the dotted line indicates the one obtained from the chorda tympani.

In the chorda tympani the response magnitude increases with an increase in NaCl concentration above 0·1 M and also with a decrease in the concentration below 0·1 M, but in the thalamus the magnitude of response

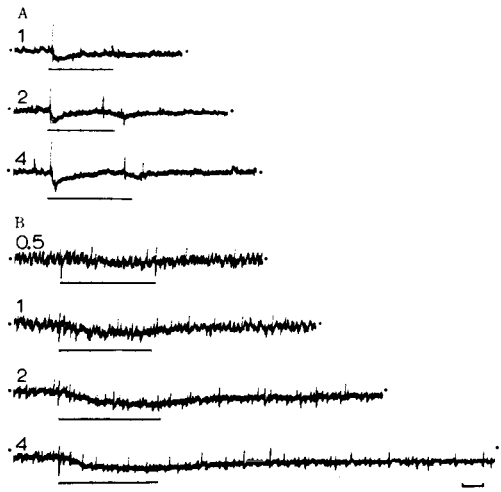


FIG. 7. Inhibitory responses to NaCl obtained by stimulation of the anterior area (A) or of the whole area (B) of the tongue. In each record, the upper trace shows the summed response, and the lower bar the duration of stimuli, while numerals represent NaCl concentration and dots indicate the level of the spontaneous activity of the first records in A and B, marked in the successive record. No adjustment of the level was made throughout the experiment. A bar in the right-hand corner shows 10 sec.

TABLE 3. CHANGES IN THE THALAMIC INHIBITORY RESPONSE TO NaCl BY A CHANGE IN THE AREA OF STIMULATION OF THE TONGUE SURFACE

NaCl concentration	Max. magnitude (%)		Rate of inhibition (sec)		Rate of recovery (sec)	
4 M	219·4	195	9·0	4·2	108·3	4·5
2 M	137·2	122·5	10·5	2·4	56·8	7·5
1 M	100	100	7·2	3	29·8	7·6
0·5 M	71·3	51	8·7	2·5	19·7	4
0·25 M	57·5		11·5		12·5	

Each value indicates the mean of 4–6 cases. In each column the preceding numerals indicate the values obtained by stimulating the whole tongue surface while the following ones show those obtained by the stimulation of the anterior two-thirds of the tongue.

changes approximately linearly with a change in the concentration; therefore, the intensity of stimulus is expressed by a single parameter, which would be easier for the animal to discriminate the intensity of stimuli.

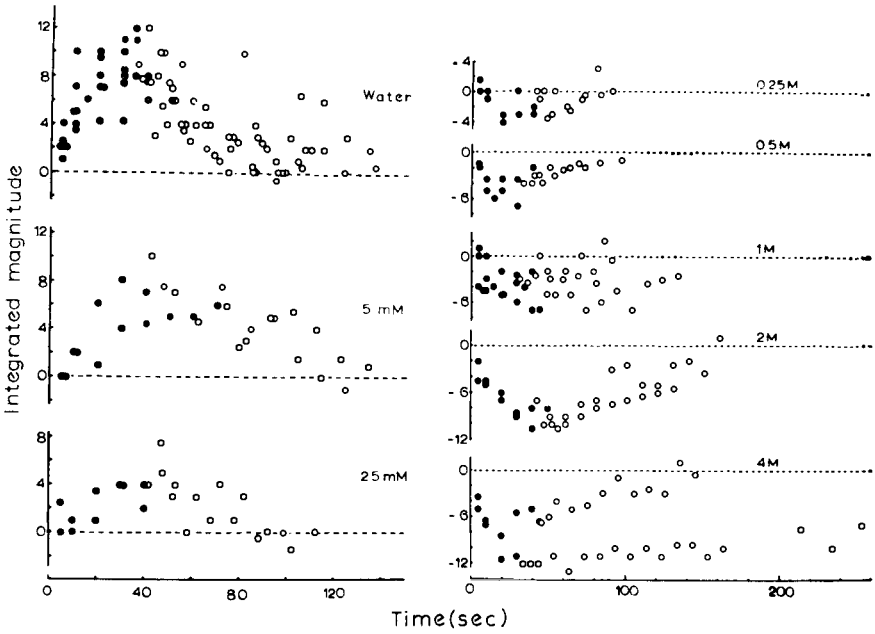


FIG. 8. Changes in the magnitude and in the type of the summed response at a recording site following a progressive increase in NaCl concentration from zero (water) to 4 M. Stimulations were repeated 2–5 times for each concentration. ● and o; the magnitude obtained during and after stimulation, respectively.

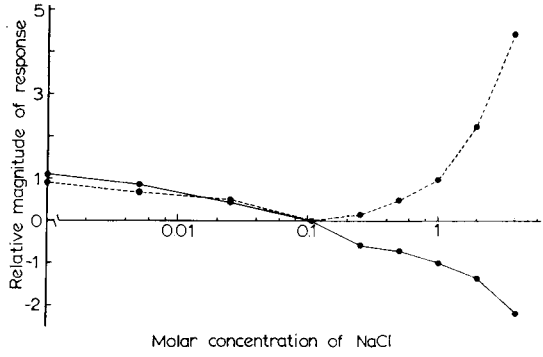


FIG. 9. Relationships between the magnitude of the summed responses relative to that of 1 M NaCl and its concentration obtained from the thalamus (solid line) and from the chorda tympani (broken line). Each point represents the mean of 2–6 cases.

ACKNOWLEDGEMENT

We wish to thank Prof. M. Sato for his helpful advice and discussions in the preparation of this paper.

REFERENCES

APPELBERG, B., LANDGREN, S. 1958. The localization of the thalamic relay in the specific sensory path from the tongue of the cat. *Acta Physiol. Scand.*, **42**, 342–357.

ISHIKO, N., AMATSU, M. 1964. Effects of stretch of the tongue on taste responses in the glossopharyngeal and chorda tympani nerves of cat. *Kumamoto Med. J.*, **17**, 5–17.

MAKOUS, W., NORDS, S., OAKLEY, B., PFAFFMANN, C. 1963. The gustatory relay in the medulla. In *Olfaction and Taste*, ed. ZOTTERMAN, Y., Pergamon Press, Oxford.

NAGAKI, J., YAMASHITA, S,. SATO, M. 1964. Neural response of cat to taste stimuli of varying temperatures. *Jap. J. Physiol.*, **14**, 67–89.

SATO, M. 1963. The effect of temperature change on the response of taste receptors. In *Olfaction and Taste*, ed. ZOTTERMAN, Y., Pergamon Press, Oxford.

SNIDER, S. R., NIEMER, W. T. 1961. *A stereotaxic atlas of the cat brain.* The Univ. Chicago Press, Chicago.

PFAFFMANN, C. J. 1955. Gustatory nerve impulses in rat, cat and rabbit. *J. Neurophysiol.* **18**, 429–440.

SPECIFIC UNITARY OSMERECEPTOR POTENTIALS AND SPIKING PATTERNS FROM GIANT NERVE CELLS*

A sensory coding in a neural *in vivo* model

A. ARVANITAKI, H. TAKEUCHI and N. CHALAZONITIS

Centre National de la Recherche Scientifique
Department of Cellular Neurophysiology, Institute of Neurophysiology
Marseille, France

UNFAVORABLE anatomical conditions have been the main obstacle to study of the primary events in the transduction of the stimulus into a coded message displayed in the individual receptor cells of the olfactory apparatus. Microelectrophysiological techniques seemed unpracticable, owing to the minute dimensions of the receptor cell bodies and to their most tiny afferent nerve fibers (*fila olfactoria)* tightly ensheathed in the mesaxons (de Lorenzo, 1963).

On the other hand, the cell membrane of relatively large sized neurons in certain Invertebrates (particularly *Aplysia*) was already recognized as endowed with highly sensitive receptor properties concerned with changes in temperature, illumination, mechanical pressure, pO_2 and pCO_2. Like specific biological transducers such neurons display conversion of different forms of energy into bioelectric "generator currents" and act, according to the case as thermoreceptors (Arvanitaki and Chalazonitis, 1963), photoreceptors (Arvanitaki and Chalazonitis, 1961, Chalazonitis, 1964), mechanoreceptors (Arvanitaki and Chalazonitis, 1956, Chalazonitis and Arvanitaki, 1961), chemoreceptors (Chalazonitis, 1959, 1963). It was then tempting to investigate to what extent such preparations would provide a useful neural model for olfactory receptor mechanisms. Moreover, anatomical

* Supported in part by the Centre National de la Recherche Scientifique, France and in part by Research grants from the United States Public Health Service under grant No. NB-03337 and the Aerospace Research of the USAF, through its European office under Grant No. AF-EOAR 63-114.

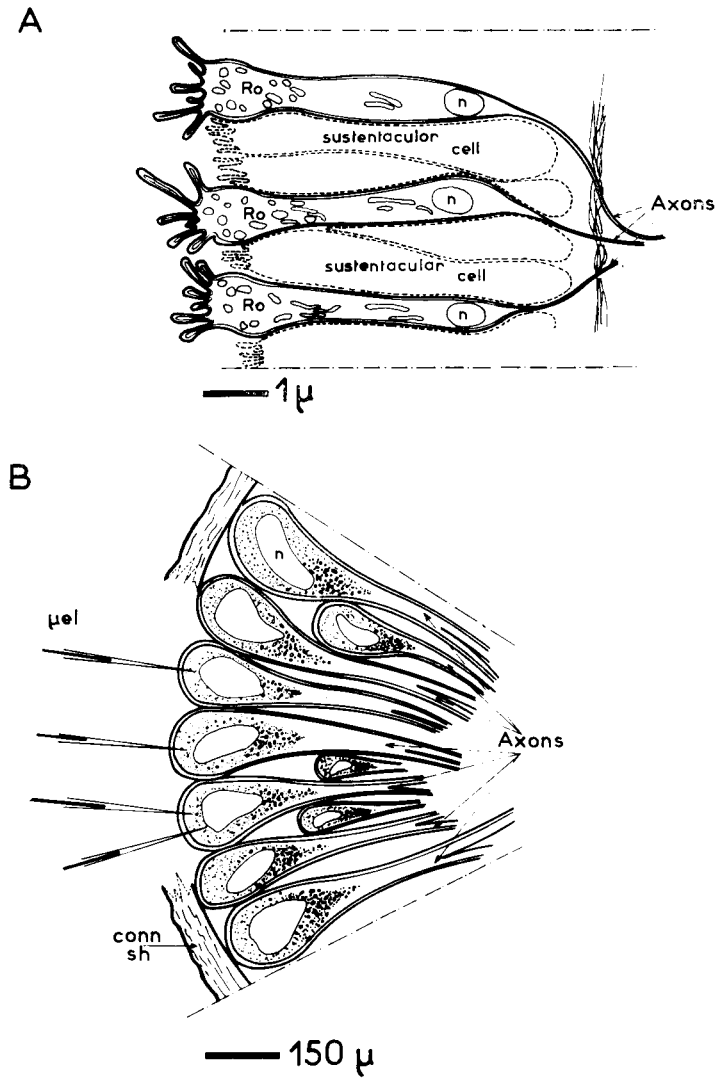


FIG. 1. Schematic representation of the anatomical organization, comparatively: in (A) the olfactory mucosa (redrawn after de Lorenzo, 1963).
In (B) the neuronal outer layer of a visceral ganglion of either *Helix* or *Aplysia*.
(A) The apical systems of the olfactory rods (*Ro*) extend as naked processes beyond the "limiting membrane" of the olfactory epithelium, in direct contact with the external milieu.
(B) The apexes of superficial neurons, in a molluscan ganglion, experimentally naked through a local narrow excision of the connective sheath (conn. sh.) simulate those of the olfactory rods but, to a 150-fold scale. They offer room for introducing many microelectrodes (μ el) and for recording unitary bioelectrical signs of activity as evoked by odorous molecules.

conditions appeared most suitable to the investigation. Tightly ordinated, the bodies of the nerve cells constitute the outer layer of the ganglia in *Helix* (Kunze, 1921) as well as in *Aplysia* (Arvanitaki and Chalazonitis, 1955) while the axon hillocks, internally oriented converge towards the central neuropile. A careful narrow excision of the superficial connective tissues —except for a very fine protective sheath—leaves thus exposed the apexes of a few nerve cells (Fig. 1B). Such an anatomical arrangement schematically reminds one of that generally encountered in the Vertebrates olfactory system (Fig. 1A) but with the advantage of approximately 150-fold dimensions.

In that way, current microphysiological techniques revealed in individual cells, as a response to transient actions of different molecules used in olfaction studies, "receptor" membrane potentials of specific sign and time course. The results provided direct evidence of peripheral discrimination mechanisms and suggested a plausible coding of representation at the sen. sory level and of transmission and instruction to the central system level-

METHODS

The experimental set up is schematically shown in Fig. 2. Sub-oesophagal ganglia of *Helix pomatia* showing, —under the narrow excision of the epithelial tissue—a few quasi naked nerve cells, was introduced in an appropriate chamber. Two nerve trunks containing afferent axons, left connected to the ganglia, were mounted over pairs of Ag–AgCl electrodes to allow orthodromic activation of the nerve cells. The chamber was closed except for two windows of equal apertures permitting the inlet and outlet of the gases without variation in the chamber pressure. Two to three glass microelectrodes (0·5 μ tip diameter) monitored by micromanipulators, entered the chamber. In some experiments they penetrated two to three naked cells for simultaneous independent recording of their individual activities (through cathode followers to the usual amplifying and recording systems). In some cases the cell was impaled with two independent microelectrodes. One of these permitted injection either of square current pulses of monitored intensity—to control changes in the cell membrane conductance—or of a direct current, in order to preset the membrane potential to a required level.

Among the different odorous substances currently used in olfaction studies, the following were mainly tried in the present investigation: methyl-, ethyl-, butyl-, propyl- and allyl-alcohols; amylacetate; acetone; benzene, benzaldehyde; xylene; ammonia; pyridine. The preparation was exposed for some seconds alternatively to an air stream containing a controlled

concentration of the odorous substance under study, then to a pure air stream. The two streams were of an equal constant flow-rate. According to the case this varied from 5 to 15 cm^3 sec^{-1}. Air streams containing the requisite concentrations of a given substance were obtained by mixing in various proportions a stream of pure air (S_{air}) to one saturated with odorous vapors (S_{od}). By suitable control (taps and flowmeters of high preci-

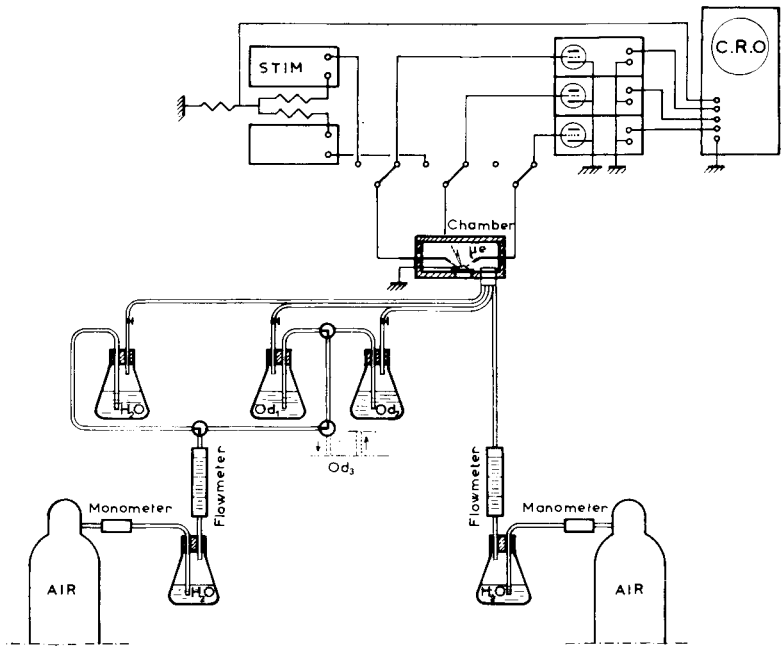


FIG. 2. Schematic representation of the experimental set up. For details, see text.

sion), any desired proportion of odorous vapor, between saturation concentration at the temperature of the experiment and zero concentration could be produced and enter at a given rate (φ) the chamber containing the preparation. In such conditions, given the saturation pressure of the odorous vapor at the temperature of the experiment (24 °C) its concentration was in each case calculated in 10^{-6} M per ml in the mixture S_{mx} streaming into the chamber. A simple manipulation of stopcocks permitted the flow at an equal rate either of the mixture $S_{mx} = S_{od} + S_{air}$ (at "on") or of pure air (at "off"). A light signal recorded the timing of action of the odorant molecules simultaneously to the bioelectrical changes in the cell membrane. An interval of 5 min in the presence of pure air was systematically managed between two consecutive trials.

It will be noted that when at "on" S_{mx} is admitted in the chamber initially containing pure air, the programmed concentration C of odorous molecules is only gradually reached. The instantaneous concentration c increases as a function of time according to

$$c = C\left(1 - e^{-\frac{t}{\tau}}\right) \quad \text{with} \quad \tau = \frac{V}{\varphi}$$

where V = the volume of the chamber (25 cm^3)
V being constant, the higher the rate φ, the lower the τ.

The adopted values permitted attainment of $\frac{2}{3}$ C in approximately 2·5 sec to 5 sec, according to the case.

The experimental set up permitted the assay successively of two to three substances, on each of the impaled cells.

After introduction of the microelectrode, some of the impaled cells appeared spontaneously active, others were inactive. In any case cells showing initially a synaptic noise, were discarded.

However, in cases where the excision of the connective epithelium was rather large—leaving thus exposed many naked cells—synaptic noise frequently arose in the impaled cell during the action of the odorant. This synaptic invadement was then ascribable to the activity of the other naked cells simultaneously reached by the stimulus. When they occur, such cases were duly discussed; as known, at the level of the primary olfactory receptors no synaptic interactions are functionally implied.

RESULTS

I. Osmereceptor Potentials of the Nerve Cell Membrane

1. Negative osmereceptor potentials. A brief action of odorous vapours under the above described conditions determined a transient change of the membrane potential. For simplicity sake initially inactive cells will be considered first.

In Fig. 3, upon admission of benzene vapours, two cells—impaled with independent microelectrodes—both reacted by a depolarization. This developed at an increasing rate, reached a maximum displacement (approximately 10 mV), then it slowly subsided.

The time course and the maximum amplitude of the depolarization depended:

(a) on the identity of the cell;

(b) on the nature, the time of action and the concentration of the odorous molecules.

FIG. 3. *Receptor depolarization of the nerve cell membrane in the presence of odorous molecules (negative osmereceptor potential).* Simultaneous recording through two independent microelectrodes in two nerve cells.
Benzene, concentration 3×10^{-6} M/cm^3, was admitted in the preparation chamber for 10 sec (timing of action as indicated by the horizontal trace under the recordings). A depolarization is initiated in both cells. The upper one reached a maximum 2·5 sec after "on" while the lower did it much later, in 7 sec.

Such a transient cannot be assigned to a summation of synaptic components. As a matter of fact it was generally devoid of any synaptic signal—even at the highest amplifications—and especially when the excision of the epithelium was narrow enough to leave exposed only very few cells. Its time course and amplitude in relation to the concentration and time of action of the stimulating molecule remind one of potentials recorded from the sensory epithelium in the Vertebrates nasal mucosa (Ottoson, 1956, 1958, 1963; Beidler and Tucker, 1955; Higashino *et al.*, 1961; Gesteland *et al*, 1963) or from olfactory sensilla in Insects (Hodgson, Lettvin and Roeder, 1955; Boeckh, 1962; Schneider, 1963).

The present transient changes of potential may then be considered as receptor potentials at the level of the nerve cell membrane. Being initiated under the action of odorous particles they will henceforth be denominated *osmeceptor* or *osmereceptor* potentials.*

The osmeceptor potential is thus the primary unitary potential change in response to odorous molecules acting on a nerve cell membrane. However, the overall shape of the osmeceptor depolarization is not always

* The suggested term, *osmeceptor* or *osmereceptor* is a combining form, from ὀσμή= *osmē* (smell, odor) and ceptor or receptor, by analogy to photo-, thermo-, mechano-, chemo-receptor (although *osmeleptic* from ὀσμή (smell, odor) and λαμβάνειν (to lay hold of) should be of a more correct construction, Incidently, one more remark could be introduced here, related to the linguistic code: currently used combining forms from ὀσμή such as osmometer, osmoscope, osmophore ..., should more correctly, be turned to *osmemeter, osmescope, osmephore* ..., in order to avoid misleading interference with ὠσμός,=osmos (impulse, driving force) as in osmosis.

as simple as that shown in Fig. 3. According to the case, two more variants were mainly observed:

(a) When a level of approximately 5 mV was reached, a rapid depolarization was triggered followed by a long lasting plateau which was not abolished with the suppression of the stimulant (Fig. 4). The sum of the amplitudes of the two negative components increased supralinearly as a

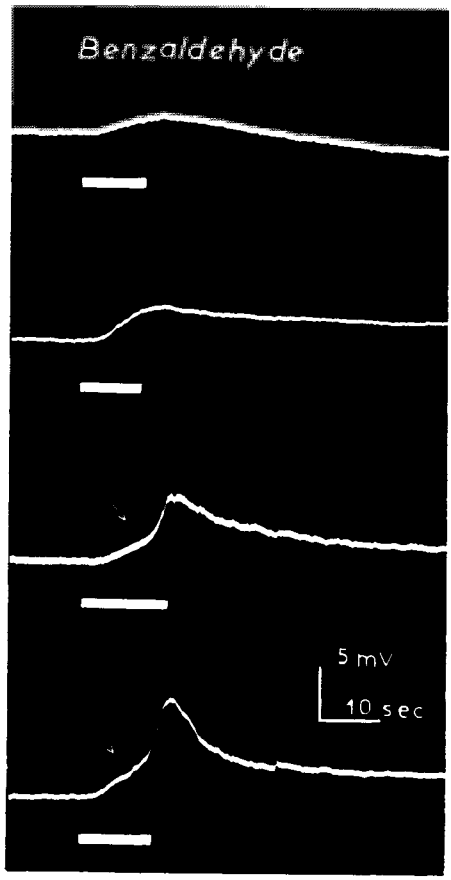


FIG. 4. *Negative osmereceptor potential as a function of the odorous molecule concentration.* A nerve cell was exposed for a few seconds (as indicated by horizontal bars under the potential traces) to benzaldehyde vapors at various concentrations, from top to bottom: 40, 46, 48 and 50 10^{-9} M/cm^3.
The rate of rise of the initial depolarization increased with the concentration until—a threshold level having been reached—a second component is evoked (arrows). The latter has the shape of a slow wave. Once triggered, its development is not abolished in spite of the outlet of the odorous vapors.

function of the stimulant concentration reaching in some cases 10 to 20 mV.

(b) A second variant of negative osmeceptor potential is illustrated in Fig. 5. Here the initial depolarization at "on", was shortened by a rapid repolarization resulting in a spike-like swing of approximately 5 sec duration.

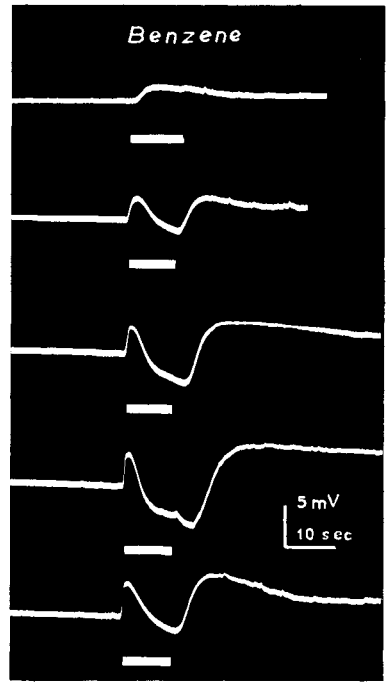


FIG. 5. *A variant of negative osmereceptor potential: early development of a hyperpolarizing after potential.* The nerve cell was exposed for a few seconds (as indicated by the horizontal bars under the potential traces) to benzene vapors at increasing then decreasing concentrations, from top to bottom: 1·8, 2·8, 3·7, 5·6, and $3{\cdot}7\ 10^{-6}$ M/cm^3.

At "on", a relatively high rate negative receptor potential. Its rate of decay and the amplitude of a positive after potential increase as a function of the concentration.

At "off", a prominent negative effect sets up the membrane to a long-lasting depolarized level.

This was followed by a hyperpolarization increasing with the stimulus strength, i.e. the concentration of the odorous particles. Upon the suppression of the odorant stimulus, a large, long outlasting depolarization

was developed. Such recordings introduced the idea of the possible occurrence in a same receptor cell of both sign potentials.

The question then arose on whether the nerve cell membrane might display hyperpolarization as a primary osmeceptor mechanism.

2. Positive osmereceptor potentials. The investigation proceeded with impaling a large number of cells and carefully trying in each of them,

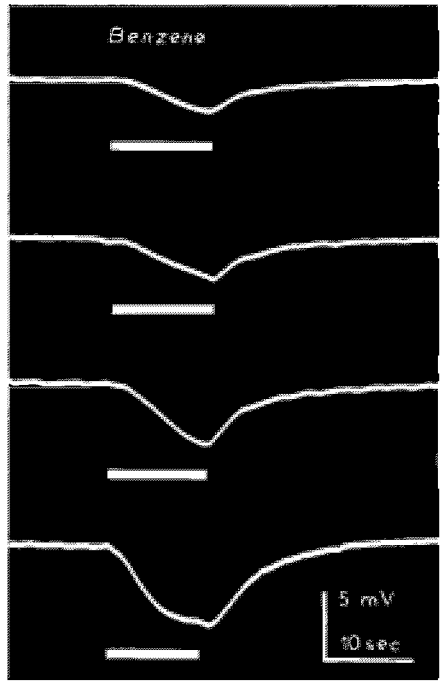


FIG. 6. *Receptor hyperpolarization (a positive osmereceptor potential).* A remarkable type of osmereceptor potential is evoked in this cell, hyperpolarization of rate and amplitude increasing with the concentration of the odorous molecules—here benzene—from top to bottom: 0·8, 1, 1·9 and 2·8 10^{-6} M/cm^3. At off, a second hyperpolarizing component is sometimes elicited at high concentration, followed by redepolarization and rebound.

successively the effects of many odors. Among 246 impaled nerve cells, 58 reacted by a primary hyperpolarization in the presence of odorized air, specifically with given molecules, mainly benzene, xylene, benzaldehyde acetone, butyl alcohol. A typical example is given in Fig. 6.

Mixed to air, benzene vapors were sent into the chamber. At once the cell membrane started hyperpolarizing. Redepolarization developed

as soon as pure air replaced the odorized one. In successive trials on the same cell the concentration of benzene was increased; so did correlatively the rate and the amplitude of the hyperpolarization. An increase of the concentration from 0·8 to 2·8 10^{-6} M/cm^3 determined an increase in the amplitude of the membrane positive transient from 2·6 to 10 mV, as a function of the benzene concentration. The positive osmeceptor potentials could not be ascribed to a summation of inhibitory post synaptic potentials (i.p.s.p.) as, in many instances, they were recorded as well in nerve cells devoid of any i.p.s.p. sign when submitted to synaptic action through afferent nerve fibers.

We are thus dealing here with a primary osmeceptor hyperpolarization of the nerve cell membrane, which does not find an analogue in the olfactory

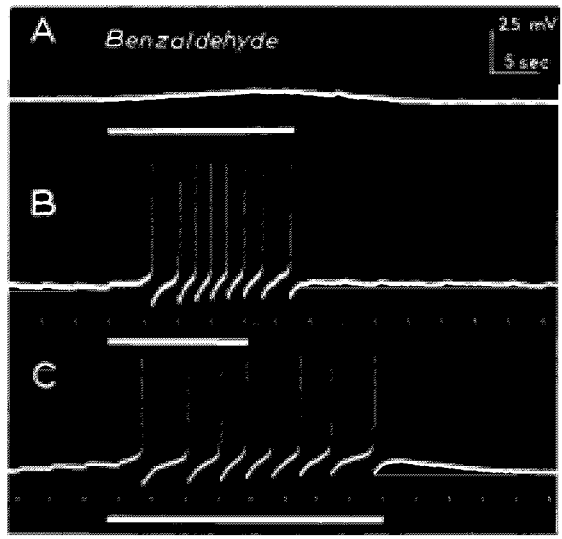


FIG. 7. *Enhancement of the synaptic activability parallel to the negative osmereceptor potential.* (A) Membrane depolarization evoked by benzaldehyde: 0·05 10^{-6} M/cm^3 concentration. Time of exposure as indicated by underlying traces.

(B) The nerve cell was submitted to a subtle synaptic activation at 0·3 sec^{-1} frequency (vertical bars under the potential trace mark timing of the afferent fiber stimulation). Of subthreshold value before, the i.p.s.p.'s became of threshold value in the presence of benzaldehyde, then autoactivity was established. At "off", synaptic activability depressed. Complete recovery 1·5 min later.

(C), as in (B) except that benzaldehyde was of lower concentration (0·04 10^{-6} M/cm^3) and its action of longer duration. Parallelly, lower but longer-lasting enhancement of the synaptic activability. At "off", total failure of the synaptic action.

Spikes truncated.

receptor potentials known from the sensory epithelium in the nasal mucosa. However, as Ottoson (1963) pointed out, the latter is a *mass response* and as such it does not inform about membrane potential changes in individual olfactory receptors. Moreover, if—as the present results in individual nerve cells do suggest—positive osmeceptor potentials occur in 20–30 per cent of the individual olfactory receptors, it should be understood why the mass olfactory potentials rather generally deal with depolarizations.

Evidence of both negative and positive primary osmeceptor potentials in the nerve cell membrane lead to the expectancy of significant concomitant changes in the membrane excitability and conductance, hence the display of functionally acting generator currents.

3. Excitability and conductance changes of the nerve cell membrane under odorant stimulation. The membrane excitability changes during the time of action of an odorant were evinced either using transmembrane electrical pulses of known constant intensity or through a controlled excitation of the nerve fibres, afferent to the impaled nerve cell. In Fig. 7, for instance, the osmeceptor negative potential evoked by benzaldehyde vapors determined parallelly an enhancement of the membrane excitability as shown by the promotion of the previously uneffective i.p.s.p., to threshold and suprathreshold transmission. The excitability enhancement varied correlatively with the concentration and the time of action of the odorous action (Fig. 7 compare B to C).

Conversely it was expected that a positive osmeceptor potential should be parallelled by a depression of the membrane excitability. This was shown by the decrease either in the effectiveness of the synaptic action (Fig. 8A) or in that of the direct electrical stimulus (Fig. 9B).

Moreover, introducing a second intracellular electrode and controlling the membrane conductance by the square current pulses method, it was shown that parallelly to the development of osmeceptor potentials, whether positive or negative, the membrane resistance decreased correlatively. This was disclosed by the decrease in the ratio V/i (i = intensity of the membrane current pulse; V = displacement in the membrane potential), and the decrease in the time constant of the displacements (Fig. 9A).

Such changes in the membrane potential and the concomitant increase in the conductance of the cell membrane areas exposed to the odorous molecule action would imply current flows over the cell body. These would then functionally act either as generators or as depressors (inhibitors) according to whether they are outward or inward at the pacemaker *loci*.

II. Transform Functions: Osmereceptor Potential Patterns into Spike Patterns

1. The "receptor function"

(a) "Representation" of the stimulus time of action and concentration at the primary receptor structure. The receptor function deals with the energy conversion of the odorant stimulus at the primary receptor membrane level. It must contain all information about the stimulus charac-

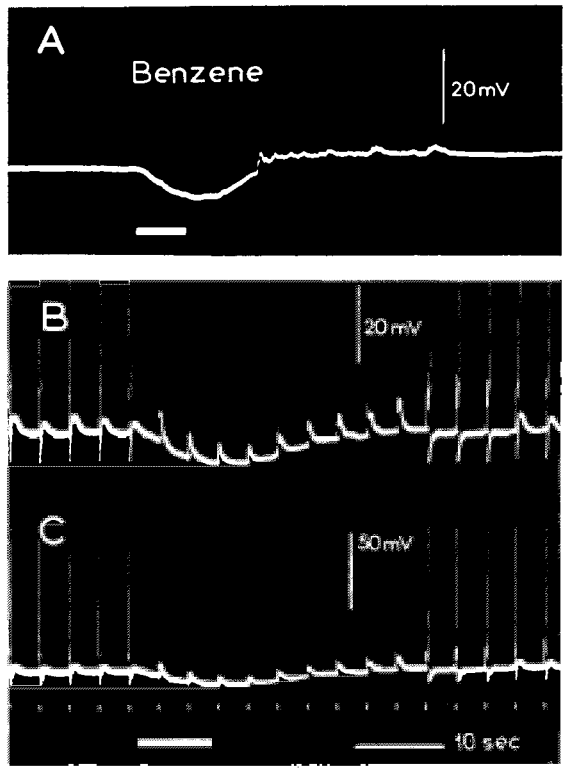


FIG. 8. *Depression of the synaptic activability parallel to the positive osmereceptor potential.* (A) Admission of benzene vapors, $3\ 10^{-6}$ M/cm^3 concentration (timing, horizontal bar under the potential trace) hyperpolarize the cell membrane. (B) and (C) simultaneous recordings at high (B) and low (C) amplifications. The nerve cell was initially submitted to threshold, 0·3 sec^{-1} synaptic bombardment (vertical bars under the potential traces mark timing of the afferent nerve fiber stimulation). Upon admission of the odorous stimulus, hyperpolarization and simultaneous depression of the synaptic activability. Recovery after "off". In (B) spikes truncated. In (C) low amplification shows full spike amplitude unchanged after benzene action.

teristics such as time of action, concentration, quality, i. e. nature of the odorant molecule. Its immediate sign is a receptor potential. But from the foregoing it comes out that the conversion may accurately be quantitized in the terms of an electrical current.

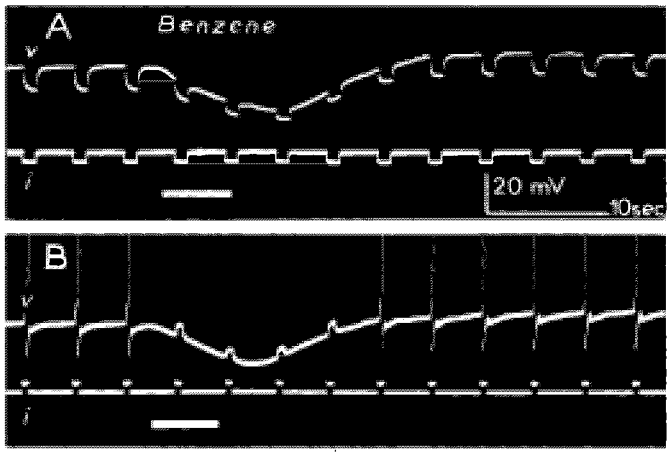


FIG. 9. *Decrease of the cell membrane electrical resistance concomitant to osmereceptor potentials.* (A) Inward square current pulses (*i*) injected at 0·25 sec^{-1} frequency, provoked membrane potential displacements (*V*). Admission of benzene vapors (timing, horizontal bar) determine immediate hyperpolarization and decay of the membrane resistance as shown by the decrease of the potential displacements. At maximum depression, during the odor stimulation, the ratio *V*/*i* reduced to one-third its initial value. Note the parallel decay of the potential displacements time constant.
(B) Outward current pulses (*i*) determined negative displacements (*V*) evoking a spike. Upon benzene admission, hyperpolarization developed, the negative displacements were depressed and the spike genesis failed. Removal of benzene vapors was followed by recovery of the excitability.

As well known, a firing of spikes is generated whenever a transmembrane outward current reaches a threshold value. This is what is observed in the nerve cell membrane under the action of an odor molecule at threshold concentration (Figs. 10 and 11). Spikes frequency as a function of time deserves particular attention. As a general rule the frequency increased continuously either supralinearly or sublinearly with time during the odorant action. Such a firing should be determined in the first case by a membrane current of intensity exponentially rising with time (Arvanitaki *et al.*, 1964) with a current intensity linearly rising with time in the second case (Arvanitaki *et al.*, 1965).

It may thus be concluded that the transduction of the odor molecules action at the receptor membrane, yields an electrical current (generator current) increasing as a specific function of time. This is the primary electrical conversion of the stimulus at the receptor level.

Moreover, when in repetitive actions of a given odorant, the pro-

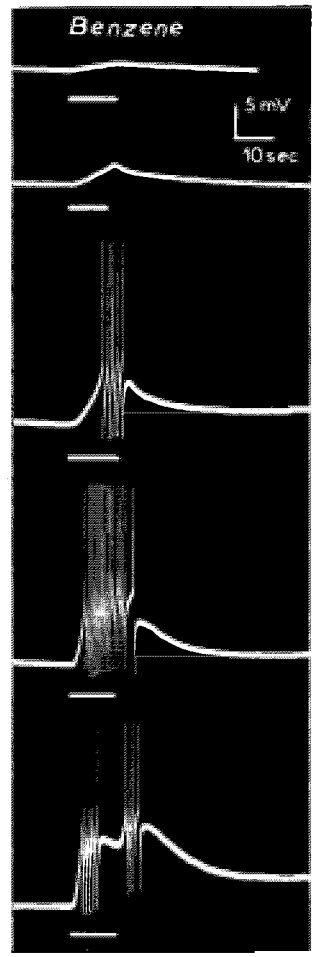


FIG. 10. *Transform from osmeceptor potential to a pulse pattern in an initially inactive nerve cell.* Benzene admitted for approximately 10 sec in the preparation chamber at increasing concentrations; final values, from top to bottom: 0·6, 1·1, 1·8, 2·8 and 3·6 10^{-6} м/см^3.

The rate of rise of the osmeceptor potential and the amplitudes reached in 10 sec, increase with the final concentration value. The spikes frequency increases as a function of the osmeceptor potential—hence with the odorant concentration.

grammed concentration C was increased, correlative changes in the frequency function disclosed the concomitant increase in the membrane generator current.

Consequently the primary receptor change, i. e. the generator current

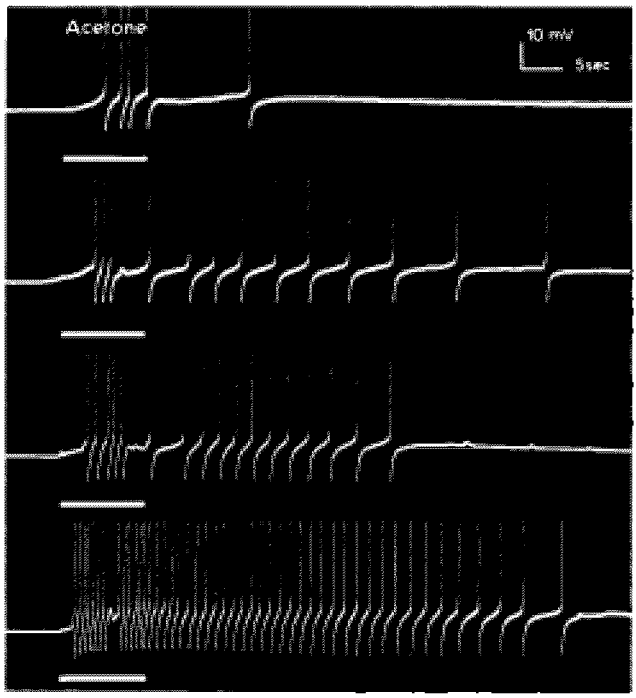


FIG. 11. *Transform from osmereceptor potential to a pulse pattern. Long lasting remanent depolarization at "off"*. Acetone vapors were admitted in the chamber for 10 sec as indicated by underlying horizontal bars. Increasing concentrations, from top to bottom: 0·4, 0·6, 1·3 and 2·6 10^{-6} M/cm^3.
During the inlet of the odorous stimulus the spikes frequency increased as a function of the concentration.
After "off", in contrast to the Fig. 10 case, the membrane stands depolarized. The amplitude and duration of this prolonged plateau as well as the frequency of the remanent spiking increase with the concentration.
Spikes truncated.

which is the "receptor function", i_{rec} encodes the time of action and the strength (i.e. the concentration) of the odorant stimulus: $i_{rec} = f(t, C)$.

Accordingly, in initially autoactive neurons, transient increases (Fig. 12) or transient decreases (Fig. 13) in the preexisting constant frequency would be indicative of the addition to the intrinsic outward generator current responsible for the autoactivity, of transient either outward

(Fig. 12) or inward (Fig. 13) i_{rec} currents due to transient odor actions, i_{rec} increasing with the odor concentrations.

(b) "Representation" of the stimulus "specific quality" (nature of the odor molecule) at the primary receptor level. The question then arises: which dimension in the receptor function would contain the information about the nature of the odor molecule?

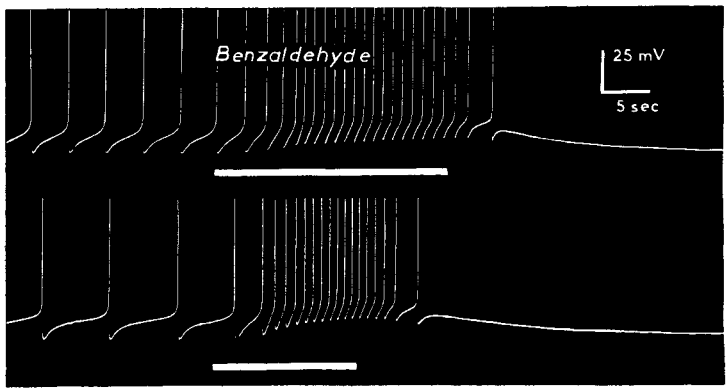


FIG. 12. *In initially autoactive cells, modulation of the spikes frequency by a negative osmereceptor potential, as a response to a transient odorous stimulus.* During the action of benzaldehyde vapors (timing as indicated by horizontal bars) depolarization and increase in frequency.
At "off", the membrane hyperpolarizes and spiking was abolished for 60 sec. Depolarization and maximum frequency increase with benzaldehyde concentration: respectively 0·04 10^{-6} M/cm^3 (upper recording) and 0·05 10^{-6} M/cm^3 (lower). Spikes truncated.

Differentiation of some types of olfactory responses evidenced at the level of the Vertebrates olfactory bulb (Adrian, 1950, 1953) and some differences in the rates of the olfactory mucosa responses or in the emerging spikes frequency to different stimuli (Ottoson, 1956, Gesteland *et al.*, 1963) were the main data available concerning discrimination of the odor stimulus.

However, to account for the selectivity to such an extensive range, as that implied in the olfactory discrimination, thorough information about the specific modes of the primary reactions to different odor molecules from many different varieties of receptor units, seemed basically required.

In this connection, data from different individual neurons might be of some interest to such an approach. As has already been pointed out each

of the examined neurons responds to certain of the odor species to which it is subjected, while it shows no response to others.

Moreover, its receptor potential as a response to the different tried

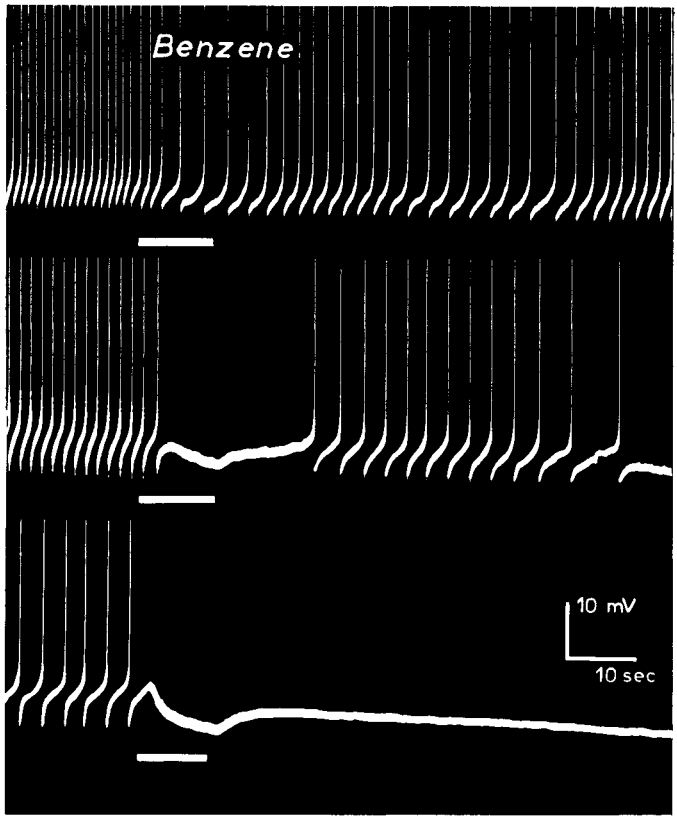


FIG. 13. *Transform of positive osmereceptor potentials in initially autoactive cells.* Benzene vapors were admitted in the preparation chamber at increasing concentrations, from top to bottom: 0·56, 1·12, 1·82 10^{-6} M/cm^3, final concentrations. According to the odorant concentration (hence the amplitude of the positive osmereceptor potential) transient decay of the frequency or transient suppression of the spiking for periods of time increases with the concentration.

odors displayed several possible shapes (Fig. 14). These may roughly be classified into 4 main types:

(1) Simple $\int$ shaped depolarization of amplitude increasing with time until a maximum, then slowly subsiding to a plateau level, as illustrated in Fig. 3.

(2) Receptor depolarization developing in two consecutive $\int$ shaped steps, the second step emerging with a higher rate, as illustrated in Fig. 4.

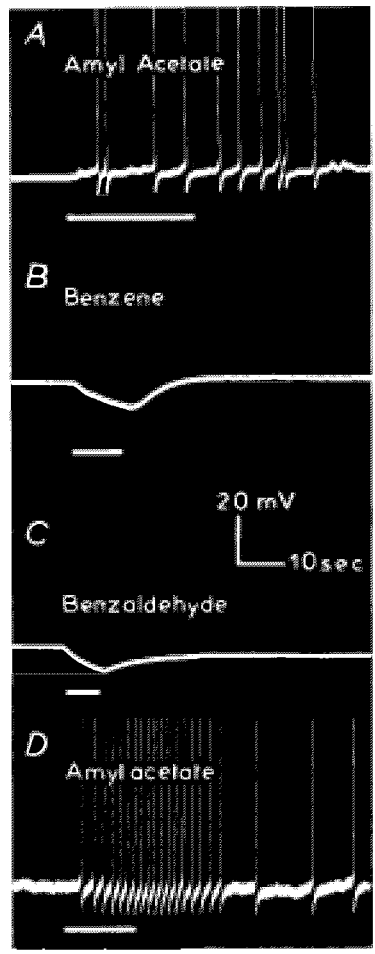


FIG. 14. *Given a cell, the type of the osmeceptor potential specifies the odorous molecule.* Submitted successively to transient actions from three odorous molecules, the same cell responded by depolarization and spiking to amylacetate (A) but by hyperpolarization to benzene and benzaldehyde (B and C). In the next action, the same cell submitted again to amylacetate reacted by depolarization (D), as it did in the first assay.
Spikes truncated.

(3) Receptor depolarization sharply reaching a maximum then rapidly subsiding beyond the initial level of the membrane potential, as illustrated in Fig. 5.

Modalities (2) and (3) were prominent when concentrations became relatively high.

(4) Hyperpolarizing receptor potentials as illustrated in Figs. 6, 8 and 9.

As receptor cells responding to a series of odors do not respond to

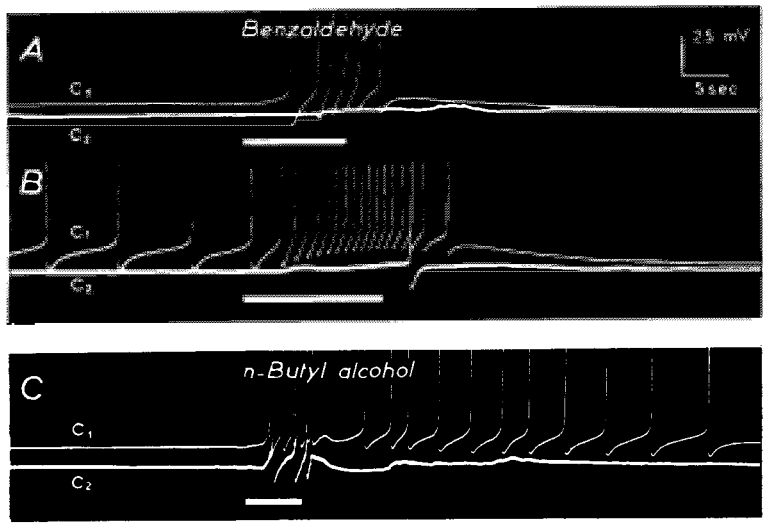


FIG. 15. *The osmereceptor pattern and the ensuing propagated signal as determined by a given molecule are specific to the receptor membrane cell.* Two cells c_1 and c_2 were simultaneously controlled by two independent microelectrodes. The two cells were submitted to benzaldehyde vapors at $0{\cdot}02\ 10^{-6}$ M/cm^3 (in *A*) at $0{\cdot}05\ 10^{-6}$ M/cm^3 (in *B*). Under both actions the cell c_1 displayed a negative osmeceptor potential and concomitant spiking, while the cell c_2 showed negligible changes at "on".

In *C* the two cells submitted to *n*-Butyl alcohol both displayed the same pattern, a negative osmeceptor potential and a spiking, however, at different frequencies. Note moreover, differences in the patterns of cell c_1 between responses to benzaldehyde (*A*, *B*) and that to *n*-Butyl alcohol. In the latter case long lasting remanent discharge at "off".

others, a fifth type should be added to the signs of specificity, i. e. the "no response" type.

Further emphasis must be given to two more facts:

(1) A given cell displays stereotypically the same type of receptor potential to a same odor molecule (Figs. 3, 4, 5 and 6).

(2) Different cells may display either the same or a different type of receptor potential when submitted to a given odor molecule (Fig. 14).

These statements would mean that the different cells have their own selectivity properties with a fairly good reliability.

Consequently some information concerning the quality of the odor-stimulus may be accurately supported at the primary cellular level by the specific time course of the receptor potential, hence its counterpart the specific time course of the underlying receptor current.

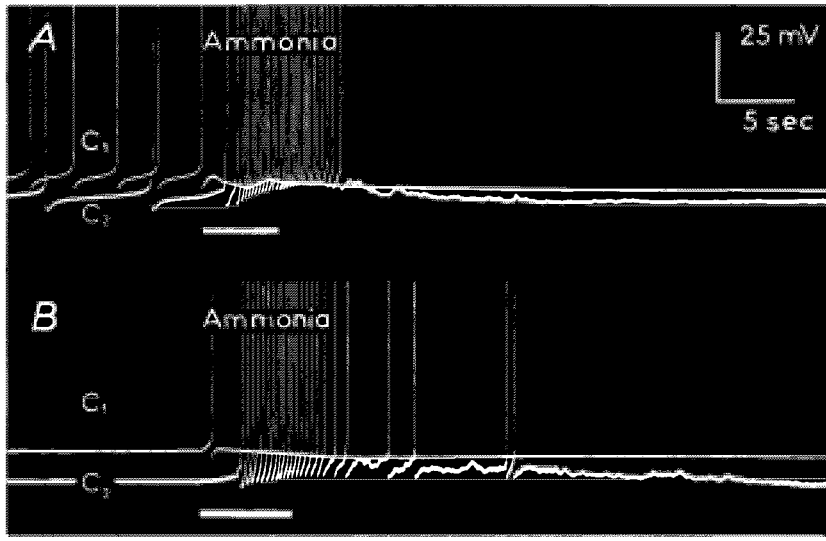


FIG. 16. *Given an odorant, the twin-motive spatio-temporal receptor pattern from two primary receptor units and the emerging twin-motive spiking pattern, reproducibly label the identity of the odor molecule.* Two cells c_1, c_2, were submitted to ammonia vapors. In *A* the two cells were initially autoactive. The receptor pattern of cell c_1 was of the hyperpolarizing mode and as such, inhibited the pre-existing activity. Simultaneously the reaction of the cell c_2 to the same molecule was of the depolarizing type and determined a high frequency discharge.
In *B* these same two cells were initially inactive. Their respective responses to the same molecule, at the same concentration, reproduced those in *A*.

However, if limited to the few different possible specific shapes in the receptor potentials and currents, the representation of quality should be of a rather poor yield. One more dimension must enter the picture if it is realized that in a given field of a receptor surface, many different unitary receptors with fixed space coordinates are simultaneously met by the odor molecules.

In that way in a field of *n* different specific receptors, *n* transient temporal patterns of receptor potentials should be displayed during the action of

a given odor molecule. The *ensemble*, a specific spatio-temporal pattern will contain all the necessary dimensions for a due representation of the timing, the concentration and the quality of the acting odor molecule. With n different receptor cells in a given receptor field and given the above cited

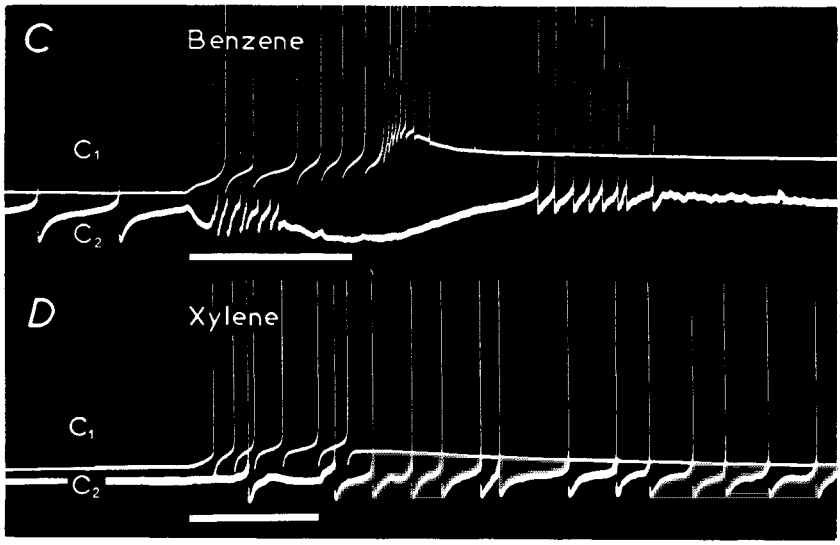


FIG. 17. *Two differenciated spatio-temporal receptor and emerging spiking patterns as responses to two different odor molecules from two primary receptor units.* Same cells c_1 and c_2 as in Fig. 16, here submitted successively to two more odorant species.

The cell c_1 which reacted to ammonia by a hyperpolarizing pattern (Fig. 16) reacts here to benzene (in *C*) by a negative osmereceptor potential and spiking with a high frequency after discharge at "off". Simultaneously, the second cell c_2 reacts to benzene by a distinct pattern, hyperpolarization (the superimposed spikes are due to a synaptic excitatory bombardment from another cell which should simultaneously react to benzene by a high frequency excitatory pattern).

To xylene (in *D*) both cells c_1 and c_2 reacted by excitatory type patterns, although of different temporal characteristics.

Compare the two differenciated spatio-temporal patterns here under the action respectively of two odor species, to that recorded in Fig. 16, from the same couple of cells c_1, c_2 but under the action of a third odor.

5 possible aspects of unitary receptor potentials, 5^n different specific spatio-temporal patterns, each able to label one odor molecule, should be expected.

2. *The "transfer function"*. The transfer function deals with the transformation of the receptor function into a propagating signal, i.e. the transform of the specific osmereceptor pattern into a specific spike pattern, emerging from the cell.

In the transforming of a membrane transient current of a given time course into a discharge of spikes, the characteristics of the current are suitably transduced in the frequency (F) of the spikes discharge as a function of time (Figs. 10, 11, 12, 13 and 14).

From each primary receptor neuron displaying a given receptor pattern $i_{rec} = f(t, C)$ originates a burst of spikes with a frequency $F = \psi(i_{rec})$. Such a spiking emerges from the receptor neuron and the information is precisely contained and conveyed by the frequency function.

As the anatomical arrangement provided in the olfactory system gives each cell a special pathway to the central nervous system, the whole spatio-temporal specific pattern of receptor potentials at the primary receptor surface is transferred to the central level (Figs. 15, 16,17). The transfer of the whole information concerning the timing, nature and concentration of the odor stimulus would thus be brought about by the built in assignment of the emerging pathways to assigned *loci* of central integrator units.

DISCUSSION

A small membrane area (of approximately 50–100 μ^2) of a common central nerve cell reacts by local receptor potentials, when reached by certain molecules which are not classifiable on the basis of their chemical function, but share the property of being perceived by the olfactory sense organ; thence their grouping as "odor molecules".

It has been shown how each of the examined cells reacts with selectivity to the different odor molecules, exhibiting receptor potentials—the so-called osmereceptor potentials (ORP)—which may affect distinct *specific* shapes according to the molecule. A feature of this sort, allows the nerve cell to generate spike discharges of *specific* frequency–time relationships, in which the ORP tenor is precisely transduced. In that way, encoding of the information—on the nature, the concentration, the timing of the acting molecule—and conveyance of the message are accurately secured.

The ORP is thus homologous to other primary generator potentials already known to occur in giant nerve cells of the same kind, proceeding from light, temperature, mechanical pressure, etc., energy conversions (Arvanitaki and Chalazonitis, 1956, 1961, 1963; Chalazonitis, 1964) but here, with the further ability of displaying sophisticated shapes.

Positive-going receptor potentials were already described in *Aplysia* giant nerve cells locally submitted to light or to temperature increase (Arvanitaki and Chalazonitis, 1961, Chalazonitis, 1964). However, shape variances in the negative-going receptor potentials were not yet encountered.

These should then be ascribed to the particular molecular mechanisms implied in the first steps of the odorants action on the cell membrane. In actual olfactory receptors it seems generally admitted that adsorption of the odorant on the cell membrane structures would be involved. The same assumption could be adopted in the present model. If so, the ORP time course should be expected to somehow deal with the kinetics of the adsorption on the cell membrane, i.e. the kinetics of the concentration of the adsorbate—here, the odor molecule—on the adsorbent surface—here the cell membrane. Kinetics of the adsorbate concentration should be correlated to the "adsorption isotherm" as, in the adopted experimental conditions, the environmental concentration of the odor molecule was increasing as a function of time. Now, adsorption isotherms may show many variant shapes (see Kipling, 1965) with conspicuous steps recalling ORP shapes as illustrated in Fig. 4. Steps occur particularly with heterogeneous adsorbent surfaces as, adsorption on chemically different sites (sites of differing energy) would imply different orientations and concentrations of the adsorbate molecules over the adsorbent field.

Such a situation should indeed be expected when adsorption is considered on the nerve cell membrane which structurally, cytochemically and functionally is an utterly organized mosaic.

So the features displayed by the osmereceptor potentials (when compared to other generator potentials) should be assigned to the particular physical dimensions of the stimuli—molecular shapes and sizes, concentration– and to the spatial distribution of specific receptor sites over the cell membrane.

To what extent conclusions drawn from the above neural model experience should be of any help in understanding mechanisms displayed in the actual olfactory function, i.e. reception, encoding, transmission, integration of the odor signal, it is difficult to decide. However, although unitary data from primary olfactory receptors are completely missing, many criteria would argue for actual analogies. For instance, specific differences in the time course of the olfactory mucosa receptor potentials (Ottoson); specific temporal patterns recognized whenever spikes discharges were recorded in individual nerve fibers (Beidler and Tucker, 1955; Gesteland *et al.*, 1963) all suggest that in the primary actual olfactory receptors, generator potentials and currents of various specific shapes should be as well displayed. Much higher sensitivity and ability in generating significantly variant osmereceptor patterns should moreover be expected there, owing to the total surface extent and the elaborated profiles displayed by the receptor membrane in the numerous hairs of each olfactory rod. Furthermore,

specific modal patterns in the spikes frequency–time relationships as detected in single taste nerve fibers by Zotterman to different gustatory stimuli should extend applicability of the present modol sensory coding to taste perception.

Anyhow, the unambiguous demonstration in a common nerve cell membrane of a keen ability for specific *direct* perception and "recognition" of various molecules should afford vistas in the mechanisms of "chemical stimulation" (Hayashi, 1959) directly displayed at the central nerve system levels, and more generally in different aspects of neuropharmacology.

SUMMARY

1. A neural *in vivo* model is proposed to search for the mechanisms of the odor perception sequential processes at the microelectrophysiological scale. The model implies the naked apexes of a few giant nerve cells of the *Helix* ganglia as primary receptors.

2. Transient actions of different odor species currently used in olfaction studies were given to the model at varying, low concentrations.

3. By intracellular recording, in any given cell when acted by a given odor molecule, a local osmereceptor potential (ORP) of specific reproducible shape was disclosed. A membrane current (i_{rec}) was concomitantly correlated.

4. Both negative and positive-going primary unitary osmereceptor potentials were recorded.

5. A given receptor neuron exhibits specific ORP shapes when acted by different odor species; ORP shapes from different receptor cells acted by the same odor are cell specific.

6. The final amplitude of the ORP, thence of the i_{rec}, varies as a function of the odor molecule concentration. Beyond a threshold value a spike discharge is elicited and emerges through the axon. The spikes frequency–time relationship suitably encodes the characteristics of the generator current, i.e. the physico-chemical dimensions of the stimulus.

7. Simultaneous recording from n nerve cells under an odor stimulus reveals a reproducible *ensemble*, a n-termed spatio-temporal receptor pattern. This is the representation at the primary receptor level of that odor stimulus; its nature, timing and concentration. Beyond the threshold the whole information encoded in a n-arrayed spatio-temporal spiking pattern in n independent emerging pathways, is conveyed to assigned second order receptor *loci*.

REFERENCES

ADRIAN, E. D. 1950. The electrical activity of the mammalian olfactory bulb. *Electroenceph. Clin. Neurophysiol.* **2,** 377–388.

ADRIAN, E. D. 1953. Sensory messages and sensation. The response of the olfactory organ to different smells. *Acta Physiol. Scand.* **29,** 5.

ANDERSEN, H. T., FUNAKOSHI, M. and ZOTTERMAN, Y. 1963. Electrophysiological responses to sugars and their depression by salt. In *Olfaction and Taste*, ed. Y. ZOTTERMAN, Pergamon Press, London, **1,** 177–192.

ARVANITAKI, A. and CHALAZONITIS, N. 1955. Potentiels d'activité du soma neuronique géant *(Aplysia)*, *Arch. Sci. physiol.*, **9,** 115–144.

ARVANITAKI, A. and CHALAZONITIS, N. 1956. Biopotentiels neuroniques à l'échelle infracellulaire. Stimulation mécanique graduée dans le soma géant d'*Aplysia*. *Bull. Inst. océanogr.*, Monaco, **53,** n°1079, 1–21.

ARVANITAKI, A. and CHALAZONITIS, N. 1961. Excitatory and inhibitory processes initiated by light and infra-red radiations in single identifiable nerve cells (giant ganglion cells of *Aplysia*). In *Nervous Inhibition*, Pergamon Press, 194–231.

ARVANITAKI, A. and CHALAZONITIS, N. 1963. Patterned activities from identifiable "cold" and "warm" giant neurons *(Aplysia)*. In *Olfaction and Taste*, ed. Y. ZOTTERMAN, Pergamon Press, London **1,** 377–380.

ARVANITAKI, A., CHALAZONITIS, N. and COSTA, H. 1964. Excitation du neurone géant par courants transmembranaires exponentiellement croissants *(Aplysia fasciata)*. *C. R. Soc. Biol.*, **158,** n°12, 2373–2377.

ARVANITAKI, A., COSTA, H. and TAKEUCHI, H. 1965. Fréquences des potentiels oscillatoires et pointes de la membrane somatique, par courants transmembranaires constants et linéairement croissants (neurone d'*Helix pomatia*). *C. R. Soc. Biol.*, **159,** n°3, 697–703.

BEIDLER, L. M. and TUCKER, D. 1955. Response of nasal epithelium to odor stimulation, *Science*, **76,** 122.

BOECKH, J. 1962. Elektrophysiologische Untersuchung einzelner Riechsensillen von Käfern, *Ver. dtsch. Zool. Ges., Wien*, 297–301.

CHALAZONITIS, N. 1959. Chemopotentiels des neurones géants fonctionnellement différenciés. *Arch. Sci. physiol.*, **13,** 41–78.

CHALAZONITIS, N. 1963. Effects of changes in pCO_2 and pO_2 on rhythmic potentials from giant neurons. *Ann. N-Y. Acad. Sci.*, **109,** 451–479.

CHALAZONITIS, N. 1964. Light energy conversion in neuronal membranes. *Photochem. Photobiol.*, **3,** 539–549.

CHALAZONITIS, N. and ARVANITAKI-CHALAZONITIS, A. 1961. Potentiels générateurs par activation mécanique de la membrane somatique. *C. R. Acad. Sci.* **252,** 4046–4048.

GESTELAND, R. C., LETTVIN, J. Y., PITTS, W. H. and ROJAS, A. 1963. Odor specificities of the frog's olfactory receptors. In *Olfaction and Taste*, ed. Y. ZOTTERMAN, Pergamon Press, London, **1,** 19–34.

HAYASHI, T. 1959. *Neurophysiology and Neurochemistry of convulsion.* Dainikon. Tosho Co. Ltd. Tokyo, Japan.

HIGASHINO, S., TAKAGI, S. F. and YAJIMA, M. 1961. The olfactory stimulating effectiveness of homologous series of substances studied in the frog. *Jap. J. Physiol.*, **11,** n°5, 530–543.

HODGSON, E. S., LETTVIN, J. Y. and ROEDER, K. D. 1955. Physiology of a primary chemoreceptor unit. *Science*, **122,** 417–418.

KUNZE, H. 1921. Zur Topographie und Histologie des Central nervensystems von *Helix pomatia* L. *Zeitschrift Wissenschaft. Zool.*, **118,** 25–203.

LORENZO, A. J. DE. 1963. Studies on the ultrastructure and histophysiology of cell membranes, nerve fibers and synaptic junctions in chemoreceptors. In *Olfaction and Taste*, ed. Y. ZOTTERMAN. Pergamon Press, London, **1,** 5–17.

OTTOSON, D. 1956. Analysis of the electrical activity of the olfactory epithelium. *Acta Physiol. Scand.* **35,** Suppl. 122, 1–83.

OTTOSON, D. 1958. Studies on the relationship between olfactory stimulating effectiveness and physico-chemical properties of odorous compounds. *Acta Physiol. Scand.* **43,** 167–181.

OTTOSON, D. 1963. Generation and transmission of signals in the olfactory system. In *Olfaction and Taste*, ed. Y. ZOTTERMAN, Pergamon Press, London, **1,** 35–44.

SCHNEIDER, D. 1963. Electrophysiological investigation of insect olfaction. In *Olfaction and Taste*, ed. Y. ZOTTERMAN, Pergamon Press, London, **1,** 85–103.

ZOTTERMAN, Y. 1936. Specific action potentials in the lingual nerve of the cat. *Arch. Physiol. Scand.* **75,** 105–119.

CHEMORECEPTORS IN BRAIN TO γ-HYDROXYBUTYRATE THROUGH CEREBROSPINAL FLUID OF DOGS

T. HAYASHI, H. HOSHINO and T. OOTSUKA

Department of Physiology, School of Medicine,
Keio University, Tokyo

INTRODUCTION

In 1950 it was first reported that gamma-aminobutyric acid (GABA) was a constituent of mammalian brain[1, 25] and in 1956 that GABA and its derivatives had inhibitory action when given directly into the brain[7, 11]. In 1960 Loborit *et al.* reported that gamma-hydrobutyrate (γOHbt) and its lactone had a sleep-producing action when administered to animals,[22] and in 1963 Bessman *et al.* found that γOHbt is a constituent of the brain.[3] Incidentally, a dipeptide of GABA and histidine, i.e. homocarnosine, was found by Udenfriend *et al.* in 1961,[24] and in the following year it was found by Hayashi *et al.*[9] that it has inhibitory action to the limbic motor system. Structures of these substances are shown in Table 1.

TABLE 1. STRUCTURES OF GABA AND ITS DERIVATIVES

Gamma-aminobutyrate (GABA)

$NH_2CH_2CH_2CH_2COOH$

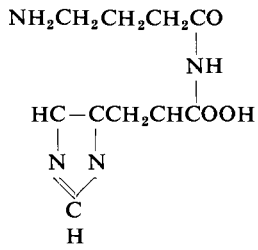
Beta-hydroxy-gamma-aminobutyrate (GABOB)

$NH_2CH_2CH(OH)CH_2COOH$

Gamma-hydroxybutyrate (γOHbt)

$OHCH_2CH_2CH_2COOH$

Gamma-aminobutyrylhistidine (homocarnosine)



We introduced γOHbt into cerebrospinal fluid of dogs through cisternal injection and confirmed that it causes behavioural sleep with a latent period of 15 min using 0·25 ml of 0·1 M solution. The sleep seemed a natural one, for the dog was awakened by a touch of the hand, a sound of clapping hands and calling its name, but soon fell asleep again.

1. BLOOD BRAIN BARRIER OF γ OHbt

We used two ways of administration, (1) via c.s.f., and (2) via the circulation, in order to compare the threshold dose causing sleep. It will be seen from Table 2 that the blood brain barrier (Bbb) in γOHbt was very high. We tried to indicate the grade of Bbb numerically by eq. (1):

$$\text{Bbb} = \frac{\text{the amount entered into the brain}}{\text{the amount remaining in the blood}} \qquad (1)$$

TABLE 2. BLOOD BRAIN BARRIER OF GAMMA-HYDROXYBUTYRATE AND OTHERS

Substance	Indicator	Introduction method	Concentration in brain	Blood brain barrier (eqn. I)
Gamma-hydroxybutyrate	Producing sleep	Via c.s.f. Via circulation	0·0036% 0·13%	$\frac{0·0036}{0·13-0·0036}=0·00389$
Beta-hydroxy-gamma-aminobutyrate	Inhibition upon conditioned reflex	Via circulation	(−) 0·00052% (+) 0·0028%	$\frac{0·00052}{0·0028-0·00052}=0·224$
Methionine sulfoximine	Producing seizure	Via c.s.f. Via circulation	0·00154 mg% 6·9 mg%	$\frac{0·00154}{6·9-0·00154}=0·00025$

One may expect that such a substance as methionine sulfoximine, which, injected into the brain, has a toxic action, does not pass easily from the blood into the brain. But γOHbt, as a naturally occurring substance which has a certain physiological action when introduced into the brain via c.s.f. to stimulate brain cells or to inhibit them, naturally has not so high Bbb.

(1) γOHbt naturally occurs in the brain. Bessman reported that it was contained in rat brain in 1·2–2·0 mM concentration, and in the human brain in 0·2–0·3 mM.[5] We have, however, found low values in rats, 0 026 mM. These diverse values must mean something, which we will discuss later.

(2) The sleep-inducing dose of γOHbt, when directly applied to c.s.f., corresponded to 0·0036% of the weight of the whole brain (see Table 2). In other words, this substance has an action of inducing sleep when its concentration in the brain attains lower values than those usually found in the brain, i.e. 0·00375–0·0091%.

From these two criteria we would presume that γOHbt might be the chemical mediator in the brain to induce circadian sleep if we had evidence that this substance is formed in the brain.

2. METABOLISM OF γOHbt IN THE BRAIN AND THE INDUCING OF SLEEP

What is the metabolism of this substance? It comes from GABA and is metabolized into succinic acid towards citric acid cycle to yield energy as shown in eqn. (2) [6] and in Fig. 1.

TABLE 3. HYDROLYTIC DEAMINATION OF GABA[5,6]

Gamma-aminobutyrate + α-ketoglutarate

→ saccinic semialdehyde + glutamate

Glutamate + $DPN^+ + H_2O$

→ NH_3α-ketoglutarate + $DPNH + H^+$

Succinic semialdehyde + $DPNH + H^+$

→ gamma-hydroxybutyrate + DPN^+

Gamma-aminobutyrate — H_2O → NH_3 + gamma-hydroxybutyrate (2)

It is known that there are two processes at work in the brain, one a positive the other a negative. We have mentioned that these two processes might depend upon the existence of two chemical transmitters which were presumed to be both derivatives from GABA. [11, 12] The evidence has been accumulated by experiments on dogs, as summarized in Fig. 1. Thus GABA has three metabolic pathways: (1) to γOHbt, (2) to chemical transmitters, and (3) to energy yielding.

As GABA has three metabolic pathways (Fig. 1), it must be expected that γOHbt production will be promoted if we block the way to succinic acid or to chemical transmitters.

(1) Working from this consideration, we introduced into dogs' c.s.f. succinic acid with glutamate which neutralized NH_3 liberated by deamination of GABA [5]. It resulted in sleep as shown in Table 4. After a latent period of 15–20 min the dog fell into behavioural sleep. The

TABLE 4. SLEEP DUE TO INJECTIONS INTO DOGS' C.S.F.

Substances	Dose	Latent period and continuation
Succinic acid + glutamic acid	0·1 M 0·5 ml 0·1 M 0·25 ml	15 min; lie down with closed eyes; fell into behavioral sleep; alert by sound; soon fell into sleep and snoring. 60 min; alert.
Succinic semialdehyde + glutamic acid	0·1 M 0·5 ml 0·1 M 0·5 ml	20 min; lie down, but not sleep 50 min; alert
NH_2OH + glutamic acid	0·1 M 0·5 ml 0·1 M 0·5 ml	No sleep
Glutamic acid	0·1 M 1~2 ml	No sleep
Succinic acid	0·1 M 1~2 ml	No sleep

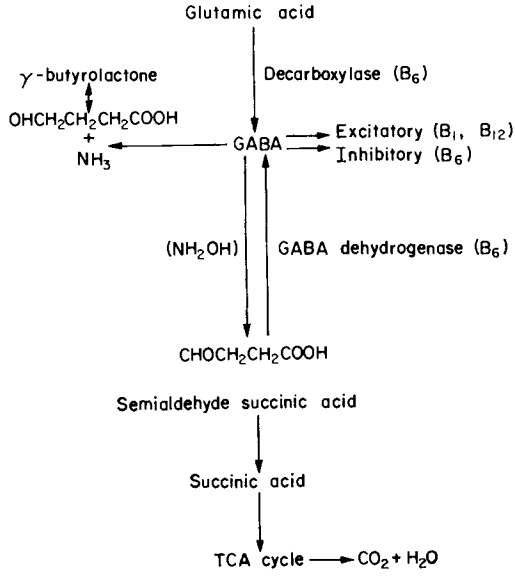


FIG. 1.

TABLE 5. SPECTROPHOTOMETRIC MEASUREMENT OF GAMMA-HYDROXYBUTYRATE

Method	Incubation	Measurement (mM)	Concentration of gamma-hydroxybutyrate (%)
Located by paperchromatography with ferric chloride	Rat's brain homogenate Incubation 30 min	0·036	0·00375
Separated into phenol solution Measurement through Hidachi EPU-2A (wavelength 540 mμ)	Rat's brain homogenate Succinic acid 0·1 ml Glutamic acid 0·2 ml Incubation 30 min	0·087	0·0091

facts strongly suggest that γOHbt can be generated in the brain, if GABA is prevented from being metabolized.

(2) Incidentally, we tested hydroxylamine which prevents the formation of succinic semialdehyde from GABA.[2] It did not cause sleep, as shown in Table 4. It showed that the pathway to γOHbt was from succinic semialdehyde, not from GABA itself, as shown previously in Table 3 and eqn. (2).

(3) Then we proceeded to experiment to detect γOHbt genesis in brain homogenate, as in the case mentioned above.

Table 5 shows the summary of these experiments. As a control, rats' brain homogenate was incubated in 38°C for 30 min. γOHbt was located chromatographically by paper separated and measured by EPU-2A Hidachi of wavelength 540 mμ[10]. γOHbt was calculated as 0·036 mM (0·00375%) which was increased to 0·087 mM (0·0091%) with the blocking of metabolism by adding succinic acid with glutamate.

3. CIRCADIAN GENESIS OF γOHbt AND THE INDUCING OF SLEEP

(1) In the next experiment we introduced the substances which prevent the production of the excitatory and inhibitory functional substances from GABA. INH is a vitamin B_6 antagonist which blocks the conversion of glutamic acid to GABA and the conversion of GABA to the inhibitory substance. Pyrithiamine is a B_1 antagonist which prevents the conversion of GABA to excitatory substance [12]. The results are shown in Table 6.

The functioning of the brain for long periods will exhaust the functional substances, especially the excitatory transmitter, and the balance of the two processes will incline to the excess of the inhibitory one. [10] Does the excess of the inhibitory transmitter produce sleep? We tested by means of introducing gamma-amino β hydroxybutyric acid (GABOB) which has been confirmed by Hayashi to be a derivative of GABA and had a strong inhibitory action on the cortex.

(2) GABOB itself has no sleep-producing action when introduced into c.s.f., notwithstanding it has a strong inhibitory action on seizure as well as on excitatory conditioned reflex.[8, 10] But when it was introduced with succinic acid and glutamate, as in eqn. (2), it produced sleep with 3–4 min latent period, as shown in Table 6.

TABLE 6. SLEEP DUE TO INJECTIONS INTO RABBITS' C.S.F.

Substances	Dose	Behavioral sleep
I N H + Pyrithiamine	0·1 M 0·2 ml 0·02 M 0·2 ml	Latent period 1·30 min; closing eyes; lie down; alert by sound 25 min: stand up
INH + Pyrithiamine	0·1 M 0·2 ml 0·03 M 0·2 ml	17–18 min: drowsy 23 min: lie down 33 min: stand up
GABOB + Succinic acid + Glutamic acid	2 M 0·25 ml 0·1 M 0·25 ml 0·1 M 0·25 ml	3–4 min: lie down; eyes closed; behavioral deep sleep 1 h 15 min: alert
GABOB	2 M 0·25 ml	No sleep

The exhaustion should be at the site of synapses and decrease production in the ganglion cells. Thus, in the evening, the concentration of γOHbt in brain cells will be increased and circadian sleep occurs.

(3) A rapid exhaustion of functional substances will result in irresistible sleep even in day time. Seizure attacks in epileptics are followed by sleep. Table 7 shows the hours of sleep after the electroshock seizure in men. The state described as a dim consciousness corresponds to light sleep.

TABLE 7. SLEEP DUE TO ELECTROSHOCK SEIZURE IN MAN

State after the seizure	Total 91	%
Sleep	29	31·9
State of dim consciousness	45	49·5
Normal state	17	18·6

4. CHEMORECEPTORS TO THE INTRINSIC γOHbt

By recording the EEG, Bremer[4] and Magoun[23] found that sleep occurs by the inhibition of reticular formation of the midbrain. Thus a sleep of high voltage and slow waves, in cortex EEG appears. Recently there were many valuable disclosures in sleep physiology, one of them being the discovery of "paradoxical sleep" by the French physiologist Jouvet and his associates.[17–21] It is a sleep of low voltage and fast waves in cortex EEG with quite atonic body muscles and rapid eye-movements. How do these two sleeps occur? The first observation was that both sleeps were elicited by γOHbt, large dosage producing paradoxical sleep.

When γOHbt is produced in nerve cells in the evening, it does not affect the cell itself, contrary to cases of intoxication or narcotization.

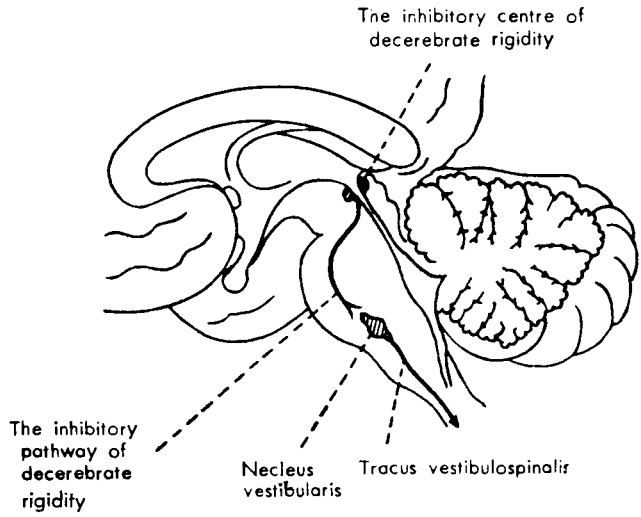


FIG. 2.

It must diffuse out. In this case it is most probable that the increased γOHbt does not diffuse into circulation, for its Bbb is very high, as already shown in Table 1. It will diffuse out into c.s.f. and affect the reticular formation of the midbrain. Thus cortical sleep will occur. Further, it diffuses into the pontile brain, and the inhibition reaches vestibular nuclei, in particular, Deiter's nuclei (Fig. 2) which is the tonic innervation centre, especially for antigravity body muscles.[14] Thus paradoxical sleep occurs. Jouvet demonstrated that when the dorsal and lateral pontile brain was electrically coagulated, γOHbt had no action to induce paradoxical sleep.[18] Involuntary rapid eye-movements will be released by the inhibition of cerebellar innervation to vestibular nuclei, due to γOHbt, and the fast EEG wave in paradoxical sleep is produced by arousal impulses from the reticular formation due to the activity of the eye muscles.

DISCUSSION

An hypothesis formed from the above experiments is that sleep is produced by γOHbt which is generated in the brain cells. The manifestation of sleep is produced by the action of γOHbt on the chemoreceptors in the reticular formation of midbrain and in the vestibular nuclei and other neighbouring structures. γOHbt is produced by the exhaustion of functional substances of the brain after the functioning of the brain cells for long periods.

The action of γOHbt upon the above receptors is not excitatory, but inhibitory. Accordingly, the above hypothesis is a hypothesis of inhibition as Pavlov postulated[10] contrary to Hess' excitation theory.[15]

(1) Hess' experiment has been confirmed by many authors, and at present it is summarized as follows: the stimulation of 4–8 per second electrical pulses causes sleep, but that of 40–60 per second pulses causes arousal state, owing to the sleep and wakefulness centres probably being situated close to each other. This is rather ambiguous.

It is probable that the arousal reaction will be produced by the excitation at the wall of the third ventricle, for there must exist excitable neurons which will be stimulated by current spread when the stimulation is frequent and strong. The question is what action has the stimulation of 4–8 per second impulses? Our tentative suggestion is as follows; hypothalamus contains much γOHbt as it contains much GABA, and, if γOHbt is connected with globulin and other nervous components, it may be released by the low frequency of stimulation. We have found in the experiment that glutamic acid mixed with a globulin *in vitro* would be easily

separated by low frequency electrical pulses through it, mainly to its negative side.[13]

(2) Recently, Monnier and his associates reported that when the Hess' centre was stimulated a special substance which is dialysable appeared in the blood. They also stated that if the blood were transfused into another animal it would fall asleep.[16] They have not yet extracted this substance, but, from the above experiments, we guess that it might be drained out into the circulation. Owing to the higher blood brain barrier of γOHbt, Monnier's substance should not be γOHbt. Their second report[16] was more doubtful, for when the stimulation of the third ventricle is more frequent the substance to appear would be expected to produce arousal.

These problems need further investigation.

REFERENCES

1. AWAPARA, J., LANDUA, A. J., FUERST, R. and SEALE, B. (1950) Free γ aminobutyric acid. *Nature*, **178**, 1052.
2. BAXISTER, C. F. and ROBERTS, E. (1959) Elevation of aminobutyric acid in rat brain with hydroxylamine. *Proc. Soc. Exper. Biol. Med.* **101**, 811.
3. BESSMAN, S. P. and FISHBEIN, W. N. (1963) Gamma-hydroxybutyrate, a normal brain metabolite. *Nature* **200**, No. 4912.
4. BREMER, F. (1935) Cerveau "isole" et physiologie du sommeil. *C. R. Soc. Biol.* **118**, 1235.
5. FISHBEIN, W. N. and BESSMAN, S. P. (1964) γ-hydroxybutyrate in mammalian brain. *J. Biol. Chem.* **239**, 357.
6. HARDMAN, J. K. and STADTMAN, T. C. (1963) Metabolism of ω-amino acids. *J. Biol. Chem.* **238**, 2081.
7. HAYASHI, T. and SUHARA, R. (1956) Substances which produce epileptic seizure when applied on the motor cortex of dogs and substances which inhibit the seizure directly. Communication of the 20th International Physiological Congress at Brussels.
8. HAYASHI, T. (1959) This inhibitory action of beta-hydroxy-gamma-aminobutyric acid upon the seizure following stimulation of the motor cortex of the dog. *J. Physiol. London* **145**, 570–578.
9. HAYASHI, T., HARA, K. and SEKI, S. (1962) Convulsions due to pallial system and limbic system in dogs. *Proceedings of the International Congress of Physiological Sciences*, vol. II, 1158, Leyden.
10. HAYASHI, T. (1965) Pavlov's sleep theory under a new light. *Keio J. Med.* **14**, 135.
11. HAYASHI, T. (1958) Inhibition and excitation due to gamma-amino-butyric acid in the central nervous system. *Nature*, **182**, 1076.
12. HAYASHI, T. (1965) Genesis of excitatory transmitter in the brain of dogs. *Essays on Physiological Evolution*, Ed. by J. W. S. Pringle, D. Sc., F.R.S., 139, Pergamon, London.
13. HAYASHI, T. (1953) A suggestion of the mechanism how electric current excites in nervous system. *Keio J. Med.* **2**, 23.
14. HAYASHI, T. (1959) *Neurophysiology and Neurochemistry of Convulsion.* Dainihon-Tosho Co. Ltd.
15. HESS, W. R. (1949) The central control of the activity of internal organs. Nobel

Lecture. Nobel lectures including presentative speeches and laureates biographies. *Physiology or Medicine* 1942–1962. Elsevier Publishing Co. Amsterdam, 1964.

16. HÖSLI, L. and MONNIER, M. (1964) Humoral transmission of sleep and wakefulness. I. Method for dialysing psychotropic humors from the cerebral blood. *Pflügers Archiv ges. Physiol.* **282,** 54. II (1964). Homodialysis of a sleep inducing humors during stimulations of the thalamic somnogenic area. *Ibid.* **282,** 60. III (1965). Homodialysis of active humors during stimulations of the midbrain reticular system. *Ibid.* **283.**
17. JOUVET, M. (1963) Étude de la dualité des états de sommeil et des mechanismes de la phase paradoxale. *Colloques intenationaux du centre Nationale de la referches scientifique* **127,** 379.
18. JOUVET, M. (1965) Behavioral and EEG effects of paradoxical sleep deprivation in the cat. *Proceedings of the International Union of Physiological Sciences*, vol IV, XXIII rd International Congress Tokyo, Lecture and Symposia.
19. JOUVET, M. (1962) Recherches sur les structures nerveuses et les mechanismes responsables des differente phases du sommeil physiologiques. *Arch. Ital. Biol.* **100,** 125.
20. JOUVET, M. (1962) Un appareil enregistreur automatique des phases rhombencéphaliques du sommeil chez le chat; l'onirographe. *Rev. Neurol.* **107,** 269.
21. JOUVET, M., JOUVET, D. and VALATIX, J. L. (1963) Étude du sommeil chez le chat pontique chronque. Sa suppression automatique. *C. R. Soc. Biol. (Paris)* **157,** 845.
22. LOBORIT, H., BRUCE, F., JOUANY, J. M., GERARD, J. and FABIANI, F. (1960) Géneralite concernant l'étude experimentale dés l'empoi clinique du gamma-hydroxybutyrate de Na. *Aggressologie.* **1,** 407.
23. MAGOUN, H. W. and MC. ISAAC, W. H. (1962) *The Waking Brain.* Springfield, Ill., USA.
24. PISANO, J. J., WILSON, D., COHEN, L., ABRAHAM, D. and UDENFRIEND, S. (1961) Isolation of γ-aminobutyrylhistidine (homocarnosine) from brain. *J. Biol. Chem.* **236,** 499.
25. ROBERTS, E. and FRANKEL, S. (1950) γ-aminobutyric acid in the brain and its formation from glutamic acid. *J. Biol. Chem.* **187,** 55.

THE ELECTRO-ODOCELL FOR INSPECTION AND GRADING OF MEAT AND FISH

N. N. TANYOLAÇ

Robert College Research Center
Bebek, Istanbul, Turkey

I. INTRODUCTION

From a scientific point of view, we know only what our senses tell us. But to give a quantitative meaning to what our senses tell us, standard units of measurements and objective measuring instruments are essential.

The aim of odor research work at the Robert College Research Center has been to develop an instrument which will measure the odor objectively, and thus replace the human nose in the detection and measurement of odor.

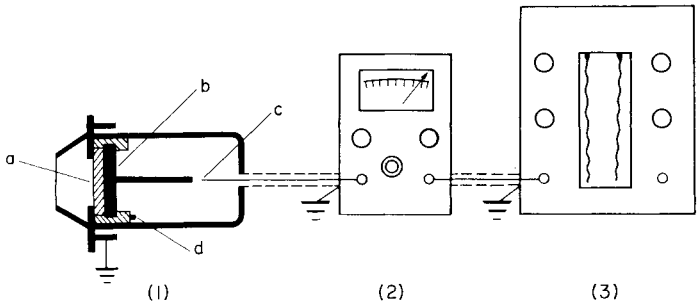


FIG. 1.

Research on the development of an objective odor-measuring instrument has been intensified since 1949.[1] Odocell, developed at Purdue University in 1948 by the writer and Eaton, was used by various investigators[2, 3, 4, 5, 6] for odor studies. Hartman[10] at Cornell University, Dravnieks[11] at IIT, Moncrieff[12] in England, and Bertan[15] in France had developed instruments to be used as objective odor-measuring devices.

In 1964 the writer presented the Electro-odocell as an instrument to detect and measure odor or micromicro amounts of material in the air.[16,17,18]

The Electro-odocell detects and measures the change in potential or current on a dielectric surface when one surface of the dielectric is contaminated by the molecules given off by odorants. The Electro-odocell consists of an odor-sensitive transducer open to odor molecules, a micro-voltmeter and an automatic recorder[16] (Fig. 1).

The experiments carried out at the Robert College Research Center to develop a method of using the Electro-odocell for the inspection and grading of meat and fish are presented in this paper.

II. EXPERIMENTAL PROCEDURE

A. Preparation of samples

1. One pound of fresh ground beef was divided into three equal groups (A, B, and C). Group (A) had no treatment. Group (B) was placed in distilled water. Group (C) was boiled in distilled water at 80 degrees Centigrade for 15 minutes.
2. Equal portions of ground beef (10 grams) were placed in conical weighing bottles. Thus twelve samples, four out of each group, were prepared. Half the samples of each group (A_1, A_2; B_1, B_2; C_1, C_2) were refrigerated for five days before being removed for testing. These samples were designated samples of condition (b). The other half of the samples of each group (A_3, A_4: B_3, B_4; C_3, C_4) were ready for experiment, and were used immediately. These samples were designated samples of condition (a).

B. Testing procedure

1. Micro-voltmeter and recorder switch of the Electro-odocell was turned on for 15 minutes before any measurements were taken.
2. Initial conditions of the Electro-odocell (initial reading of the voltage, temperature, barometric pressure and relative humidity) were recorded and the initial voltage read was reduced to zero.
3. Micro-voltmeter scale was set to the lowest value.
4. The stop watch was started and the cover of the sample bottle was removed at 45 seconds. Recording started at 55 seconds and at 60 sec-

onds the bottle of the sample was placed under the detector of the Electro-odocell (Fig. 2). The recorder plotted voltage–time curve due to odor of the sample.

5. The scale of the recorder was increased when necessary.
6. After 5 minutes of recording the sample was removed from the detector. There was a 5 minute wait to record the drop in the voltage–time curve.
7. The second test was not begun until the reading of the micro-voltmeter had lowered to zero.

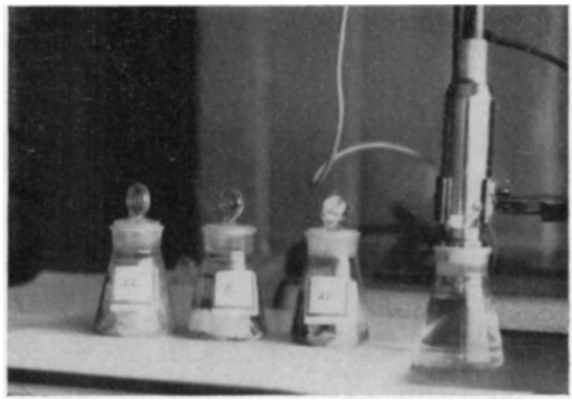


FIG. 2.

Fish samples were prepared and tested under the same procedures as ground beef. Flat fish, mackerel and sea bream were the types of fish available for the experiments. Instead of using a pound of fish, one live fish was used to prepare twelve samples.

III. TEST RESULTS

In order to compare the recordings of Electro-odocell, the readings of the same sample at different ages are plotted on the same graph paper using logarithmic scale in micro-volt axis. Thus the readings of various degrees of spoilage are on one paper, making it easier to see the differences between the graphs. But (y) scale being in logarithms of microvolts, the existing differences in voltage for any specific time (t) of two different samples does not appear to be very great.

A) Test made with ground beef samples

Those of condition (a) are indicated in Fig. 3. Curves A'_{3-1}, A'_{4-1}, B'_{3-1}, and C'_{3-1} indicate that after aging the ground beer of group (A) about 25 hours, the Electro-odocell indicates a voltage change of about (-600) microvolts within 3 minutes of test and the odor of the meat resembles the odor of H_2S.

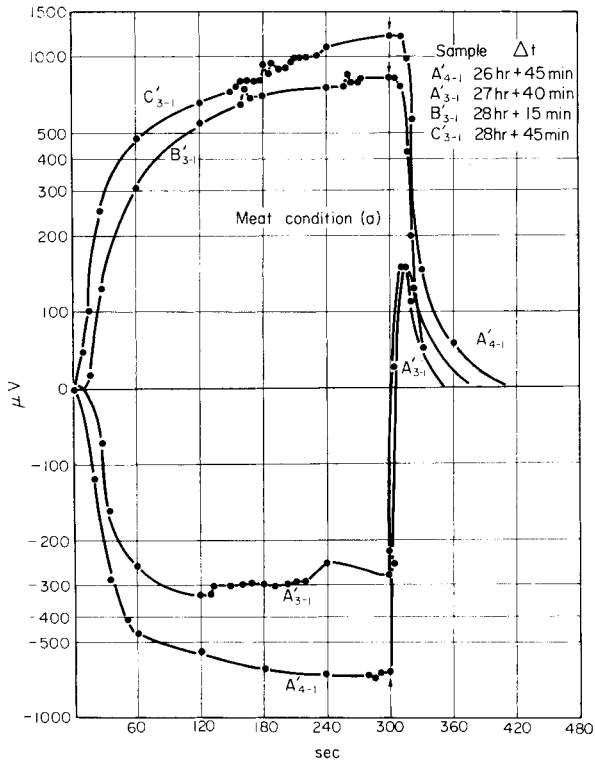


FIG. 3.

For samples of group (B) this change is also about 600 microvolts, but in positive value; where for samples of group (C) the change is like (B) but higher value.

Tests made with ground beef of condition (b) are indicated in (Fig. 4). Curves A_{2-2}, A_{1-3}, B_{1-2}, B_{2-3}, C_{1-2}, and C_{2-3} indicate that in all cases for the first sixty seconds the change in voltage indicated by the Electro-odocell is negative and after a short time the change becomes positive. Samples of group (A) have higher values of voltage than groups (B) and (C).

For groups (A) and (C) as the age of the sample increases the change of voltage also increases, but for group (B) it shows a decrease in voltage as the age of the sample increases. These changes are indicated for each group separately in (Fig. 5a), (Fig. 5b), and (Fig. 5c).

The difference of curves as the age of sample increases are more noticeable for group (A) and group (B). This can be related to the spoilage of boiled meat group (C) with time being generally less than meat in water group (B) or meat in the air group (A).

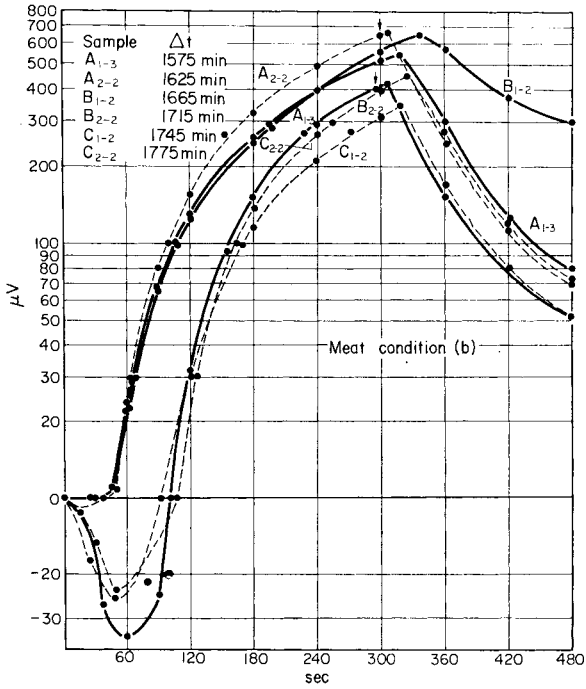


FIG. 4.

B) Tests made with two different kinds of fish (flat fish and mackerel)

Taken from the refrigerator, under three different treatments (A, B, C), (Figs. 6a, 6b, 6c, 7a, 7b, and 7c), indicate that as the aging of the fish increases, Electro-odocell indicates higher voltage. For a twenty-four hours of aging increase in voltage is about 43%. Experiments made with *fresh fish samples*, without putting to ice box at all, indicates both increase and decrease in voltage as the age of the fish increases; the number of samples which increase in voltage are more than the number of samples

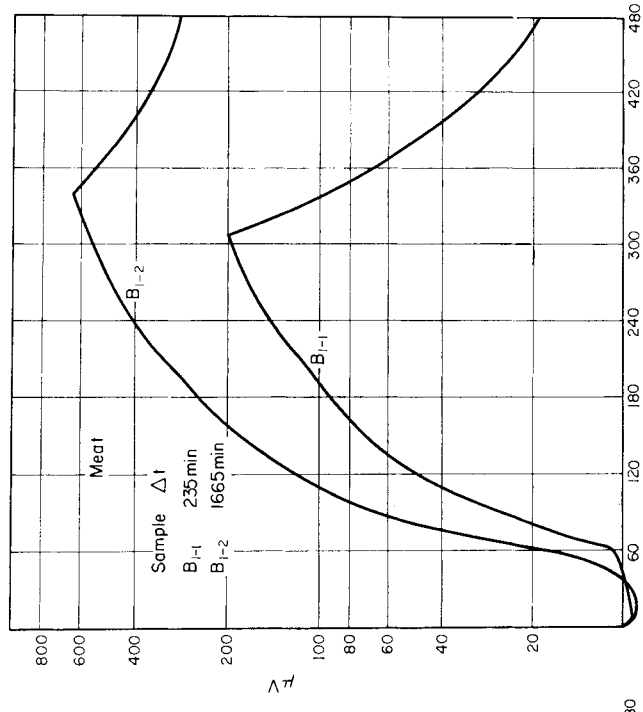

Meat
Sample Δt
B1-1 235 min
B1-2 1665 min
B1-2
B1-1
μV
sec

FIG. 5b.

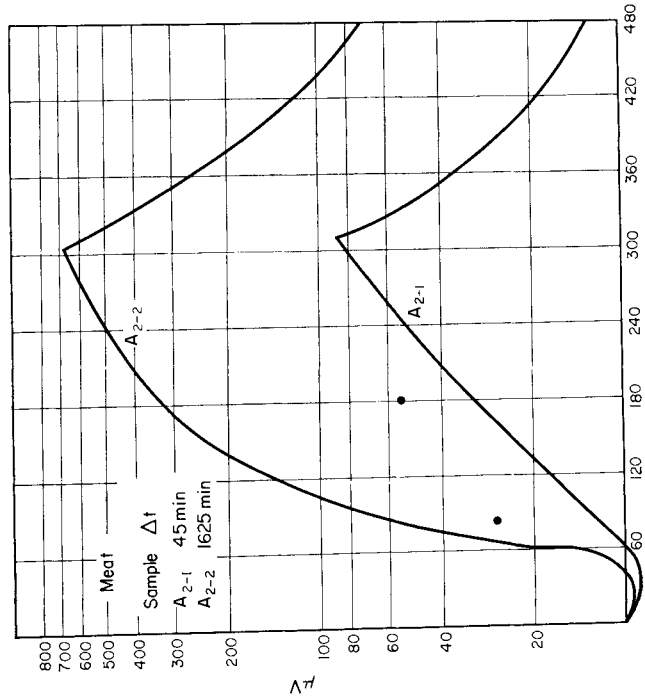

Meat
Sample Δt
A2-1 45 min
A2-2 1625 min
A2-2
A2-1
μV
sec

FIG. 5a.

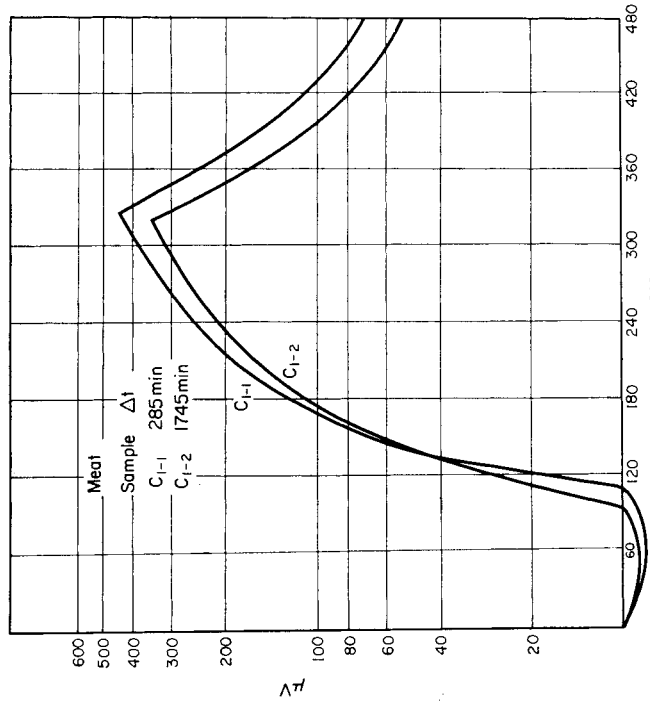

Meat
Sample Δt
C1-1 285min
C1-2 1745min
C1-1
C1-2
600
500
400
300
200
100
80
60
40
20
μV
60
120
180
240
300
360
420
480
sec

FIG. 5c.

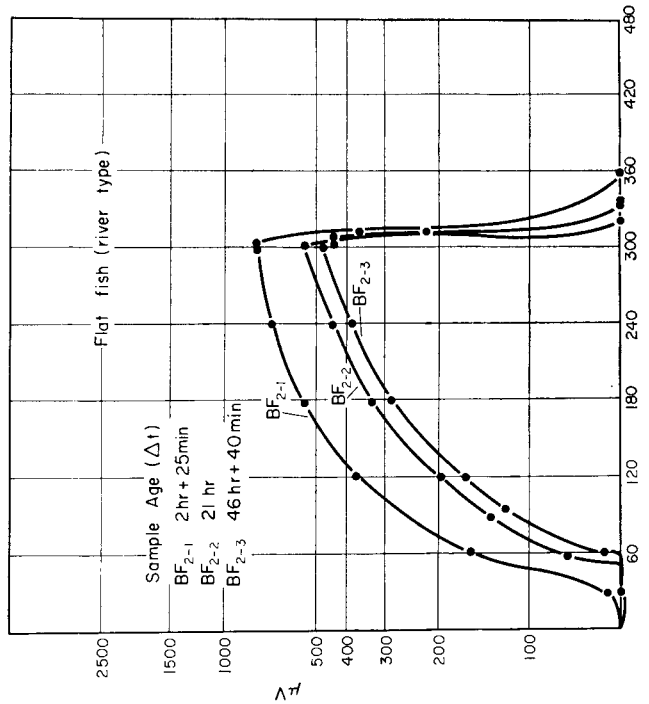

Flat fish (river type)
Sample Age (Δt)
BF2-1 2hr + 25min
BF2-2 21 hr
BF2-3 46 hr + 40 min
BF2-1
BF2-2
BF2-3
μV
sec
2500
1500
1000
500
400
300
200
100
60
120
180
240
300
360
420
480

FIG. 6b.

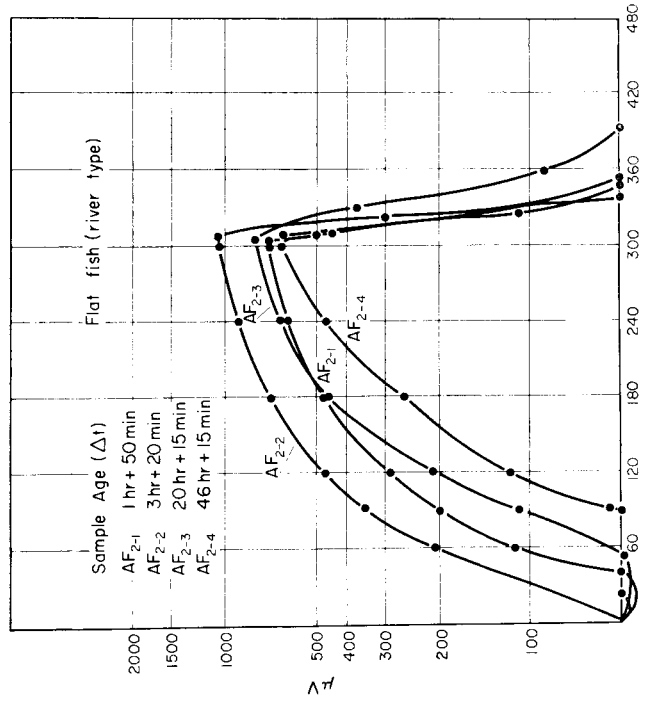

Flat fish (river type)
Sample Age (Δt)
AF2-1 1 hr + 50 min
AF2-2 3 hr + 20 min
AF2-3 20 hr + 15 min
AF2-4 46 hr + 15 min
AF2-1
AF2-2
AF2-3
AF2-4
μV
sec
2000
1500
1000
500
400
300
200
100
60
120
180
240
300
360
420
480

FIG. 6a.

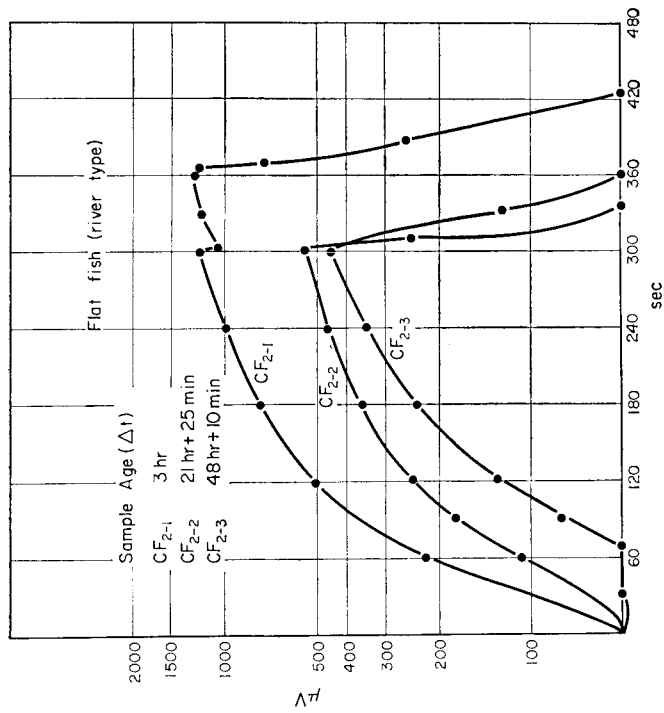

Flat fish (river type)
Sample Age (Δt)
CF2-1 3 hr
CF2-2 21 hr+25 min
CF2-3 48 hr+10 min
CF2-1
CF2-2
CF2-3
μV
2000
1500
1000
500
400
300
200
100
60
120
180
240
300
360
420
480
sec

FIG. 6c.

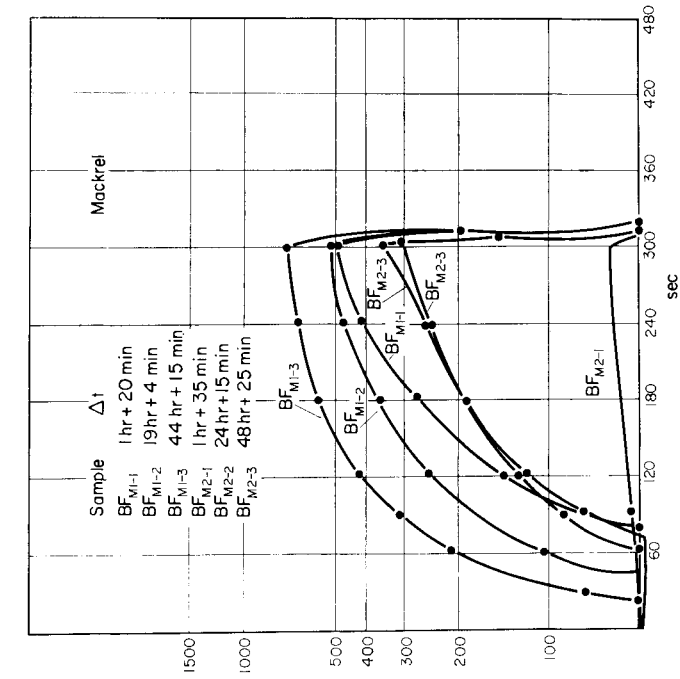

Mackrel
Sample Δt
BF$_{M1-1}$ 1 hr + 20 min
BF$_{M1-2}$ 19 hr + 4 min
BF$_{M1-3}$ 44 hr + 15 min
BF$_{M2-1}$ 1 hr + 35 min
BF$_{M2-2}$ 24 hr + 15 min
BF$_{M2-3}$ 48 hr + 25 min
BF$_{M1-3}$
BF$_{M1-2}$
BF$_{M2-3}$
BF$_{M1-1}$
BF$_{M2-1}$
μV
1500
1000
500
400
300
200
100
60
120
180
240
300
360
420
480
sec

FIG. 7b.

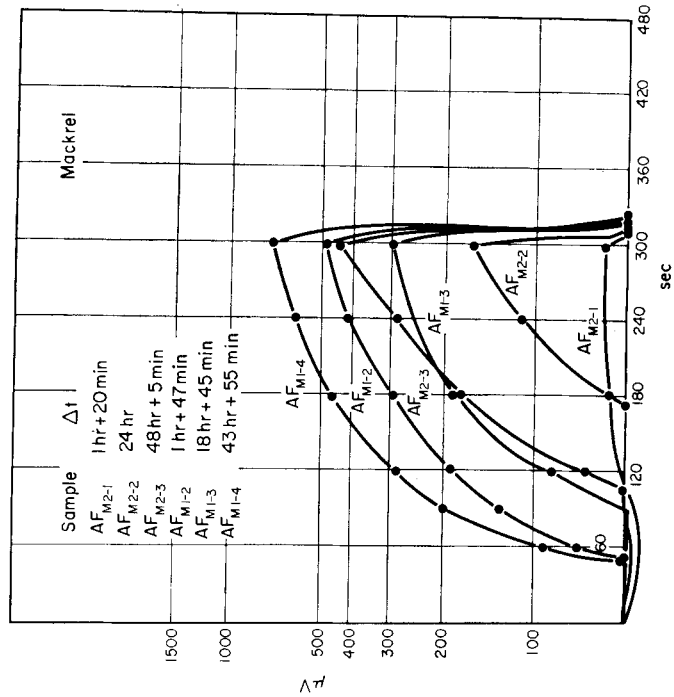

Mackrel
Sample Δt
AF$_{M2-1}$ 1 hr + 20 min
AF$_{M2-2}$ 24 hr
AF$_{M2-3}$ 48 hr + 5 min
AF$_{M1-2}$ 1 hr + 47 min
AF$_{M1-3}$ 18 hr + 45 min
AF$_{M1-4}$ 43 hr + 55 min
AF$_{M1-4}$
AF$_{M1-2}$
AF$_{M2-3}$
AF$_{M1-3}$
AF$_{M2-2}$
AF$_{M2-1}$
μV
1500
1000
500
400
300
200
100
60
120
180
240
300
360
420
480
sec

FIG. 7a.

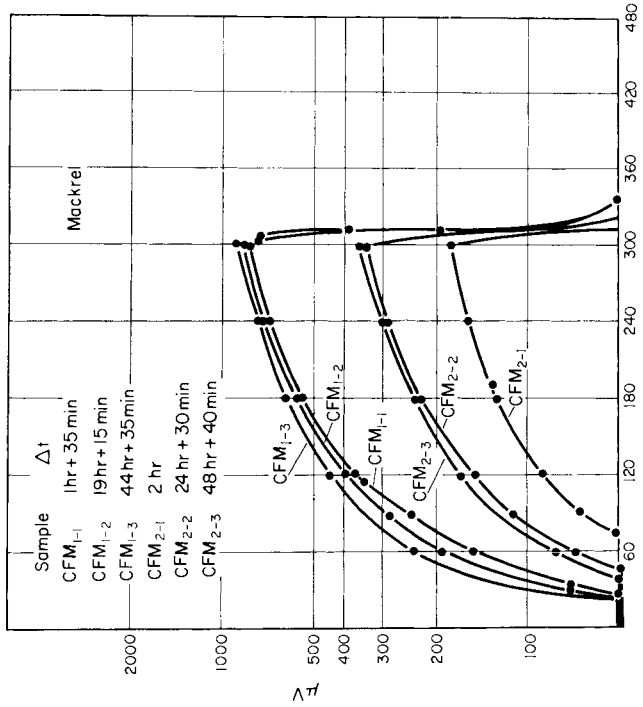

Sample
Δt
CFM1-1
1hr + 35 min
CFM1-2
19hr + 15 min
CFM1-3
44hr + 35min
CFM2-1
2 hr
CFM2-2
24 hr + 30 min
CFM2-3
48 hr + 40 min
Mackrel
μV
2000
1000
500
400
300
200
100
60
120
180
240
300
360
420
480
sec
CFM1-3
CFM1-2
CFM1-1
CFM2-3
CFM2-2
CFM2-1

FIG. 7c.

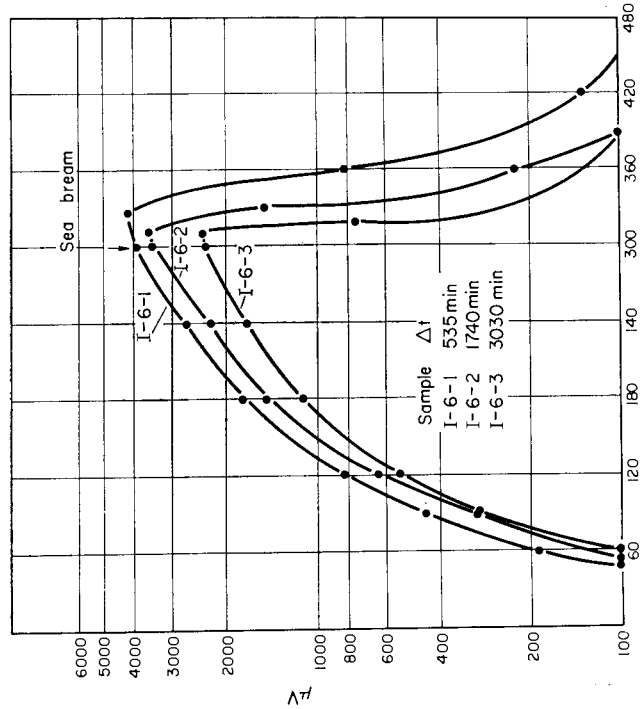

Sea bream
I-6-1
I-6-2
I-6-3
Sample Δt
I-6-1 535 min
I-6-2 1740 min
I-6-3 3030 min
μV
sec

FIG. 8b.

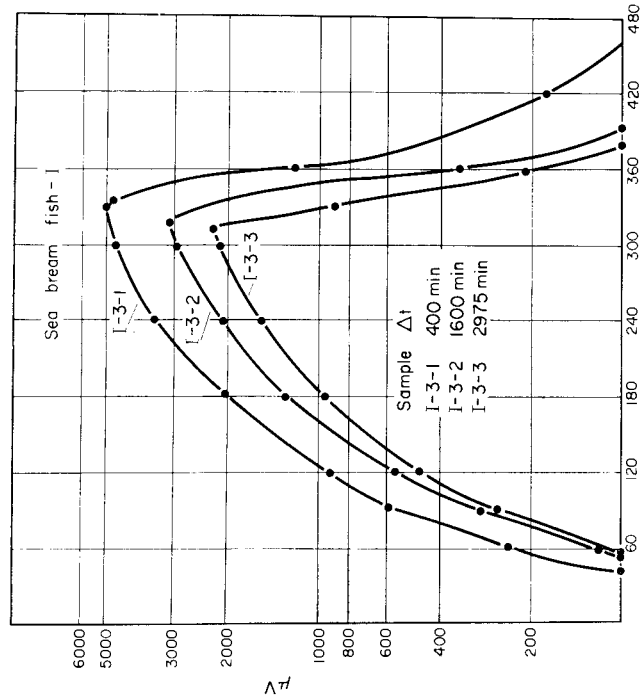

Sea bream fish-1
I-3-1
I-3-2
I-3-3
Sample Δt
I-3-1 400 min
I-3-2 1600 min
I-3-3 2975 min
μV
sec

FIG. 8a.

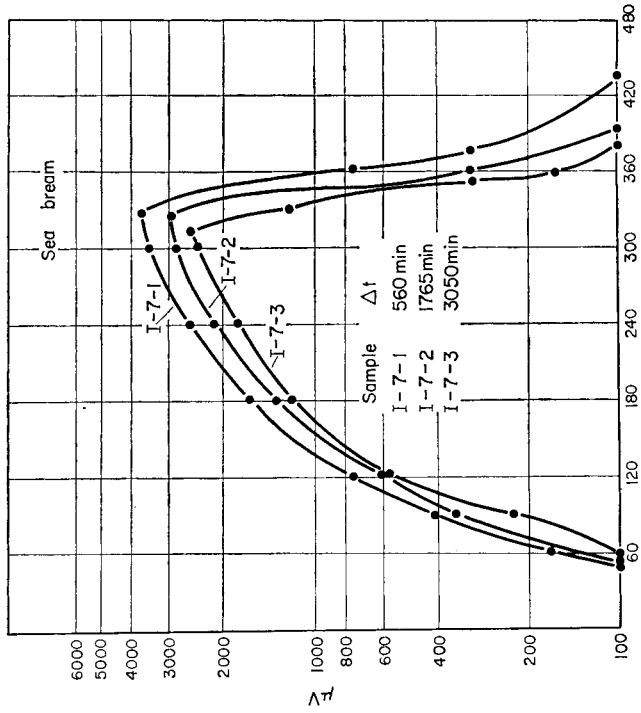

Sea bream
I-7-1
I-7-2
I-7-3
Δt
Sample
I-7-1 560 min
I-7-2 1765 min
I-7-3 3050 min
μV
6000
5000
4000
3000
2000
1000
800
600
400
200
100
60
120
180
240
300
360
420
480
sec

FIG. 8c.

which indicate decrease in voltage. Comparison of groups (A), (B) and (C) in both methods, indicate that samples of group (B) are more uniform; that is, voltage indicated by the Electro-odocell increases as the age of the fish samples increases.

These preliminary experiments indicated that fish samples kept in water give more uniform results and can be recommended for future research work as a suitable method of testing of fish.

Another series of tests were made with *sea bream* samples prepared as group (B). Experiments with sea bream samples (Figs. 8a, 8b, 8c) indicate that, where the initial slopes are compared, out of 16 experiments, 5 showed irregular increase, 3 showed irregular decrease and 8 showed regular decrease as the age of sample increases. If the points in curves at the time of removing of samples are compared, out of 16 experiments, 1 showed a regular increase, 2 showed irregular increase, 4 showed irregular decrease and 9 showed regular decrease.

These experiments with sea bream indicate that voltage changes indicated by Electro-odocell decrease as the aging of sea bream increases.

CONCLUSIONS ABOUT THE EXPERIMENTS ON MEAT AND FISH

(i) Experiments carried out with various samples of ground beef meat under three different groups (A, B, C) and two different conditions (a, b) indicate that, as the age of the meat increases, the voltage–time curves recorded by the Electro-odocell will change, thus the shape of the curve recorded by the Electro-odocell will be different at different ages of the same meat sample. These measurements and curves can be used to differentiate a fresh meat sample with aged meat sample or with spoiled meat, by recording the curve of the fresh meat first and using as a reference curve. But, to set up a standard method of testing, extensive experimental work with various parts of the meat of a given animal and animals of different locations and different kind must be carried out in various laboratories, with Electro-odocell.

(ii) Experiments carried out with various samples of flat-fish, mackerel and sea bream under three different groups (A, B, C) and two different conditions (a, b) indicate that Electro-Odocell records a specific curve for a given fish at a specific age. As the age of the fish increases, that is, as the fish becomes spoiled, the characteristic curve of the fresh-fish also changes. To find out the spoilage of a fish, it is possible to compare the curves of Electro-odocell of a given kind of fish at a given age with the

curves of the fresh fish of the same kind. But to set up a standard method of testing, again extensive measurements with Electro-odocell of various kinds of fish must be carried out by various laboratories.

REFERENCES

1. TANYOLAÇ, N. N. Study of Odors Ph.D. Thesis, Purdue University (1949).
2. TANYOLAÇ, N. N. and J. R. EATON. Study of Odors, *Journal of the American Pharmaceutical Association*, Scientific edition, Vol. XXXIX, No. 10, October 1950.
3. CAMPBELL, J. A., Z. F. AHMED, J. E. CHRISTIAN and J. R. EATON. A Study in the Measurements of Odors and the Deodorizing Effects of Certain Materials, *Journal of the American Pharmaceutical Association*, Scientific Edition, Vol. XLII, No. 4, April 1953.
4. AHMED, Z. F., J. A. CAMPBELL, J. E. CHRISTIAN and J. R. EATON. A Proposed Method for Determining the Effect of Deodorants on the Elimination of Alcohol Breath, *Journal of the American Pharmaceutical Association*, Scientific Edition, Vol. XLII, No. 4, April 1953.
5. EATON, J. R., J. E. CHRISTIAN and J. A. CAMPBELL. Rapid Surface Tension determination using a modified pendant drop technique, *Journal of the American Pharmaceutical Association*, Scientific Edition, Vol. XLIV, No. 8, August 1955.
6. EATON, J. R., J. E. CHRISTIAN and J. A. CAMPBELL. Surface phenomena related to Odor Measurements, *Annals of the New York Academy of Sciences*, Vol. 58, Art. 2. Basic odor research correlation.
7. CHAPMAN, C. R., J. R. EATON. *Electric Potential Changes at Surfaces as a Means of Measuring Odorous Atmospheric Contamination*. AIEE Conference paper CP56-97.
8. KOPPLIN, J. O., J. R. EATON and J. E. CHRISTIAN. Studies on the Adsorption of Odorous Materials I. 1, Surface Potential Changes of Solid and Liquid Absorbing Surfaces. *Journal of the American Pharmaceutical Association*, Scientific Edition, Vol. XLVIII, No. 8 August 1959.
9. KOPPLIN, J. O., J. R. EATON and J. E. CHRISTIAN, Surface Potential Changes due to the Adsorption of Alcohol Vapors, *Journal of the American Pharmaceutical Association*, Scientific Edition, Vol. XLVIII, No. 9 September 1959.
10. HARTMAN, J. D. and E. W. TOLLE, An apparatus designed for the rapid electrochemical estimation of flavors in vegetables, *Food Technology*, 1957, Vol. XI, No. 2, p. 130–132.
11. DRAVNIEKS, DR. ANDREW. ARF Lays Groundwork for Synthetic Nose, *C & En* April 3, 1961, p. 56.
12. MONCRIEFF, R. W. An Instrument for Measuring and Classifying Odors, *Journal of Applied Physiology*, Vol. 16, No. 4, July 1961.
13. GRANZEIER, F. J. Chemo-Electric Odor Detection, *Industrial Research*, July–August 1962.
14. AMOORE, J. E., J. W. JOHNSON, JR. and MARTIN RUBIN, The Stereochemical Theory of Odor, *Scientific American*, February 1964.
15. KUEHNER, R. L. Recent Advances in Odor: Theory, Measurement and Control, Annals of the *New York Academy of Sciences*, Vol. 116, Art. 2, July 30, 1964.
16. Patent No. 12287 of Turkish Ministry of Industry on Electro-Odocell.
17. TANYOLAÇ, N. N. Electro-Odocell for Odor Measurement, *International Rhinology*, Vol. III, No. 3, January 1965, pp. 129–134.
18. TANYOLAÇ, N. N. The Electro-Odocell for odor measurement and surface effects. In the book of J. I. BREGMAN, and A. DRAVNIEKS, *Surface Effects in Detection*, Spartan Books, Inc., 1965, pp. 89–102.

SOME EFFECTS OF OLFACTORY STIMULATION ON LOCOMOTOR PATTERNS IN FISH*

H. KLEEREKOPER

McMaster University, Hamilton, Ont., Canada

INTRODUCTION

The existence of a well-developed olfactory sense in fishes has been documented in an extensive literature which has been reviewed in part by Hasler (1957), Teichmann (1959) and Kleerekoper and Mogensen (1963). Many species use olfaction to procure and recognize food. While the role of one or more specifically active substances in the odour of food or prey was suspected (Sheldon, 1911; Chidester, 1924; van Weel, 1952) a specific attractant was isolated for the first time by Kleerekoper and Mogensen from the scent of trout (1963).

Detection of alarm substances at very low threshold concentrations and the capability to discriminate components of odour mixtures have been shown for various species of fish by different authors.

Less-well-documented has been the role of olfaction in orientation in fish and the results of experimentation in this area have been contradictory even where closely-related species are concerned. These unsatisfactory results may be due largely to inadequate experimental methods. Direct observations in uncontrolled environment for relatively short periods are not likely to produce statistically reliable data on locomotor response to olfactory stimulation. The effect of the presence of the observer on the behaviour of the animals often has not been assessed. In quantitative work experiments have generally been carried out with conditioned animals. While this approach is appropriate for the determination of threshold values of sensitivity it cannot provide information on the behaviour of the unconditioned animal in response to olfactory stimulation.

* This report deals with the partial results of an investigation carried out under contract Nonr-3391(00) NR 104–560 between the U.S. Office of Naval Research and McMaster University, Hamilton, Ontario, Canada.

In 1963 some results obtained with a different experimental method, developed by the author, were reported (Kleerekoper and Mogensen, 1963). The method allowed for long term, indirect observations in controlled and undisturbed environment through the automatic recording of movements of the unconditioned animal.

During 1964–65 an investigation was made at the Stazione Zoologica, Naples, of the effect of olfactory stimulation on orientation in several species of marine fish using the above recording technique with a number of important modifications. In another paper, to be presented elsewhere, the data and results on orientation in the absence of olfactory stimulation will be treated in detail. In the present paper the effects of such stimulation on orientation will be emphasized but it will be necessary to discuss a limited number of data on orientation in the absence of olfactory stimulation for comparative purposes.

METHODS OF RECORDING

In each experiment the locomotor activity of a single animal was recorded automatically and remotely before and during olfactory stimulation. The equipment was designed so as to provide a continuous record of di-

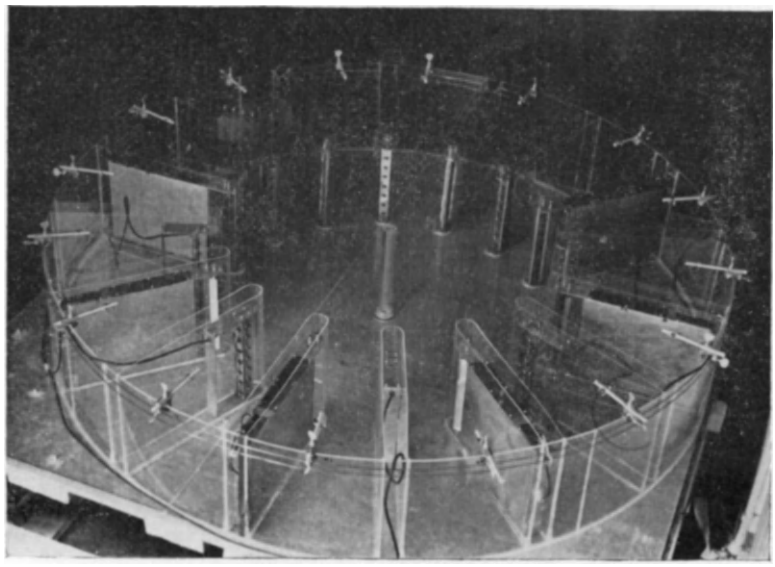


FIG. 1. View of the experimental tank. The double-walled partitions house, alternately, sources of infrared light and banks of photocells. The water supply system is not shown.

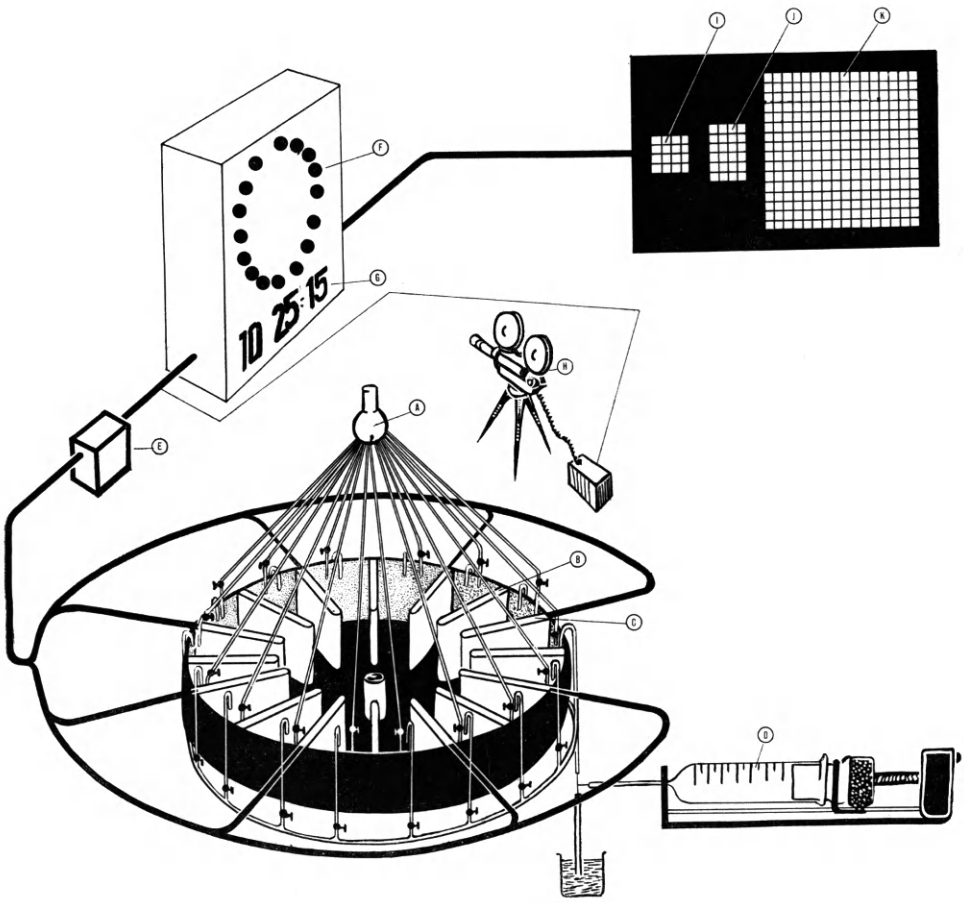


FIG. 2. Diagram of recording system.

a—manifold of water supply system.
b—one of eight dividing walls containing source of infrared light emitting 8 beams.
c—one of eight dividing walls containing bank of 8 photoconductive cells.
d—in fusion–withdrawal pump.
e—amplifier system.
f—display of animal's movements by neon bulbs.
g—timer.
h—motion picture camera exposes one frame for each displacement of light on panel.
i—counters indicate number of seconds animal remains in each compartment.
j—counters indicate hourly frequency of displacements of animal during 24 hours.
k—counters display direction and frequency of movements from any one compartment into any other compartment (see text).

rection, distance, frequency and hourly distribution of movements, time of permanence at each destination, and the distribution of left-hand and right-hand turns. The experimental tank, 200 cm in diameter and 30 cm high, is shown in Fig. 1 and is an improved version of the tank described earlier (Kleerekoper and Mogensen, 1963). Housed in an isolated chamber under constant conditions of lighting it is constructed of "plexiglas" and divided into 16 compartments by 15 double-walled dividers, 50 cm long, which leave open a central area 100 cm in diameter. The hollow dividers house alternately sources of infrared light whose eight beams are directed laterally onto banks of photoconductive cells of the polycrystalline type housed in the neighbouring dividers. Passage of the fish through a beam increases the resistance of the corresponding photo cell. This variation in resistance is used to trigger electronic circuits outside the experimental chamber (Fig. 2). They command a panel displaying the movements of the animal by means of neon light bulbs. Single frame photographs can be made automatically with a 16 mm motion picture camera whenever a change in position of the light source occurs. A timer is placed in the optical field of the camera so that time and position are recorded simultaneously. High speed negative film is used providing 4000 frames (entries) per spool of 100 ft.

Three counter systems are connected with the above circuits. They provide information on: (1) the cumulated time in seconds spent by the animal in each compartment; (2) the hourly distribution of movements during 24-hour periods and (3) the directions (with their frequencies) of these movements. The latter system provides immediate and continuous visual information on preferred routes and patterns. The circuitary and technical details of the versatile system will be presented elsewhere.

Water is supplied to the tank through a glass manifold suspended in central position above the tank. From this manifold 16 calibrated glass tubes lead to the periphery of each compartment. The supply can be regulated and the rate of flow into the individual compartments controlled. A central standing pipe and peripheral outlets can be controlled to allow for a variety of flow patterns.

The experimental chamber could be lighted with "blue light" fluorescent tubes for which the photo conductive cells were not sensitive. Artificial day–night sequences could be produced by means of a switch clock but continuous lighting or darkness were used mostly. A plywood cover provided shade in the compartments leaving the centre part of the tank exposed.

In most experiments fresh animals were used which had been captured

in the Gulf of Naples or in its vicinity. The fish were kept in a holding tank without food for a varying number of days prior to experimentation. Recording of movements began 12–24 hr after the introduction of the fish into the monitor tank after tests had shown that this period was sufficient for the animal to become used to its new environment and to establish its typical pattern of locomotion (see below).

STIMULATION

The scent substances used in these experiments fall into two categories: "whole" odours derived from live food organisms and pure components of fish body odour isolated in the laboratory. The "whole" odours were obtained by confining live organisms in aquaria with or without continuous renewal of water and using aliquots of the water as the source of odour substances. In some instances live food organisms were squashed and the filtrate of the material used for stimulation. The chemical isolates consisted of a series of substances obtained from the body odour of fish mainly by gas chromatographic methods (Gorecki and Kleerekoper, in preparation). "Whole" odours were introduced mainly by gravity through teflon tubing into the general water supply or into the individual compartments. All required manipulations were made outside the experimental chamber which was never entered during an experiment. To avoid variations in rate of flow water containing odour substances was introduced always in substitution of a flow of sea water delivered at the same rate previous to stimulation. Introduction of the chemical isolates of known concentration was done by means of a double-barreled physiological pump of the infusion-withdrawal type whose rate of delivery could be widely varied. The glass syringes could be connected to any one or several of the compartments of the tank by means of permanently installed glass tubes whose outlets in the compartments adjoined those of the tubes supplying the sea water. During the "no stimulation" period preceeding the "stimulation" period the pump system was operated with sea water from the general supply system so as to avoid the variables of rate of flow and vibration in these comparative studies of locomotor behaviour. Animals were made anosmic, when necessary, by occlusion of the narines.

EVALUATION OF DATA

Significance tests were applied to all the above data. Early in the investigation it became clear that, in general, a minimum of 1000 movements had to be recorded for each of the two periods observed (before stimulation

and during stimulation) in order to obtain statistically significant data. Consequently, most of the results presented are based on a minimum of 1000 recorded movements and in several experiments twice or more this number was used. Unless otherwise stated, in all results presented $p<0{\cdot}01$.

RESULTS

In figures which indicate the frequency of the movements of the animal between any two compartments the points of departure are shown on the vertical, those of arrival on the horizontal axis (e.g. Fig. 3). The value of each dot may vary from graph to graph and depends on the maximum number of entries which had to be recorded in a square. This value is indicated with each figure. The direction of movement in the shaded areas of the figures is left-handed, that in the non-shaded areas right-handed. In some figures the largest frequency of both left-and right-handed movements is marked by a circle.

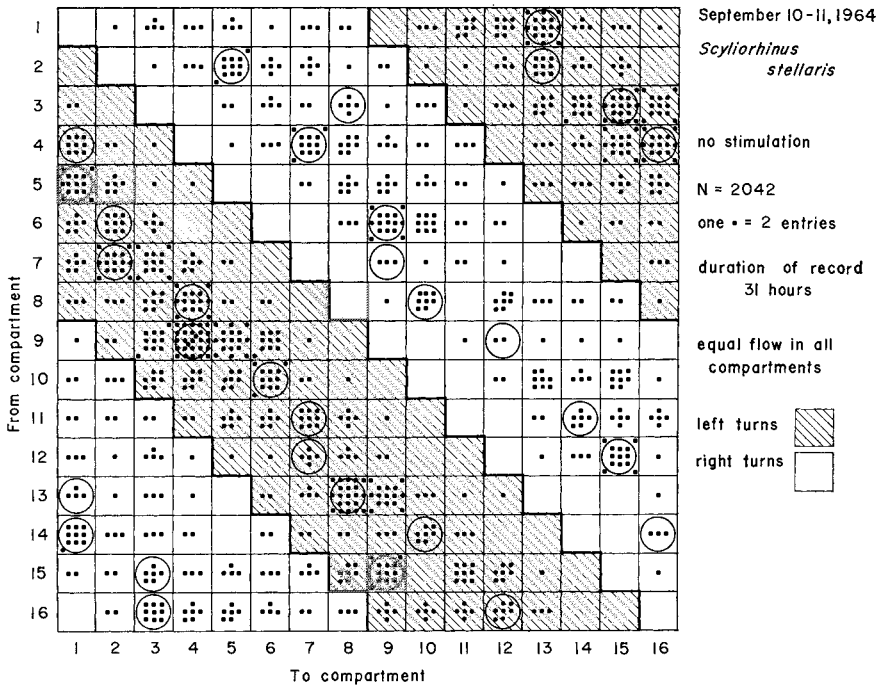


FIG. 3. *Scyliorhinus stellaris*. September 10–11, 1964. Before stimulation. Frequency of movements between individual compartments. The left-hand numerals on the vertical axis indicate the compartment of origin of the movement, those on the horizontal axis (numerals at bottom) the compartment entered by the animal. Destinations with greatest frequency are marked by a circle.

Orientation in the Absence of Olfactory Stimulation

In the experiments reported here, unless otherwise stated, the flow of water was from the periphery of the tank towards the centre with equal rate of flow in all compartments. The effects of differential flow and lighting conditions on orientation will be presented elsewhere.

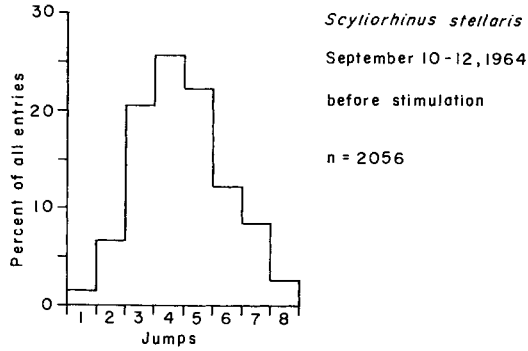


FIG. 4. *Scyliorhinus stellaris.* September 10–12, 1964. Before stimulation. Frequency distribution of the number of compartments by-passed ("jumps") per cent of all recorded movements.

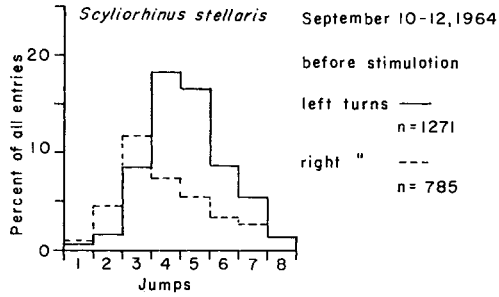


FIG. 5. *Scyliorhinus stellaris.* September 10–12, 1964. Before stimulation. Frequency distribution of "jumps" by left- and right-hand movements percent of all movements.

Scyliorhinus stellaris. September 10, 1964. Figure 3 shows that the movements of the animal were not random but consisted of a series of preferred pathways which are indicated by circles in the squares containing the largest numbers of entries. A complete sequence of these pathways will thus show the route preferred by the animal. There was a preponderance of left-hand turns in this record.

In moving from one compartment to another the animal rarely entered neighbouring compartments but by-passed or "jumped" a number of compartments. The frequency distribution of this number of "jumps" for all movements recorded is presented in Fig. 4.

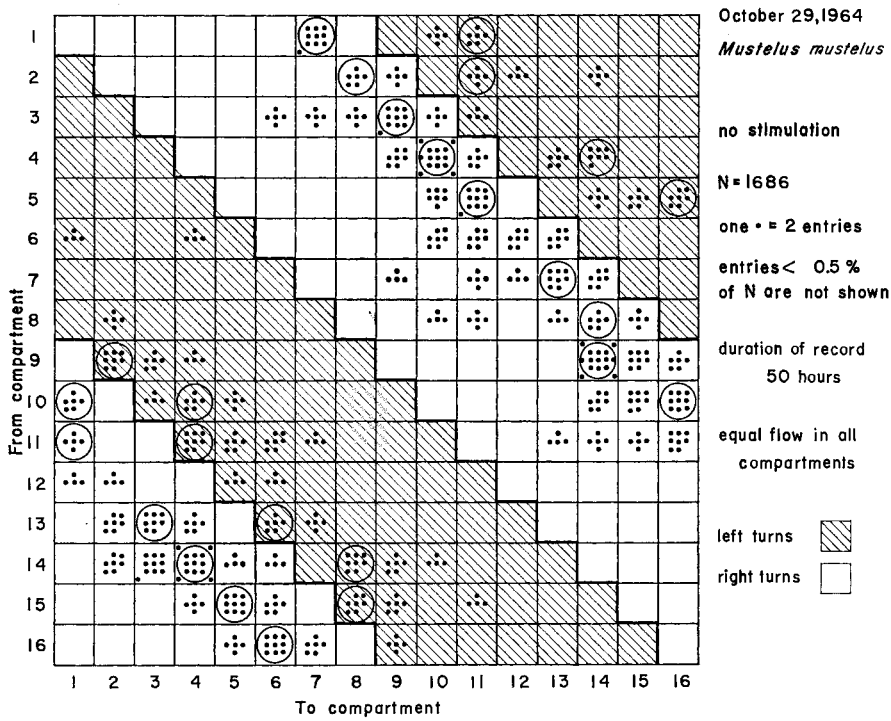


FIG. 6. *Mustelus mustelus*. October 29, 1964. Before stimulation. Frequency of movements between individual compartments. See Fig. 3 for explanation.

From the histograms as well as from the straight lines formed by the encircled frequencies in Fig. 3 it is seen that the frequency distribution was not random but had a pronounced peak. In about 25% of all movements the preferred jump was 4. In Fig. 5 the frequency distribution of the jumps of all movements is broken down into left and right turns; 31% of all right-hand movements had a 3-jump characteristic while 30% of the left-hand movements made four jumps.

Mustelus mustelus. October 29, 1964. This species, although little active, displayed a pattern of preferred pathways shown in Fig. 6. Right-hand turns occurred in 63% of all movements. Figure 7 presents the frequency distribution of the jumps and shows a sharp peak at 6 jumps. In Fig. 8 the fre-

quency distribution of the jumps is shown in left-and right- hand turns. In spite of the graphic similarity of the histograms in Fig. 8 their difference is considered significant ($p < 0{\cdot}05$).

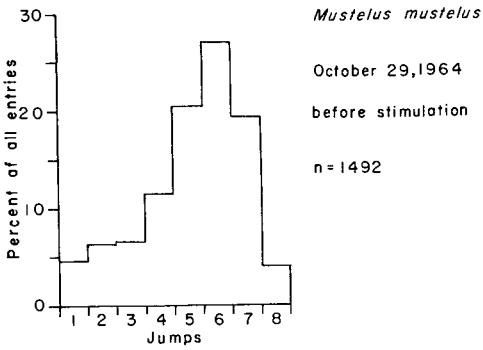


FIG. 7. *Mustelus mustelus*. October 29, 1964. Before stimulation. Frequency distribution of "jumps". See Fig. 4 for explanation.

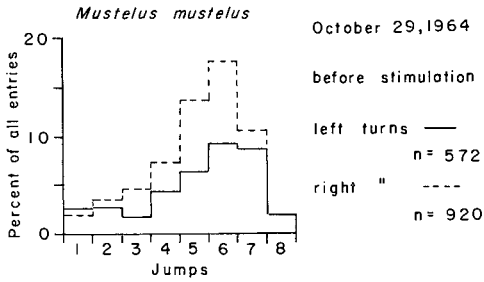


FIG. 8. *Mustelus mustelus*. October 29, 1964. Before stimulation. Frequency distribution of "jumps" by left- and right-hand movements.

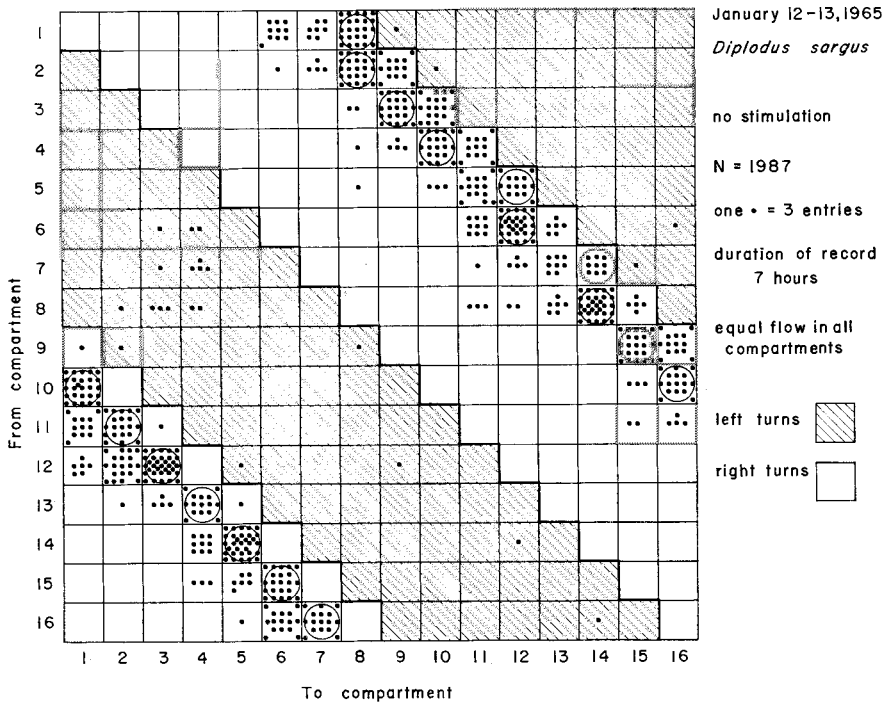


FIG. 9. *Diplodus sargus*. January 12–13, 1965. Before stimulation. Frequency of movements between individual compartments. See Fig. 3 for explanation.

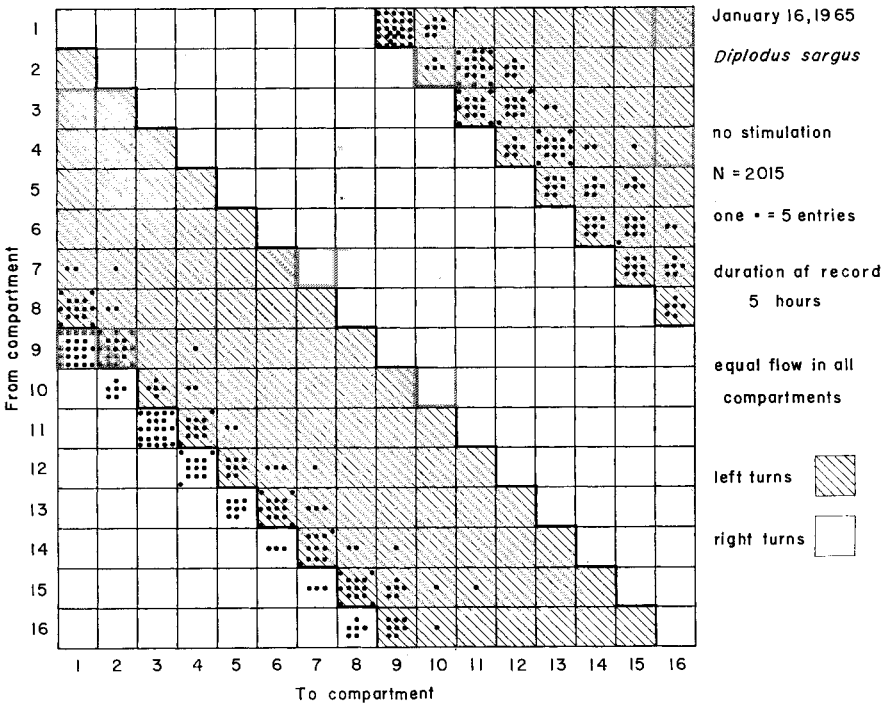


FIG. 10. *Diplodus sargus*. January 16, 1965. Before stimulation. Frequency of movements between individual compartments. For explanation see Fig. 3.

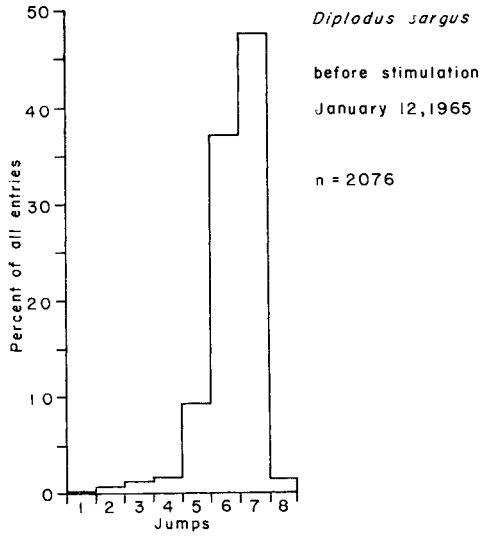


FIG. 11. *Diplodus sargus*. January 12–13, 1965. Before stimulation. Frequency distribution of jumps. See Fig. 4 for explanation.

Diplodus sargus. This is an active swimmer which displayed a particularly regular pattern of movements in the experimental tank (Fig. 9: January 12, 1965; Fig. 10: January 16, 1965). In Fig. 9 the right-hand turns ac-

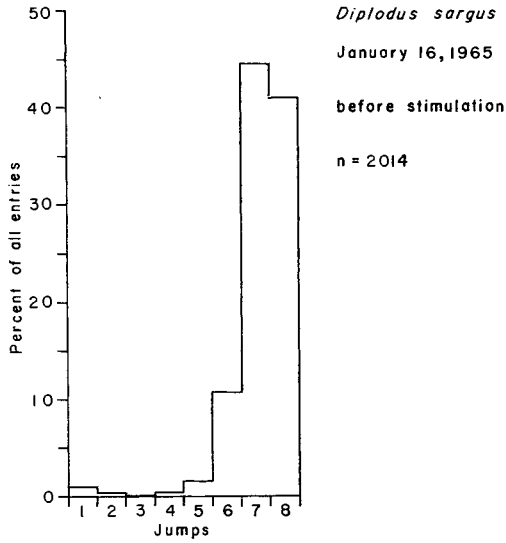


FIG. 12. *Diplodus sargus*. January 16, 1965. Before stimulation. Frequency distribution of "jumps". See Fig. 4 for explanation.

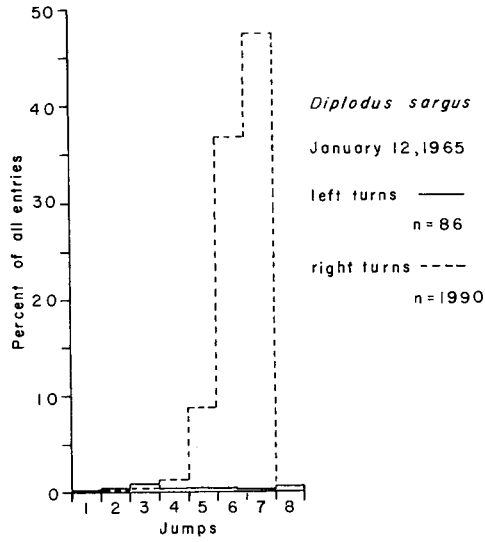


FIG. 13. *Diplodus sargus*. January 12–13, 1965. Before stimulation. Frequency distribution of "jumps" by left- and right-hand movements percent of all movements.

count for 95·8% while in Fig. 10 left-hand turns prevail with 78% of all movements. The frequency distribution of the jumps is presented in Figs. 11 and 12. In the former, 85% of all movements covered 6 and 7 jumps while

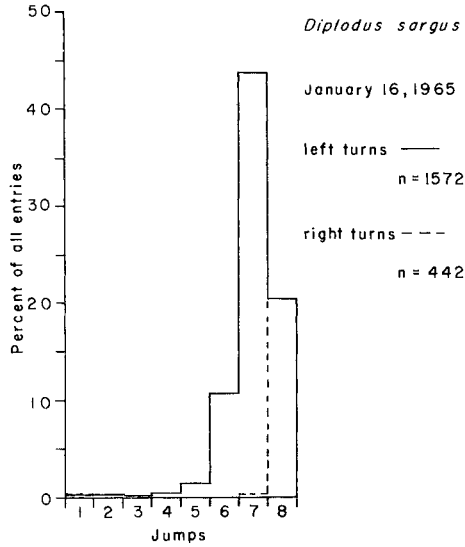


FIG. 14. *Diplodus sargus*. January 16, 1965. Before stimulation. Frequency distribution of "jumps" by left- and right-hand movements percent of all movements.

in Fig. 12, 7 and 8 jumps made for 85% of all movements. In both cases 7 jumps prevailed.

The frequency distribution of the jumps in left- and right-hand turns is shown in Figs. 13 and 14.

The Effects of Olfactory Stimulation on Orientation Patterns

Scyliorhinus stellaris. September 11, 1964 (continuation of record of 9.10.64, Fig. 3). Stimulation with scent of *Scyliorhinus stellaris* in compartment 1. Stimulation with scent of its own species does not significantly affect the relative frequency of movements between compartments but affects slightly the frequency distribution of jumps (Fig. 15; p is 0·05). The participation by left- and right-hand turns in this change of frequency distribution is shown in Fig. 16. The proportion of both turns in all movements changed from

$$\frac{n \text{ left before}}{n \text{ right before}} = \frac{1271}{785} \quad \text{to} \quad \frac{n \text{ left after}}{n \text{ right after}} = \frac{840}{1020}.$$

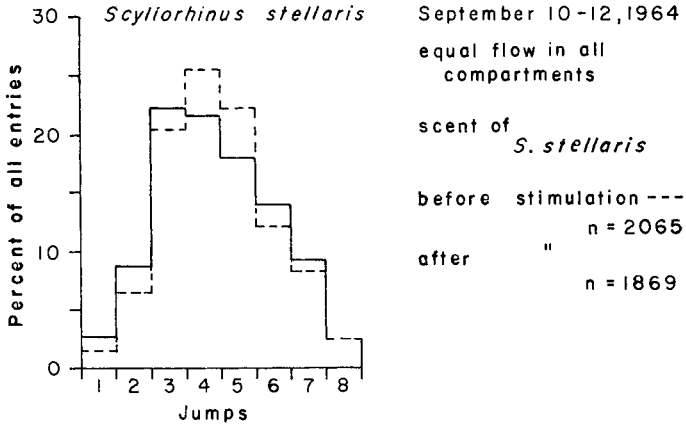


FIG. 15. *Scyliorhinus stellaris*. September 10–12, 1964. Frequency distribution of "jumps" before and after stimulation with scent of *S. stellaris*.

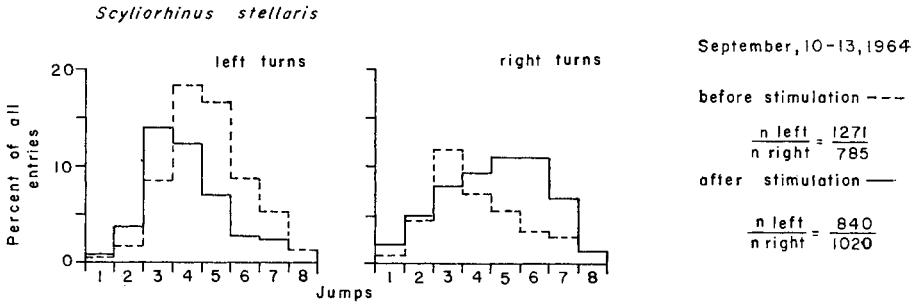


FIG. 16. *Scyliorhinus stellaris*. September 10–13, 1964. Frequency distribution of "jumps" by left- and right-hand movements before and after stimulation with scent of *S. stellaris*.

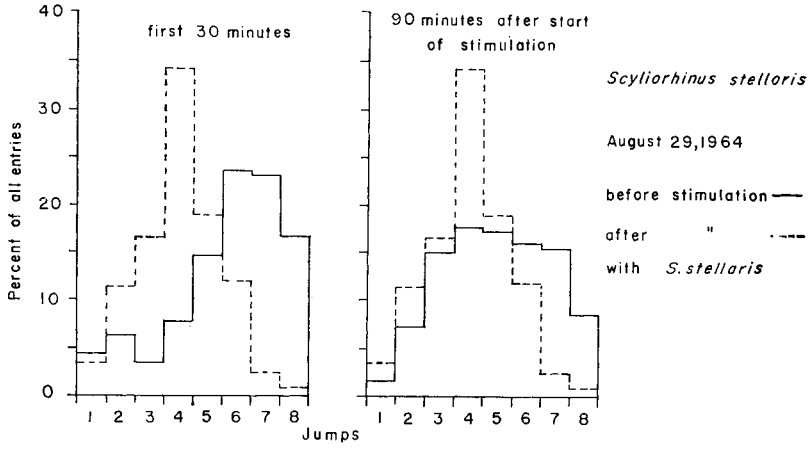


FIG. 17. *Scyliorhinus stellaris*. August 29, 1964. Frequency distribution of "jumps" by all movements 30 and 90 minutes after the onset of stimulation with scent of *S. stellaris*.

August 29, 1964. Stimulation with scent of *Scyliorhinus stellaris*. In this experiment, not previously referred to in this paper, the effect of duration of stimulation on the frequency distribution of jumps was ascertained. The results are shown in Fig. 17 which presents the observations during the first 30 min of stimulation and 90 min after the beginning of stimulation. Repetition of the experiments with anosmic animals failed to produce any of the above changes.

Mustelus mustelus. November 1, 1964 (continuation of record started on October 29, Fig. 5). Stimulation with scent of *Anemonia sulcata* into compartment 9. The frequency distribution of movements before the introduction of the odour was referred to above and shown in Fig. 6. The effect

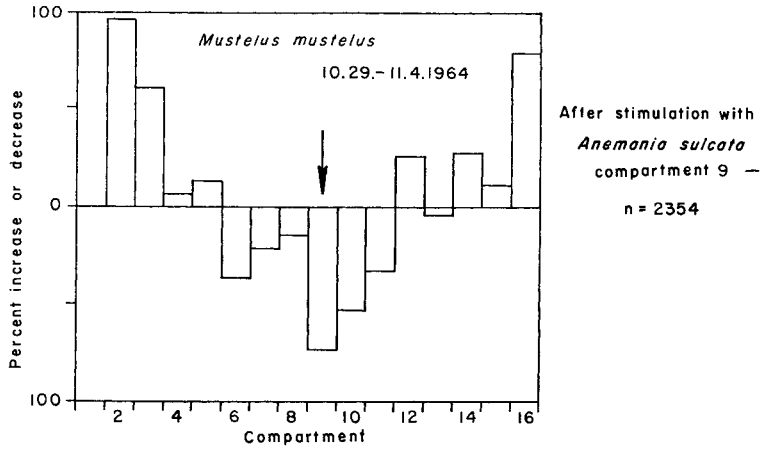


FIG. 18. *Mustelus mustelus*. October 29–November 4, 1964. The increase of decrease in the relative number of entries into the 16 compartments is presented percent of the number of entries into each compartment previous to the introduction of scent of *Anemonia sulcata* into compartment 9.

of the scent of this obnoxious animal on the frequency of accumulated entries from all directions into the 16 compartments is presented in Fig. 18. In this and similar following figures the increase or decrease of the number of entries following stimulation is expressed in per cent of the number of entries previous to stimulation. Figure 18 indicates a general decrease of movements into compartments 6–11 with the greatest relative decrease pertaining to compartment 9. The frequency distribution of jumps changed as indicated by Fig. 19 which presents the distribution of all jumps (left and right) before and after the introduction of the odour. The peak of the distribution is displaced from 6 to 2 jumps. Left and right turns do not

participate proportionally in this change as is indicated in Fig. 20. Not only is the change more pronounced in the right-hand movements but the proportion of left- and right-hand turns changes from

$$\frac{n \text{ left}}{n \text{ right}} = \frac{38}{62} \text{ before stimulation to } \frac{61}{39} \text{ after stimulation.}$$

Occlusion of the narines abolishes all changes referred to above.

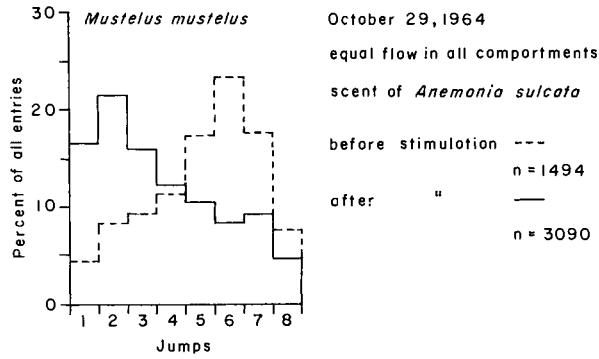


FIG. 19. *Mustelus mustelus*. October 29–November 4, 1964. Frequency distribution of "jumps" before and after stimulation with scent of *Anemonia sulcata*.

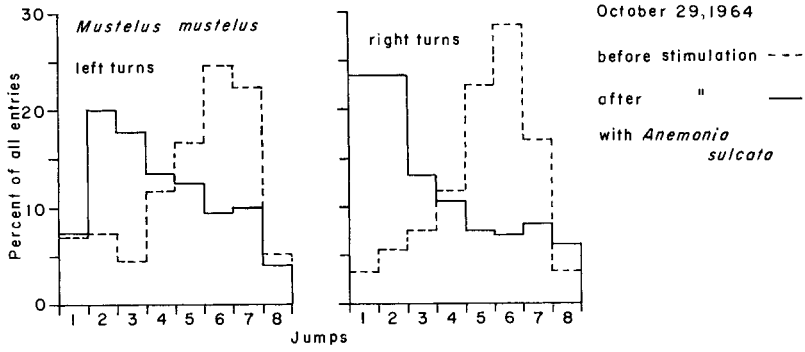


FIG. 20. *Mustelus mustelus*. October 29–November 4, 1964. Frequency distribution of "jumps" by left- and right-hand movements before and after stimulation with scent of *Anemonia sulcata*.

Diplodus sargus. January, 1965 (continuation of record of January 12, Fig. 9). Stimulation with scent of *Anemonia sulcata* in compartment 5. The effect of this odour on the frequency distribution of jumps for all

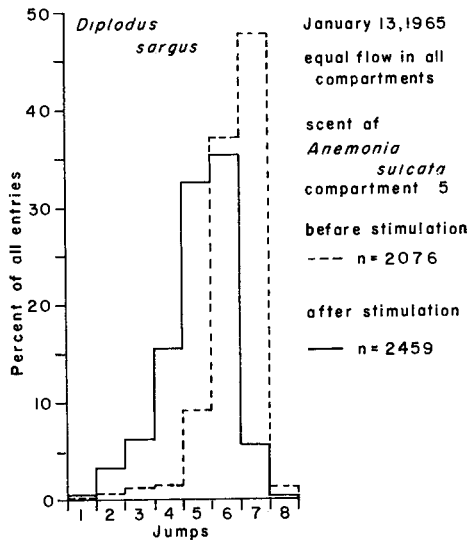


FIG. 21. *Diplodus sargus*. January 12–13, 1965. Frequency distribution of "jumps" before and after stimulation with the scent of *Anemonia sulcata*.

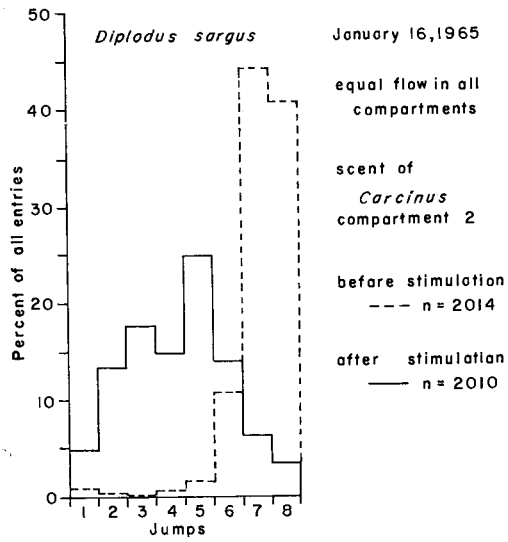


FIG. 22. *Diplodus sargus*. January 16, 1965. Frequency distribution of "jumps" before and after stimulation with the scent of *Carcinus*.

movements before and after stimulation is shown in Fig. 21. The peak is displaced from 7 jumps before to 6 jumps after the introduction of the scent. Since right turns predominated in this experiment the change in the value of the jumps is brought about almost exclusively by right-hand turns. No significant change in the ratio left-hand: right-hand movements occurred.

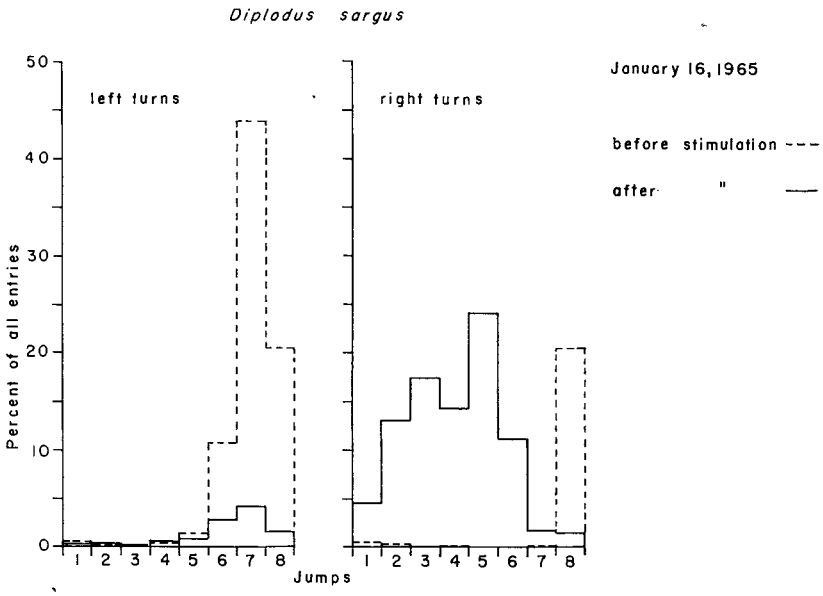


FIG. 23. *Diplodus sargus*. January 16, 1965. Frequency distribution of "jumps" by left-and right-hand movements before and after stimulation with scent of *Carcinus*.

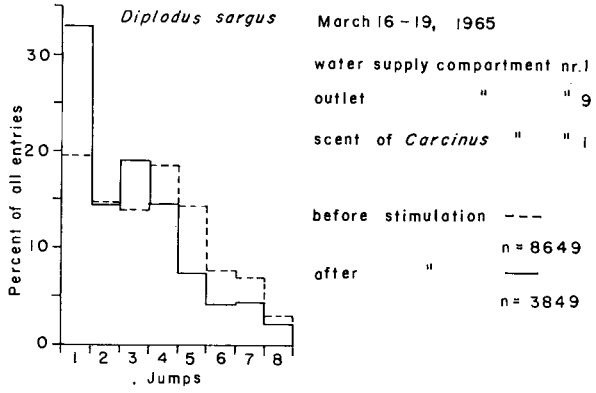


FIG. 24. *Diplodus sargus*. March 16–19, 1965. Frequency distribution of "jumps" before and after stimulation with scent of *Carcinus*.

January 16, 1965 (continuation of record of January 16, Fig. 10). Stimulation with the scent of live *Carcinus* in compartment 2. The odour of this natural food organism effected a change in the frequency distribution of jumps as indicated in Fig. 22. From a sharp peak at 7 jumps before stimulation the distribution shifted to a peak at 4 jumps at introduction of the scent of the food organism. The ratio of total left- to right-hand movements changed from $\frac{1572}{442}$ before, to $\frac{235}{1775}$ after stimulation while the jump distribution between left-and right-hand turns changed as indicated in Fig. 23.

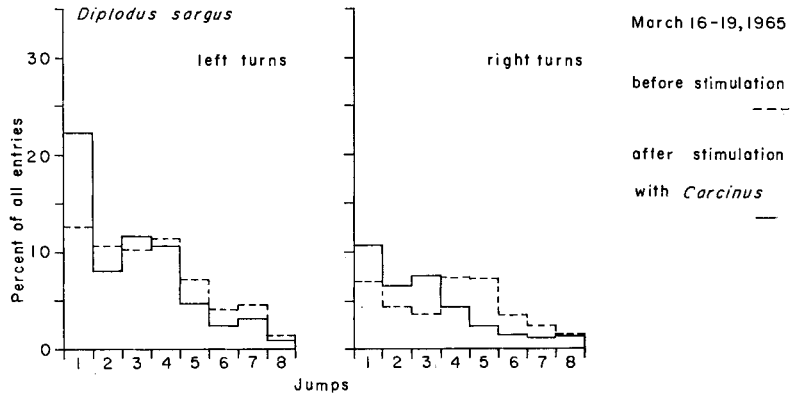


FIG. 25. *Diplodus sargus*. March 16–19, 1965. Frequency distribution of "jumps" by left- and right-hand movements before and after stimulation with scent of *Carcinus*.

March 16–19, 1965. The animal was stimulated with the scent of live *Carcinus* with the results shown in Figs. 24 and 25. None of the above changes occurred when the experiments were carried out with anosmic animals.

DISCUSSION

The results presented above are a few of those obtained in a large number of experiments carried out to ascertain the relationships between olfactory stimulation and locomotor patterns in a variety of environmental conditions in the three species of fish.

The most striking characteristic of the patterns in the absence of stimulation was that the movements were not random in direction. Since this

paper does not deal primarily with orientation mechanisms (these will be discussed elsewhere) it will suffice to state that also in the absence of any water current randomness did not occur. It should be remembered in this connection that there were no light or temperature gradients in the experimental tank and that the size of the animals had no apparent effect in the locomotor pattern observed. Analysis of the locomotor pattern in all three species revealed that the animals by-passed a preferred number of compartments on each leg of their route. In other words, on leaving a compartment the animal turned a number of degrees. The angle might be different for left- and right-hand turns but the frequency distribution of its value always showed a distinct peak. In most instances the ratio of left- and right-hand turns was not unity so that the characteristics of the whole locomotor pattern were determined by the prevalent values of left and right angles and the frequency ratio between left and right turns.

In the great majority of all experiments performed one or both of the above parameters were modified when stimulation with biologically significant odours occurred. This was even the case when the perception of the odour did not lead to localization of the source or of the general area of the source. These modifications may be considered "alarm reactions". Be it sufficient for the purposes of this paper to state that in the present investigation a number of experiments on orientation through olfaction have shown that in the absence of differential water currents the above species are unable to localize the source of the odour even when the latter consists of freshly-squashed food organisms.

Without exception, the modification of the frequency distribution of jumps (i.e. of the angle of departure from a compartment) was a reduction in the number of jumps, that is, in the number of compartments by-passed. The angle has, therefore, been decreased. The net result was a more intensive "coverage" of the area; a larger number of compartments was entered on a complete "round".

Kleerekoper and Mogensen (1963) reported the isolation of an attractant to *Petromyzon marinus* in the scent of trout. The substance, codenamed "amine *F*", has been identified recently. The identity and isolation are the subjects of two communications presented elsewhere. The reponse to "amine *F*" by *Diplodus sargus* was studied in the present series of investigations by the above method of monitoring 1150 movements before and 1070 movements after the introduction of the substance (May 28–30, 1965). Figure 26 presents the effect on the relative frequency of movements into the 16 compartments while Fig. 27 shows the now familiar displacement in the frequency distribution of jumps.

The described response to odour substances by unconditioned animals of these species provides an experimentally reliable method to ascertain perception and to establish threshold values.

The author suggests that long term monitoring, of locomotor patterns, in controlled environment, may reveal, even in the absence of directional

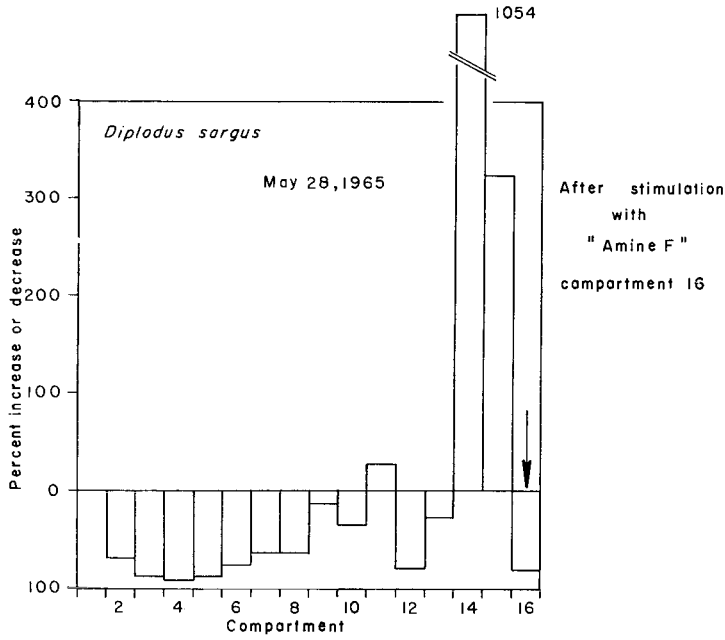


FIG. 26. *Diplodus sargus.* May 28–30, 1965. The increase or decrease in the relative number of entries into the 16 compartments after introduction of "amine *F*" into compartment 16 (see Fig. 18 for explanation). Equal flow in all compartments.

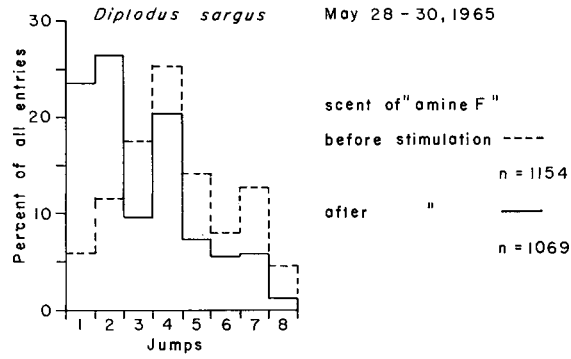


FIG. 27. *Diplodus sargus.* May 28–30, 1965. Frequency distribution of "jumps" before and after stimulation with "amine *F*".

movements, response mechanisms to olfactory stimulation in unconditioned animals generally. Such response would remain almost surely unobserved in traditional, direct, short term observations. This would be particularly so if the response were of an "unexpected" nature such as the locomotor responses described in the present paper.

ACKNOWLEDGEMENT

The author is grateful for the use of the facilities and for the extensive technical assistance received from the staff of the Stazione Zoologica di Napoli, Italy, where the above experiments were performed. The labourious statistical evaluation of all data was carried out by Mrs. H. Kleerekoper.

REFERENCES

CHIDESTER, F. E. 1924. A critical examination of the evidence for physical and chemical influences on fish migration. *J. Exp. Biol.*, **2**, 79–118.

HASLER, A. D. 1957. Olfactory and gustatory sense of fishes. In: *The physiology of fishes. II.* Academic Press Ind., N.Y. 526 pp.

KLEEREKOPER, H. and J. MOGENSEN. 1963. Role of olfaction in the orientation of *Petromyzon marinus*. I. Response to a single amine in prey's body odour. *Physiological Zoology.*, **4**, 347–360.

SHELDON, R. E. 1911. The sense of smell in Selechians. *J. Exp. Zool.*, **10**, 51–61.

TEICHMANN, H. 1959. Über die Leistung des Geruchssinnes beim Aal (*Anguilla anguilla* (L). *Zeitschr. f. vergl. Physiol.*, **42**, 206–254.

VAN WEEL, P. B. 1952. Reaction of tuna and other fish to stimuli. Part II. Observations on the chemoreception of tuna. U.S. Dept. of the Interior. Fish and Wildlife Service. *Special Science Report*, **91**, 8–35.

INVESTIGATIONS OF EXTERNAL CHEMORECEPTORS OF FISHES

J. BARDACH, M. FUJIYA and A. HOLL

School of Natural Resources,
The University of Michigan,
Ann Arbor, Michigan*

INTRODUCTION

There have been few electrophysiological studies of the olfactory and gustatory receptors of fishes, and there is only one thorough investigation of gustatory nerves in a fish, that of Konishi and Zotterman (1961, 1963) dealing with the palatal organ of the carp. The paucity of such investigations is surprising because fishes offer a multiplicity of opportunities for comparative studies of odor and taste, aside, of course, from the practical implications of such research with this economically most important class of vertebrates.

Two recent review papers on the chemical senses of fishes stress that certain species show very high chemical sensitivity: The eel detects a variety of aromatic substances, even those in such weak concentrations as to permit only a few molecules to have entered the nasal chamber, and blind cavefishes respond to stimulation by conventional taste substances in less than 0·00001 molar concentrations (Teichmann, 1962). Hasler (1957) emphasized that certain species or larger taxonomic groups are able to discriminate very specific substances such as the "fright substance" from the slime cells of minnows or rinses from the mammalian skin, and that certain fishes have the olfactory ability to discriminate between related complex scents such as the rinses from different aquatic plants and animals.

The olfactory sense has received more attention than the gustatory one, probably because it functions at a distance, so to speak, and has practical

* We gratefully acknowledge the assistance of the staff of the Biological Laboratory of the Bureau of Commercial Fisheries and the Marine Biological Laboratory at Woods Hole, where the work with the marine species was done. The work was supported by P.H.S. grant 04687.

applications to the management of commercial and recreational fisheries. The two sensory modalities overlap, to some extent, in that some substances elicit responses from both types of receptors. However, smell and taste in fishes can be distinguished anatomically and physiologically.

SCOPE AND OBJECTIVES

Our work dealt with fishes which possess gustatory chemoreceptors on the surface of their bodies, as well as in the mouth. These receptors are often concentrated on fins and/or barbels; we used the chemosensitive nerves in both types of appendages for electrophysiological recordings, employing the technique of Bardach and Case (1965). The experiments were designed (1) to obtain information on the gamut of responses of these nerves to different compounds, (2) to ascertain whether or not there are differences between external chemoreceptors of different groups of fishes and between external and oral chemoreceptors (Konishi and Zotterman, 1961, 1963), and (3) to compare the external taste sense of marine and freshwater fishes (the two environments have certain different properties, such as their buffering action, which might affect receptor responses to chemicals). Finally we hoped (4) to shed some light on the mode of interaction between chemical stimulus and response, that is on the events that occur at the chemosensitive sites of the receptor membrane and that initiate the generator potential in the receptor cell.

MATERIALS AND METHODS

The Fishes and Their Characteristics

Table 1 lists the species used in our study as well as some important characteristics of their chemosensitive appendages. These species can be classified according to the innervation of these appendages: (1) those whose barbels have cranial innervation only (e.g. catfishes, Ictaluridae), (2) those whose fins have both cranial and spinal innervation (e.g. hakes and tomcod, Gadidae) and (3) those with modified fins innervated by spinal nerves only (e.g. searobins, Triglidae). We will describe some differences in the reactions of representatives of each of these types. Detailed descriptions of the gross neuroanatomy and the histology of the appendages may be found in Herrick (1904) for the bullhead, in Bardach and Case (1965) for the tomcod and the hake, and in Morill (1899), Scharrer *et al.* (1947), and Scharrer (1963) for the searobin; Freihofer (1963) reviewed

TABLE 1. SPECIES OF FISHES USED FOR CRO RECORDINGS FROM CHEMO-SENSITIVE NERVES OF THEIR APPENDAGES

Common and scientific names	Habitat	Kind of appendage and type of chemoreceptor	Innervation
Yellow bullhead (*Ictalurus natalis* (Lesueur))	Fresh water	Barbels on head with taste buds	Cranial nerve VII only
Brown bullhead (*Ictalurus nebulosus* (Lesueur))	Fresh water	Barbels on head with taste buds	Cranial nerve VII only
Tomcod (*Microgadus tomcod* (Walbaum))	Sea water	Pelvic fins with taste buds	Cranial nerve VII and spinal nerves
Searobin (*Prionotus carolinus* (Linneaus))	Sea water	Anterior pectoral finrays with free nerve endings, no taste buds	Spinal nerve III only

the distribution of cranial nerves on the fish body from a taxonomic point of view. One point not mentioned by these authors is that the taste buds of fishes are surrounded by a number of fine nerve fibers which terminate just beneath the surface of the skin. This nerve network is better developed in the lower fishes such as Acipenser (Dogiel, 1897) than in the higher ones such as Barbus and Conger (von Lenhossek, 1893). Most, if not all, of these fibers appear to join the bundle of nerves that emerges from the taste cells. When recording impulses from a single nerve in a taste bud preparation one cannot tell whether that nerve belongs to the bundle emerging from a taste cell or to the epidermal network. Some network nerves are presumably sensitive to touch and/or pain while others may be sensitive to temperature changes (Whitear, 1952). In the free finrays of the searobin, the fibers of the thick third spinal nerve branch into small wart-like dermal and epidermal protuberances, and some, at least, end in club-shaped swellings in the outermost layer of epithelial cells (Fig. 1); we have no indication which of these many fibers may be chemically sensitive and which are sensitive to touch and/or temperature.

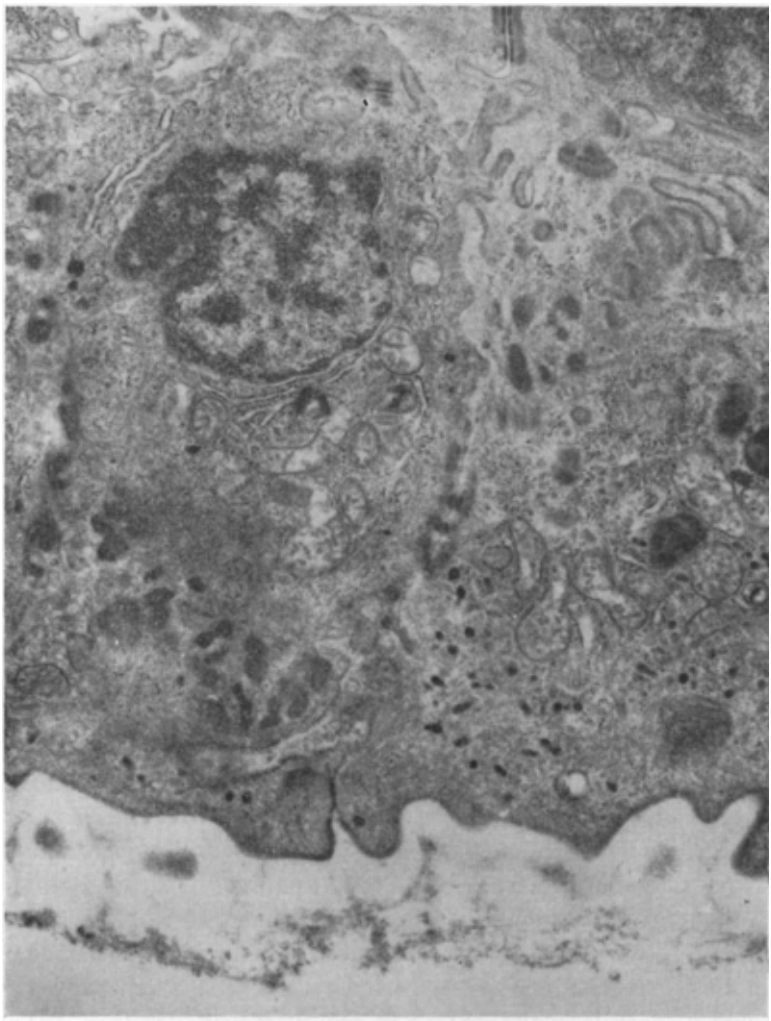


FIG. 1. Sagittal section through the epithelium of a fin ray of the searobin, *(Prionotus carolinus)* × 18,880. Unmyelinated nerve fibers, some of which at least are chemosensitive, frequently seen near the outer epithelial border terminate on cells that contain clusters of mitochondria and dense granules. The significance of this arrangement to the process of chemoreception has not as yet been clarified. Unpublished electron micrograph, courtesy of Dr. E. Scharrer, Dept. of Anatomy, Albert Einstein College of Medicine, New York.

The Recording Technique

An appendage was cut off as close as possible to the body wall and placed in a wax-coated plastic tray (Fig. 2), partitioned by a wax dam into a small shallow portion and a larger one that sloped away from the partition with a drain at the end of the slope. A small platinum plate in the shallow portion formed the indifferent electrode. The fin or barbel lay with

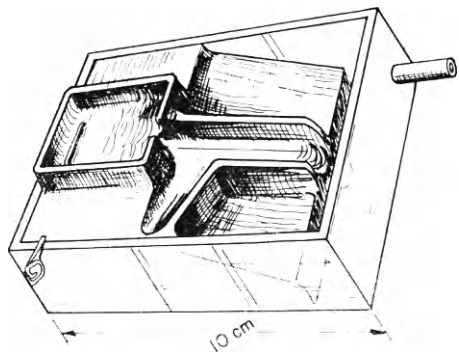


FIG. 2. Wax-covered lucite tray for electrophysiological preparations of isolated fish fins or barbels.

its proximal end in the shallow portion of the tray while the distal end reached, through a notch in the dividing dam, onto the inclined part of the tray. After the preparation was pinned into position, the notch was closed with vaseline and fish saline solution* was poured into the shallow tray to cover the proximal part of the appendage. The distal part was kept moist by repeated rinsing. After the nerve trunks were laid bare, small bundles of nerves were dissected and lifted onto a platinum–iridium external recording electrode. The functional state of the preparation was ascertained by stimulation with drops of tissue extract. If the preparation responded it was rinsed with aquarium water and various chemicals applied to it. The rinse was applied with aquarium water several times between the applications

* Fresh Water Fish:

NaCl	6·50 g/l.
KCl	0·14 g/l.
$CaCl_2$–$2H_2O$	0·16 g/l.
$NaHCO_3$	0·20 g/l.

Marine Fish:

NaCl	7·772 g/l.
KCl	0·185 g/l.
$CaCl_2$	0·165 g/l.
$MgCl_2$	0·094 g/l.
NaH_2PO_4	0·060 g/l.
$NaHCO_3$	1·260 g/l.

of the various chemicals. Such preparations could be kept alive in a cool room (*ca.* 15°C.) for nearly 2 hours.

After amplification with a Tektronix 122 preamplifier the impulses were monitored with a Tektronix 502 A oscilloscope. They were simultaneously recorded on a Tandberg Model 7 stereo tape recorder for possible later photography with a Grass C. 4 oscilloscope camera. Integrated records were obtained by interposing an integrating circuit with time constants of 0·02, 0·05, 0.2, 0·5, and 2.0 seconds between the CRO and a Brush Mark II inkwriter recorder.

Substances Tested

Table 2 lists the various chemicals we applied to the external chemoreceptors of the three species of fishes. Amino acids were selected according to ascending-column paper chromatography of buffalo fish tissue extract, the most frequently used complex taste stimulus; cystine, glutamine, glutamic acid and/or serine, threonine and/or aspartic acid, alanine and/or tyrosine, and leucine and/or isoleucine were found in it.

Cystine, which is relatively insoluble, was tested only on bullhead barbels; it failed to stimulate the chemoreceptors on these structures; but cysteine, the most effective single substance tested, evoked very strong reactions. Several fiber preparations responded to even a 0·00005-molar solution of free-base cysteine (Fig. 3). We therefore concentrated on this

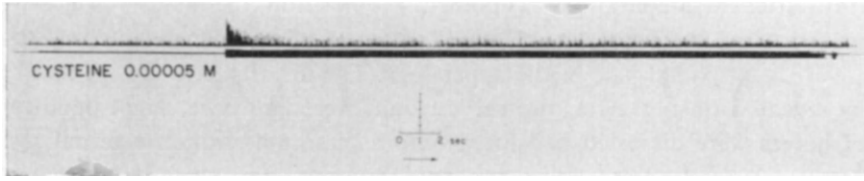


FIG. 3. Response of a few fiber preparations from a bullhead barbel nerve to stimulation with 0·00005-molar l. cysteine.

amino acid and its related substances (Table 2). Glutathione, which contains cysteine, was tested because it evokes feeding responses in certain invertebrates (Loomis, 1955), and methionine because it is another sulfur containing amino acid; only l. isomers were used.

Indole and related compounds were tested because the former has a strong odor and taste: on the human tongue it evokes first a tingling,

then a numbing sensation and one of bitter taste. In previous experiments with squirrel hake (Bardach and Case, 1965), a relative of the tomcod, and with searobins, indole sometimes evoked strong responses, sometimes equivocal ones, and sometimes inhibition. Because indole is a decomposition product of tryptophane, we tested the various progressive breakdown products of this amino acid.

The lipid substances were selected by thin-layer chromatographic analysis of buffalo fish tissue extract. After ascertaining that only charged lipids were effective stimulants, we applied some of those found in the tissue juice (lecithin, cholesterol, inositides) to the bullhead barbels.

OBSERVATIONS AND DISCUSSION

Differences between Fresh Water and Marine Fishes

Responses to acid stimuli require the presence of free H ions. More acid must be added to sea water than to fresh water to lower the pH to the same level in both media: an 0·0008-molar concentration of acetic acid in fresh water will produce a pH of 4, but an 0·008-molar concentration of acetic acid is necessary to produce a pH of 4 in salt water. Bullhead preparations of few fibers responded to the 0·0008-molar concentration,

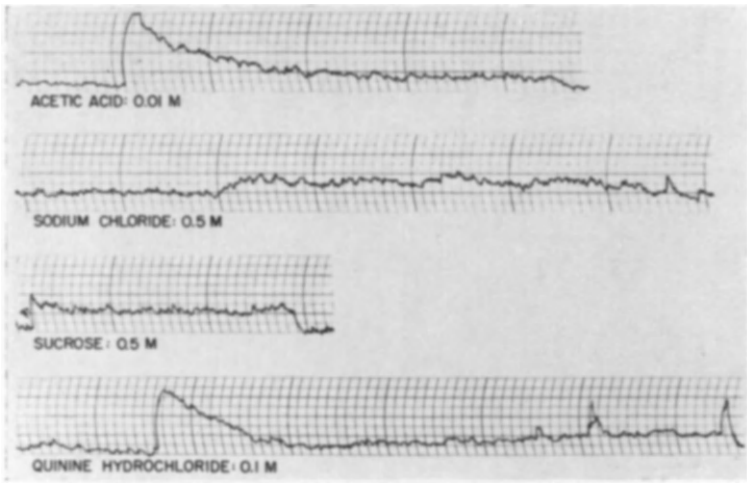


FIG. 4 a–d. Integrated responses to conventional taste substances of a several nerve fiber preparation from a bullhead barbel. Time constant – 0·02 sec; Recorder speed – 5mm/sec, equals one chart division.

TABLE 2. SUBSTANCE TESTED ON ELECTROPHYSIOLOGICAL TASTE NERVE PREPARATIONS OF BULLHEADS. *(Ictalurus natalis, I. nebulosus)* TOMCOD, *(Microgadus tomcod)* AND SEAROBINS, *(Prionotus carolinus)*

Substances	Bullhead	Tomcod	Searobin
Tissue extract	+++(51/51)*	++ (12/12)	++ (32/32)
Acetic acid	+++(48/51)** (0·0008 M)	++ (10/12) (0·008 M)	+ (29/32)
Sodium chloride	+ (35/51) (0·05 M)	++ or (10/12)	++ or (28/32)
Sucrose	+ (3/51) (0·05 M)	+ (1/12)	o (32/32)
Dextrose	+ (1/51) (0·05 M)	+ (1/12)	o (6/6)
Quinine hydrochloride	++ (6/20) (0·003 M)	++ (10/12) (0·006 M)	++ (2/6)
Fresh water	O (51/51)	— (10/12)	— (30/32)
Cysteine	++ (41/41) (0·00005 M)	++ (12/12)	++ (32/32) (0·00005 M)
Homocysteine	? (3/21)	? (12/12)	? or o (32/32)
Methionine	O (3/3)	o (5/5)	o (32/32)
Cysteine hydrochloride	++ (41/41) (0·00005 M)	++ (12/12)	++ (32/32)
Cysteic acid	+++(21/21) (0·0005) M)	? (12/12)	? (3/32) or o (29/32)
Taurine	+ (9/12)	+ (4/12)	+ (12/32)
Glutathione (red.)	+ (2/3)	+ (2/5) or ? (3/5)	? (10/10)
Alanine	++ (12/12)	+ (5/5)	+ (10/10) (0·005 M)
Serine	o (12/21):	o (5/5)	o (10/10)
Phenylalanine	+ (12/12)	+ (3/5)	+ (10/10)
Aspartic acid	? (8/12)	+ (3/5)	o (10/10)

Leucine	+ (3/12)	o (5/5)	o (10/10)
Tyrosine	o (12/12)	o (5/5)	o (10/10)
Glutamine	o (3/3)	o (5/5)	o (10/10)
Glutamic acid	+ (3/3)	+ (5/5)	o (10/10)
Tryptophane	O (3/5) or ? (2/5)	o (2/3) or ? (1/3)	? (5/7) or o (2/7)
Tryptamine	o (3/5) or ? (2/5)	o (2/3) or ? (1/3)	+ (7/7)
Skatol	+ (5/5)	+ (3/3)	+ (7/7)
Indole acetic acid	o (5/5)	o (3/3)	o (7/7)
Indole	++ (5/5)	++ (3/3)	+++(7/7)
Lecithin	see text		
Cholesterol	see text		
Inositide	see text		

* The first numbers in parenthesis refer to positive responses, the second to the number of preparations tested with the substance.
** Critical concentrations for few-fiber preparations, where ascertained, given below character of response.
+ = positive.
o = negative.
? = questionable.
— = inhibitive effects.

but preparations from marine species did not respond until the concentration was increased by a factor of almost 10. This difference between marine and fresh water species in responses to acid concentrations does not imply that marine fish are less sensitive to acids, but it does mean that marine species are less able than fresh water species to detect acid in the same dilutions. This difference could, therefore, have an important bearing on water pollution problems. A typical response to acids is illustrated in Fig. 4a.

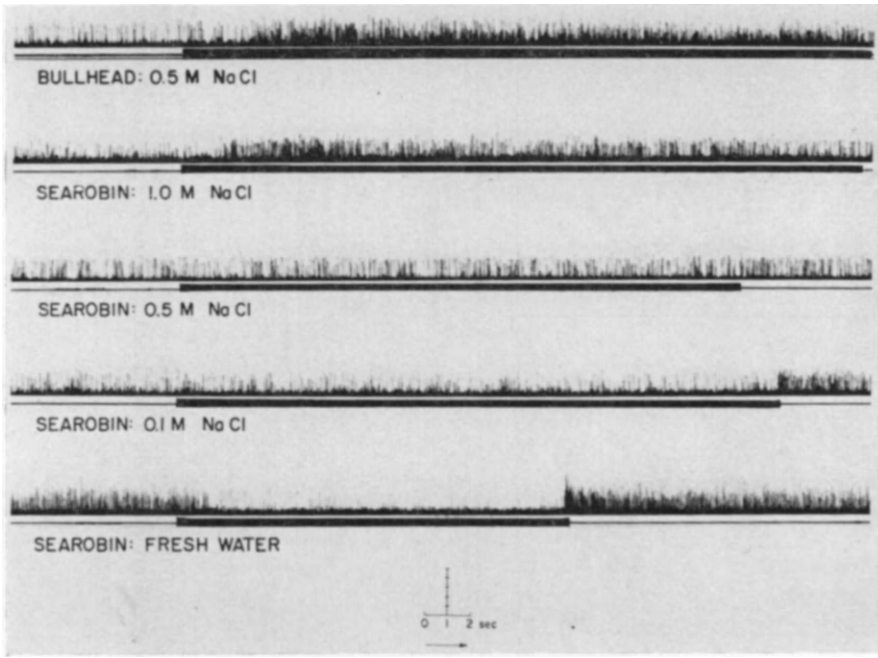


FIG. 5. Comparison of responses to sodium chloride of barbel fibers of a bullhead and fin fibers of a searobin; a 0·5-molar solution of sodium chloride has the approximate salt content of sea water.

NaCl elicited a more gradual, more sustained nerve activity from bullhead barbel fibers (Fig. 4b) than did acids (Fig. 4a). Both marine and fresh water species responded with increased discharges to a salt concentration higher than that of their natural surroundings, but a salt concentration lower than that natural environment evoked inhibition in the marine species. Sea water has approximately a 0·5-molar concentration of NaCl; when the concentration was lowered to between 0·4 and 0·3 molar, we noted a reduction of spikes from several-fiber preparations of tomcod

and/or searobins. Progressive dilution of sea water or of an NaCl solution led to progressively stronger inhibition; discharges almost ceased when fresh water applied (Fig. 5). The inhibition was followed by discharge activity greater than that which prevailed before stimulation. After placing some searobins in a 1-molar NaCl solution for 4 hours, we noted that subsequent stimulation with sea water or 0·5-molar NaCl resulted in inhibition (see also Konishi and Zotterman, 1963).

Choline chloride produced similar effects to those of sodium chloride (Fig. 6). Konishi, in the proceedings of this symposium, reports on receptors

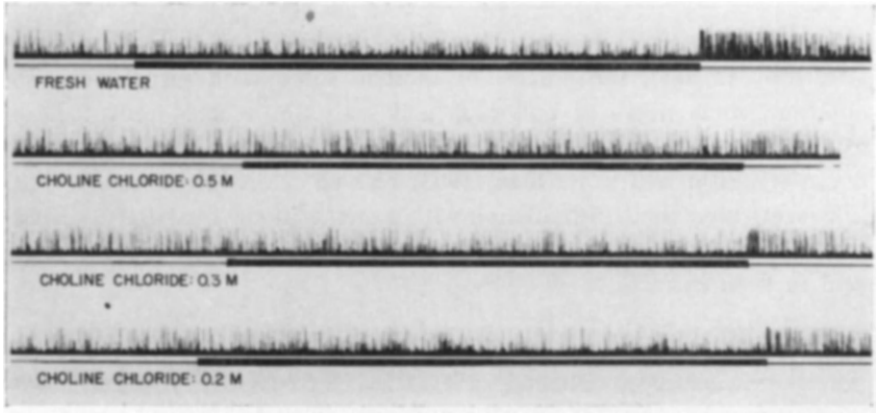


FIG. 6. Responses of searobin fin nerves to variations in choline chloride; note the similarity in response to that obtained when diluting a common salt solution (Fig. 5).

of fishes responding to dilute salt solutions. His data make them appear to be different units from those responding to concentrated salt solutions, though there are to be noted similar actions of sodium and choline chloride on both receptor types. Inasmuch as the negative ions of both these salts are identical, while the cations differ from one another in some respects, critical experiments would be of interest on anionic as opposed to cationic effects in the stimulation of salt receptor sites.

In all three species, varying numbers of fibers responded to stimulation with quinine hydrochloride but not with quinine sulfate, probably because the latter is relatively insoluble (Table 2). Less than 10% of all fibers tested responded to sugars, and fibers from the marine searobin did not respond at all to sugars. We believe that this difference in sensitivity is correlated with the type of innervation and the anatomy of the chemo-

sensitive sites; it will be discussed in this context later. Integrated responses to sugar and to quinine are illustrated in Figs. 4c and d.

The number of fibers that responded to acid and to salt, respectively, was similar in all three species. The majority of the fibers responded to acids: Bullhead *ca.* 95%, tomcod 80% and searobin nearly 90% (Table2). Salt also elicited responses from a majority of fibers: Relatively fewer bullhead fibers responded (*ca.* 70%) compared to over 80% in the tomcod and searobin.

Responses to Other Than Conventional Tastes

Some single-fiber preparations from bullhead barbels responded selectively to acid, salt, sugar, or quinine stimulation respectively. In addition, some fibers of bullheads and searobins reacted only to flesh extracts in much the same way that fibers isolated from the carp palatal organ (Konishi and Zotterman, 1963) reacted selectively to saliva and silkworm pupa fluid. Stimulation with cysteine alone further suggested that the selective response could be caused by the presence of this amino acid in flesh extracts.

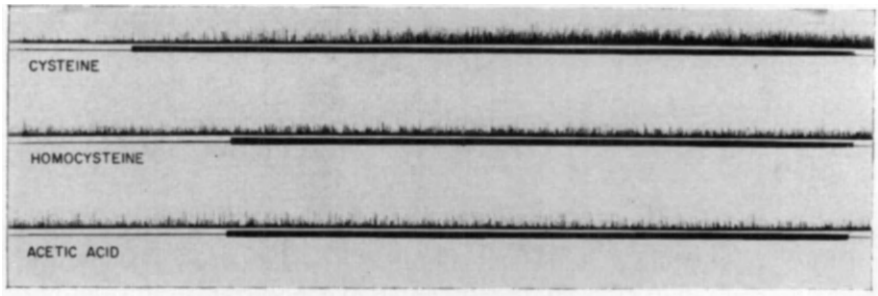


FIG. 7. Comparison of responses of bullhead barbel fibers to 1. cysteine, 1. homocysteine and acetic acid. Note the absence of responses to the two latter compounds; scale as in Fig. 5.

It is noteworthy that homocysteine, which is but one carbon atom longer than cysteine, produced no response from few-fiber preparations of searobins (Fig. 7), and that bullhead and tomcod fibers tested with homocysteine and then rinsed again responded to cysteine. This suggests that the correspondence between specific stereo and/or electrochemical properties of the substance and the villar site is responsible for the reaction (Beidler, 1961). Cysteine hydrochloride, cysteic acid, and taurine, all

related to cysteine, were also tested. The first two yielded strongly acid solutions: a 0·1-molar fresh water solution of either compound has a pH of about 2. Bullheads responded to both of them strongly, while the marine species reacted only to cysteine hydrochloride. When cysteine hydrochloride was applied to a few-fiber preparation of the bullhead barbel in a buffered solution (pH of about 6), the response was similar to that obtained by stimulation with free base cysteine, that is, an increased

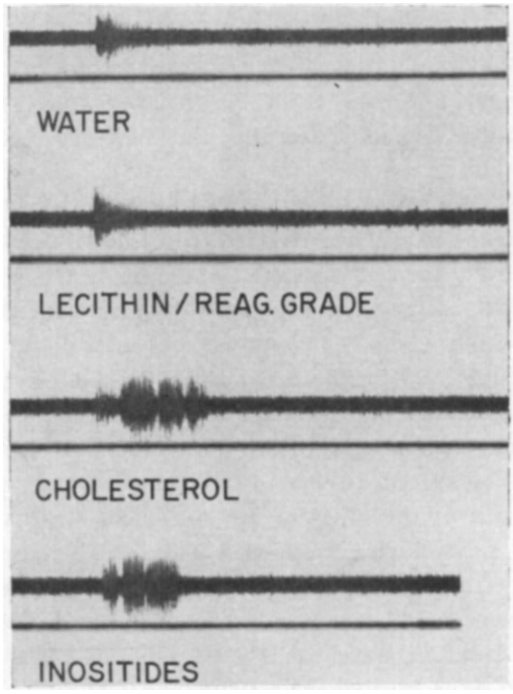


FIG. 8. Comparison of responses from a several fiber preparation of a bullhead barbel to charged lipid compounds; camera speed 1 cm/sec.

firing rate lasting 30 or more seconds. Unbuffered cysteine hydrochloride or cysteic acid also elicited a prolonged response, but the response began with a strong, quickly subsiding burst, characteristic of reactions to acids. In the buffered system the acid response is slight or absent. Some of the cysteine-sensitive fibers failed to respond to acids (Fig. 7); they may indeed be quite specific for cysteine.

Cysteine is a highly reactive compound and a strong reducing agent. The other organic sulfur compounds we used are less reactive chemically

as well as in their ability to evoke responses in taste cells. Indole, another electrochemically active substance, also had a strong stimulating action on our preparations, while indole acetic acid, although structurally related to indole, failed to elicit responses.

These reactions to cysteine are of additional interest in light of the high olfactory sensitivity of salmon to serine reported by Idler *et al.* (1956). In behavior experiments designed to test avoidance reactions these salmon did not respond to cysteine or to other amino acids. We stimulated the olfactory organ of the bullhead with serine, alanine, cysteine, recording the responses with external electrodes applied to the olfactory tract; all three 3-carbon amino acids elicited positive responses. Similar exploratory experiments with brook trout *(Salvelinus fontinalis)*, a member of the salmon family, also indicated that the olfactory receptors react to serine, alanine and cysteine.

The taste receptors of the three species tested responded more strongly to cysteine than to alanine; the response to cysteine was strongest in the bullhead. However, these species, like the carp tested by Konishi and Zotterman (1963) gave no taste responses to serine. Leucine elicited a few responses from bullhead fibers, but similar responses could not be observed from the taste fibers of the marine species. Unfortunately, nothing is known of the taste responses of trout and salmon.

These few data demonstrate that the olfactory and gustatory chemoreceptors of fishes overlap to some extent, and that certain sensitivities are specific to one or the other of the two chemosensitive organs. The olfactory electrophysiological responses of bullheads to serine, however, raise certain questions: Do many or all fishes smell serine? If so, is the perception of serine equally acute in all fishes: that is, do they have equally many "serine sites" or have the Pacific salmons developed a higher sensitivity to serine as part of their avoidance reaction to this substance which occurs in the rinse from mammalian skin? Brett (1954) has suggested that the avoidance is an adaptation against predation by bears, to which this group of fishes is particularly subject. Furthermore, do all fishes that smell serine avoid the substance, or do only salmon? The popular belief of anglers that bait handled by man is often avoided by fish may suggest a rather general avoidance of mammalian skin scent. Perhaps serine is the agent in mammalian skin scent which produces an avoidance reaction in many, if not all, fishes.

Though fish can both smell and taste certain amino acids, other organic substances are probably only tasted. Lipids are of interest here inasmuch as some of them elicited taste responses from the fibers of bullhead barbels.

Among the lipids isolated from fish tissue extracts (see Substances Tested, above), inositides and cholesterol were effective, but reagent grade lecithin was not; however, responses similar to those elicited by acids were sometimes obtained with commercial grade lecithin, probably due to impurities. These reactions are of further interest because their general pattern differed from all others: their integrated trace appeared like a bell-shaped curve and lasted but a few seconds (Fig. 8). Konishi and Zotterman

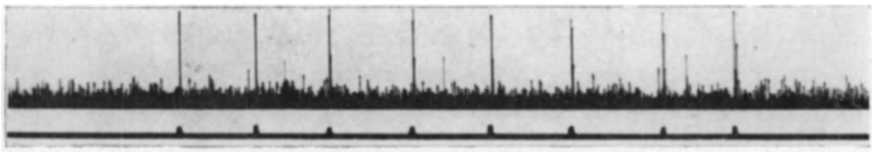


FIG. 9. Responses of intermittently stimulated phasic mechanoreceptors in the bullhead barbel.

(1963) suggested that glycerophospholipids, among other substances, were the active stimulants in carp taste-fiber responses to saliva and silkworm pupa extract; inositides are glycerophospholipids.

Lytic agents and surfactants that affect the lipid-protein complexes of the villar membrane impair or abolish taste responses (digitonin, Konishi and Zotterman, 1963; detergents, Bardach, Fujiya, and Holl, 1965). The sites on the taste cells that respond to fat-soluble substances may have a different location from those that respond to water soluble stimulants, though the relation between these sites has to be unimpaired for responses to occur to either class of chemicals.

Scanty though the evidence may be to date, we think that fishes have more taste responses than the four conventional ones: carp and bullhead fibers respond to lipids, and some bullhead fibers respond exclusively to "tissue juice", and possibly to cysteine. Furthermore, the quantitative and qualitative differences in the taste responses of Swedish and Japanese carp (Konishi and Zotterman, 1963) suggest a high degree of biochemical specialization of chemoreceptor membranes, similar to that of mammals (Beidler, 1961), as the genetic mechanisms of the species or races were influenced by ecological pressures throughout their evolution.

Besides the evidence for more fibers or sites in fishes than the four conventional tastes, we were impressed by the consistent and typical differences in the integrated patterns of the various responses (Fig. 4 a–d). Although only bullhead fibers were sufficiently explored, the reac-

tions from the other species also point to the important role which integrated response patterns must play in enabling the animal to differentiate between different stimuli.

Differences between Taste Buds and Free Nerve Endings

In addition to species or group-specific differences in gustatory responses among fishes, further differences are revealed by the responses of cranially, as opposed to spinally, innervated receptor sites. These are illustrated by comparing members of the cod family (Gadidae) and searobins (Table 3); only marine species have been selected for this comparison because inclusion of fresh water fishes might confound the picture (see above).

Unfortunately the dual innervation, spinal and cranial, of hake and tomcod fins makes a clear-cut comparison to the spinally innervated searobin fin somewhat difficult; a comparison of the searobin's oral

TABLE 3. COMPARISON OF TASTE RESPONSES OF HAKE AND TOMCOD FINS AS OPPOSED TO THOSE OF THE SEAROBIN[1]

	Hake[2] or Tomcod (Taste buds; spinal and cranial innervation)	Searobin[2] (No taste buds; spinal innervation)
Acetic acid	+	+
Sodium chloride	+	+
Sucrose	+	O
Quinine hydrochloride	+	+
Cysteine	+	+
Homocysteine	?, O	O
Methionine	O	O
Cysteine hydrochloride	+	+
Cysteic acid	?, O	?
Glutathione	+	+
Aspartic acid	+	O
Alanine	+	+
Serine	O	O
Leucine	O	+
Glutamic acid	+	O
Tryptophane	?, O	?, O
Tryptamine	?, O	+
Phenylalanine	+	+
Glycine	+	O

[1] Data from more than three observations.
[2] Some data from Bardach & Case, 1965.

receptors with its fin receptors would have been more effective. Nevertheless, some differences are clearly noticeable: Free chemosensitive nerve endings of searobins do not respond to sugars. In behavior tests with sharks (Uexkuell, 1895) and with various fresh water fishes (Wunder, 1927) the sugar response was also missing.

Searobin fins respond to a smaller variety of substances than do the spinally and cranially innervated fins of the cod family. As Table 3 shows, the cod failed to respond to only three of the substances listed, the searobin failed to respond to seven substances. For instance, searobins showed no response to aspartic and glutamic acids and to glycine. However, they responded to tryptamine (7 preparations) to which tomcod gave no (3 preparations) or uncertain (1 preparation) responses.

It appears that free gustatory nerve endings, in the fins of the searobin at least, are fairly sensitive and respond to a substantial variety of chemicals, far beyond the range ascribed to the so-called chemical sense of the general body surface which also relies on spinal innervation. The investigation of comparable structures in other fish species is clearly required, as is a comparison of taste buds in the searobin's mouth with its free finrays.

Taste and Touch in Specialized Fins of Fishes

Fishes have tonic and phasic mechanoreceptors (Bardach and Case, 1965), some of the latter resembling those of higher vertebrates (Bardach and Loewenthal, 1961). Such touch fibers appear in all nerve preparations from appendages or from other chemically sensitive sites of fishes (Hoagland, 1933; Konishi and Zotterman, 1961; Bardach and Case, 1965). Stimulation with a drop of water or with a few hairs of a brush produces a short rapid burst of impulses (Fig. 9). We know that in taste-bud-bearing fishes, touch fibers terminate in the epithelium surrounding the buds because their function is not impaired when taste buds themselves are damaged by detergents (Bardach, Fujiya, and Holl, 1965). Only with increased damage, when the surfactant apparently penetrates the epithelium, do touch responses cease as well.

Fishes with external taste structures rely less on sight than do other fishes (Teichmann, 1962), and the importance of combined mechanical and chemical exploration of their environment has been stressed in observations of their behavior (Herrick, 1904; Bardach and Case, 1965). Earlier neuroanatomical investigations (summarized by Kappers, Huber, and Crosby, 1936) showed that, in the catfish and cod families especially, the central connections between different taste and touch fibers "provide

the most important central correlations." Electrophysiological investigations now illustrate that these two senses are also most closely associated peripherally, thus supplementing information derived from anatomical and behavioral studies.

REFERENCES

BARDACH, J. E. and L. A. LOEWENTHAL. 1961. Touch receptors in fishes with special reference to the moray eels *(Gymnothorax vicinus* and *G. moringa)*. *Copeia* 1961 (1): 42–46.

BARDACH, J. and J. CASE. 1965. Sensory capabilities of the modified fins of squirrel hake *(Urophycis chuss)* and searobins *(Prionotus carolinus* and *P. evolans)*. *Copeia* 1965 (2): 194–206.

BARDACH, J. E., M. FUJIYA and A. HOLL. 1965. Detergents: Effects on the chemical senses of the fish *Ictalurus natalis* (Le Sueur). *Science* **148,** No. 3677: 1605–1607.

BEIDLER, L. M. 1961. Taste Receptor Stimulation. In *Progr. Biophysics and Biophys. Chem.* Pergamon Press, London, pp. 107–151.

BRETT, J. R. and D. MacKINNON. 1954. Some aspects of olfactory perception in migrating adult Coho and Spring salmon. *J. Fish. Res. Bd. Can.* **11**(3): 310–318.

DOGIEL, A. S. 1897. Ueber die Nervenendigungen in den Geschmacks-Endknospen der Ganoiden. *Arch. Mikrosk. Anat.* **49,** 769–790.

FREIHOFER, W. C. 1963. Patterns of the Ramus Lateralis Accessorius and their systematic significance in teleostean fishes. *Stanford Ichthyol. Bull.* **8**(2): 81–189.

HASLER, A. D. 1957. Olfactory and Gustatory senses of fishes. In M. E. BROWN, Ed., *The Physiology of Fishes*, Vol. 2, Acad. Press, New York, pp. 187–210.

HERRICK, C. J. 1904. The organ and sense of taste in fishes. *Bull. U.S. Fish. Comm.* **22,** 239–272.

HOAGLAND, H. 1933. Specific nerve impulses from gustatory and tactile receptors in catfish. *J. gen. Physiol.* **16,** 685–693.

IDLER, D. R., U. H. M. FAGERLUND and H. MAYOH. 1956. Olfactory perception in migrating salmon. I. 1-Serine, a salmon repellent in mammalian skin. *J. gen. Physiol.* **39,** 889–892.

KAPPERS, C. U. A., C. C. HUBER and E. C. CROSBY. 1936. The Comparative Anatomy of the Nervous System of Vertebrates Including Man. Vol. I, pp. 347, 358, 360–362. The MacMillan Co. New York.

KONISHI, J. and Y. ZOTTERMAN. 1961. Taste functions in the carp. *Acta. Physiol. Scand.* **52,** 160–161.

KONISHI, J. and Y. ZOTTERMAN. 1963. Taste functions in fish. In *Olfaction and Taste*, Proc. 1st. Int. Symp. Wenner-Gren Center, Stockholm. The MacMillan Co., New York, pp. 215–233.

LENHOSSEK, M. von. 1893. Der feinere Bau und die Nervenendigungen der Geschmacksknospen. *Anat. Anz.* **8,** 121–127.

LOOMIS, W. F. 1955. Glutathione control of the specific feeding reactions of hydra. *Ann. N.Y. Acad. Sci.* **62,** 209–228.

MORILL, A. D. 1899. The pectoral appendages of *Prionotus* and their innervation. *J. Morphol.* **11,** 177–192.

SCHARRER, E. 1963. Intraepithalial nerve terminals in the free finrays of the searobin, *Prionotus carolinus* L. Anat. Rec. **145**(2), 367–368.

SCHARRER, E., S. W. SMITH and S. L. PALAY. 1947. Chemical sense and taste in the fishes *Prionotus* and *Trichogaster*. *J. comp. Neurol.* **68,** 183–198.

TEICHMANN, H. 1962. Die Chemorezeption der Fische. *Erg. d. Biol.* **25,** 177–205.

UEXKUELL, J. von. 1895. Vergleichende sinnesphysiologische Untersuchungen. 1. Ueber die Nahrungsaufnahme des Katzenhaies. *Z. Biol.* **32,** 548–599.

WHITEAR, M. 1952. The innervation of the skin of teleost fishes. *Quart. Jour. Microsc. Sci.* **93,** 289–305.

WUNDER, W. 1927. Sinnesphysiologische Untersuchungen ueber die Nahrungsaufnahme bei verschiedenen Knochenfischarten. *Z.* vergl. *Physiol.* **6,** 67–98.

STUDIES ON THE STIMULATION OF CHEMORECEPTORS OF FRESHWATER FISH BY DILUTE SOLUTIONS OF ELECTROLYTES

JIHEI KONISHI

Laboratory of Physiology and Ecology,
Faculty of Fisheries, Mie Prefectural University, Tsu, Japan

KRINNER (1935) established a fairly low gustatory threshold for salt (M/20480 for NaCl) in a freshwater fish by using trained minnows *(Phoxinus)*. One of the attractive problems in the study of the chemoreceptive functions of freshwater fish concerns the sensitivity to dilute electrolyte solutions, especially since the chemoreceptors are constantly exposed to a very hypotonic aqueous environment.

Several years ago Konishi and Zotterman (1963) had noticed the peculiar phenomenon that the palatal chemoreceptors of the carp are highly sensitive to very dilute salt solutions, whereas those of higher concentration produced little response. Further studies on this subject provided more detailed information on the chemoreceptor responses of freshwater fish to dilute electrolyte solutions (Konishi and Niwa, 1964). Extremely dilute solutions of inorganic electrolytes, especially those with polyvalent anions such as Na-citrate and Na_2HPO_4, produced strong integrated responses. However, the response was depressed with increasing concentration, although the receptor activity increased again at much higher concentration, thus displaying an inversely-*S*-shaped curve of response against concentration. The application of distilled water, immediately after the receptors had been irrigated by a salt solution at the concentration where the chemoreceptor activity was depressed, elicited a remarkable response. This effect was called tentatively the "distilled water effect (DWE)". Such an effect was not observed so long as the receptors had been strongly responsive to the salt solution previously applied. In this communication, an attempt will be made to analyze further the chemoreceptor activity to dilute

electrolytes in freshwater fish by using carp; and a hypothesis which explains the mechanism of the stimulation of the chemoreceptor by dilute electrolytes will be presented.

The data of the response–concentration curve obtained by recording integrated responses from the whole palatine nerve immediately raises the question of whether or not the high sensitivity at a very dilute saline range has its origin in the activity of the specific receptor groups, being distinguished from those responding to hypertonic saline. The results from single fiber analyses clearly demonstrated the existence of two distinct receptor systems which are active in different regions of the sensitive saline range; one of them being characterized by high sensitivity to a dilute solution of an ordinary electrolyte with a monovalent cation, as well as not being stimulated by pure water. Cohen *et al.* (1955) suggested such a dual receptor mechanism in the cat, subserving discrimination within an entire salt stimulus concentration range, with one of them responding to pure water. The present results also may offer another case of the dual receptor systems involved in the salt discrimination mechanisms. It also appeared that the DWE was assigned to the activity of the same receptor system as that which is stimulated by dilute electrolytes.

As shown in Fig. 1, neural activity from such a receptor system is completely depressed above M/512 in $Na_4Fe(CN)_6$ and M/192 in NaCl. Salts with polyvalent cations such as $CaCl_2$ and $LaCl_3$ and an organic electrolyte like quinine-HCl did not stimulate this fiber at any concentration. Integrated responses to dilute electrolyte solutions are characterized by slowly rising to a peak, a long time interval between stimulus application and peak response magnitude (peak time), while the peak time of the response to a high stimulus concentration is faster. Thus the peak time vs. concentration curve for $Na_4Fe(CN)_6$ shows a discontinuous change at around M/512 (Fig. 3); this may be easily explained by assuming two distinct receptor systems which respond in opposite regions. As will be described later, the existence of distinct receptor systems will be strongly supported by the data of the effects of a polarizing current on the receptor activity. The peak time of DWE for $Na_4Fe(CN)_6$ is shorter, and no abrupt change, at least, in the peak time is seen, although it tends to lengthen somewhat above M/512. A comparison of time characteristics between integrated and single fiber responses showed an agreement; the maximum of the spike frequency/time differential in the response to M/4096 $Na_4Fe(CN)_6$ is between 1 and 2 sec., and this almost coincides with the peak time of the integrated response (cf. Figs. 2 and 3). The peak time of the integrated record of DWE (0·5 sec) also coincides with the maximum of the spike frequency/time

differential (0–1 sec). It seems most likely from these findings that the responses to dilute electrolyte solutions obtained from the whole nerve preparation represent a summated activity of the specific receptor group re-

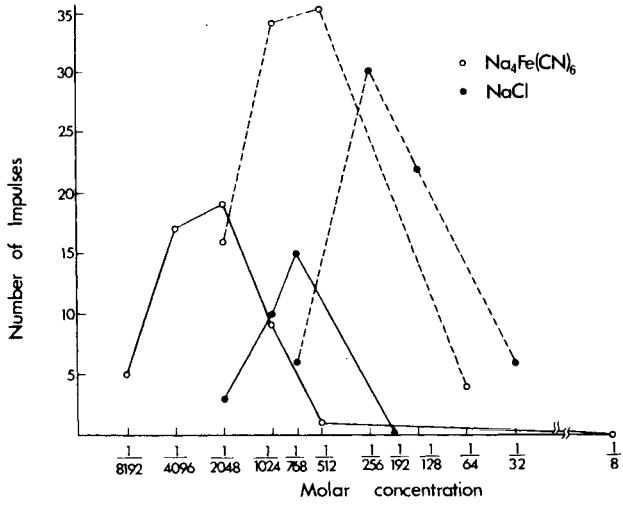


FIG. 1. Response of the single palatine nerve fiber responding specifically to dilute solutions of salts with monovalent cations ($Na_4Fe(CN)_6$ and NaCl) to varying concentrations (solid curve). Distilled water effects (broken curve) obtained from the same fiber after previous adaptation to the salt solutions of the concentrations of abscissa. Each point represents the number of impulses during first three seconds after stimulation.

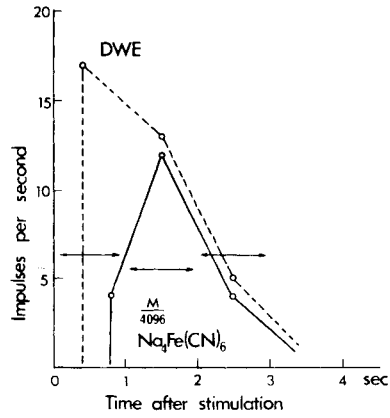


FIG. 2. Time-frequency curves for the response to M/4096$Na_4Fe(CN)_6$ solution and the distilled water effect after previous adaptation to M/512$Na_4Fe(CN)_6$ solution. Each point represents the number of impulses during a one second interval.

sponding to weak electrolyte solutions. In view of a similarity in shape of both response–concentration curves obtained by recording integrated response and in single fiber studies, it may be possible to discuss the excitability of the receptor system responding specifically to dilute electrolyte solutions, using the data obtained with whole nerve studies.

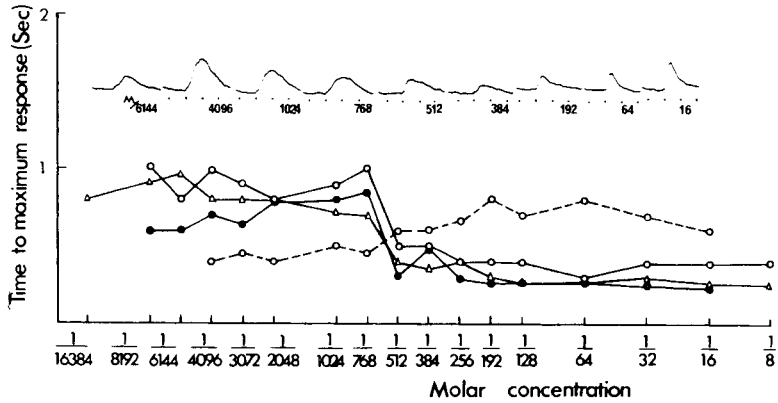


FIG. 3. Time to maximum integrated response magnitude. Three curves for the response to $Na_4Fe(CN)_6$ (solid curves) were obtained with different preparations. Each point of the distilled water effect curve (broken curve) represents time to maximum magnitude of the effect after previous adaptation to $Na_4Fe(CN)_6$ solution of the concentration of abscissa. Inserted oscilloscope traces represent integrated records of the response to $Na_4Fe(CN)_6$ solution of various concentrations. Time mark one second.

In Fig. 4A, response–concentration curves are reproduced for three typical sodium salts, $Na_4Fe(CN)_6$, Na_2So_4 and NaCl, obtained by recording integrated response. The curves for these salts have peaks at different concentrations (peak concentration) and a further raise of the concentration markedly depressed the response. The peak concentrations for these salts were at about M/4096 for $Na_4Fe(CN)_6$, at M/2048 for Na_2SO_4 and at M/1024–M/768 for NaCl, which shows that there is a relationship between the normality of the solutions and the peak concentrations. The response magnitude differs with the salt employed, and increases with the valency of the anion. The effects of different monovalent cation series are illustrated in Fig. 4B, showing all salts producing maximal responses at M/1024–M/512. Similar results were also obtained by different monovalent anion series; all salts tested gave maximal response at around M/1024, indicating uni-univalent electrolytes behave almost quantitatively like NaCl (Fig. 4C). These findings strongly suggest that the concentration where the magni-

tude of the response to a salt solution reaches a maximum depends on normality, regardless of the kind of salt, and that the response magnitude may be chiefly determined by the anion species, probably by its valency. But dilute solutions of polyvalent cation salts like $CaCl_2$, $MgCl_2$ and $LaCl_3$

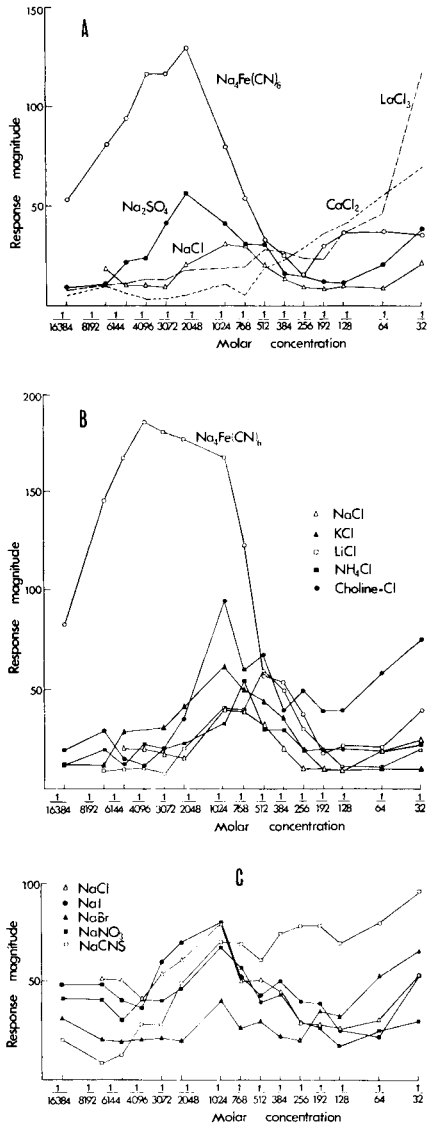


FIG. 4. Comparison of integrated responses of the whole palatine nerve to varying concentrations of various salts. Magnitude of response is in arbitrary units.

did not produce appreciable integrated responses (Fig. 4A; cf. Konishi and Niwa, 1964), though the response–concentration curves for these salts were raised sharply at high concentration ranges where an apparent depression of the response by the salts with monovalent cations was seen. These findings again suggest that anions play an important role in the stimulation of the receptors which respond specifically to dilute solutions of electrolytes having a monovalent cation.

As stated above, the DWE is the activity of the same receptor that is stimulated by the dilute electrolyte solution. Therefore, it seems to be important to analyze DWE in parallel with the stimulatory response. The magnitude of the DWE increases with an increase in concentration of the solution previously applied (in other words, with the progress of the depression of the response) and reaches a maximum at a certain concentration, after which a further increase in the concentration causes diminution of the DWE magnitude. DWEs elicited after previous adaptation to various electrolytes are illustrated in Fig. 5. Maxima of DWE curves for $Na_4Fe(CN)_6$, Na_2SO_4, NaCl and other uni-univalent inorganic electrolytes are at M/512, M/256 and M/128–M/192 respectively. The normality relationship can be seen in the DWE also. Salts with polyvalent cations ($CaCl_2$ and $LaCl_3$) in any concentration failed to produce a DWE.

Some of the outstanding properties of the receptor system responding to dilute electrolyte solution may be: (1) the receptors are strongly stimulated by extremely dilute solution of electrolytes; (2) the magnitude of response largely depends on the valency of the ions; (3) a slight change in salt concentration sharply influences response magnitude; (4) response–concentration curves have peaks at a certain dilute concentration, and a further increase of the concentration markedly depresses the response. In view of these findings, it may be rather difficult to explain adequately the behavior of the specific chemoreceptors (found in freshwater fish) in response to varying concentrations of a stimulus solution, by similar concepts to that which have been advanced to interpret the mechanisms of the chemoreceptor stimulation by hypertonic electrolyte solutions in mammals (Renqvist, 1919; Lasareff, 1922; Beidler, 1954, 1961). The response–concentration curves obtained by various dilute electrolyte solutions bear a striking resemblance to the curves obtained by Loeb, studying the influence of electrolytes on the cataphoretic charges of collodium (1922–23a) and protein (1922–23b) particles, and also to those found in the anomalous osmosis phenomenon of a collodium membrane (Kakiuchi, 1930) (Fig. 6). Furthermore, a phenomenon analogous to that found in the present experiments on the chemoreceptor activity will also be noticed in the data on

electrokinetic potential changes at glass/liquid interfaces (Furutani, 1929). The cataphoretic P.D. of colloidal particles are very much influenced by minute traces of electrolytes. The addition of little salt with a monovalent

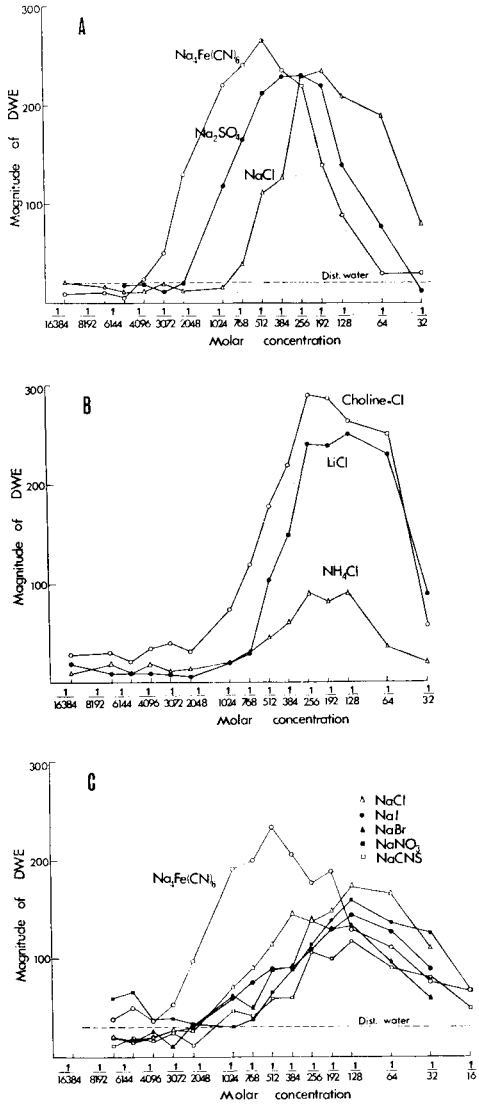


FIG. 5. Comparison of distilled water effects for various salts. A and B were obtained from the same preparation as that of Fig. 4A, and C was obtained from the same preparation as in Figure 4 C. Abscissa: concentration of the salt solution previously applied.

cation raises the cataphoretic P.D. negatively, as a result of the increase of the negative charge of the particles by specific adsorption of anions to the inner region of the electrical double layer. This process is more

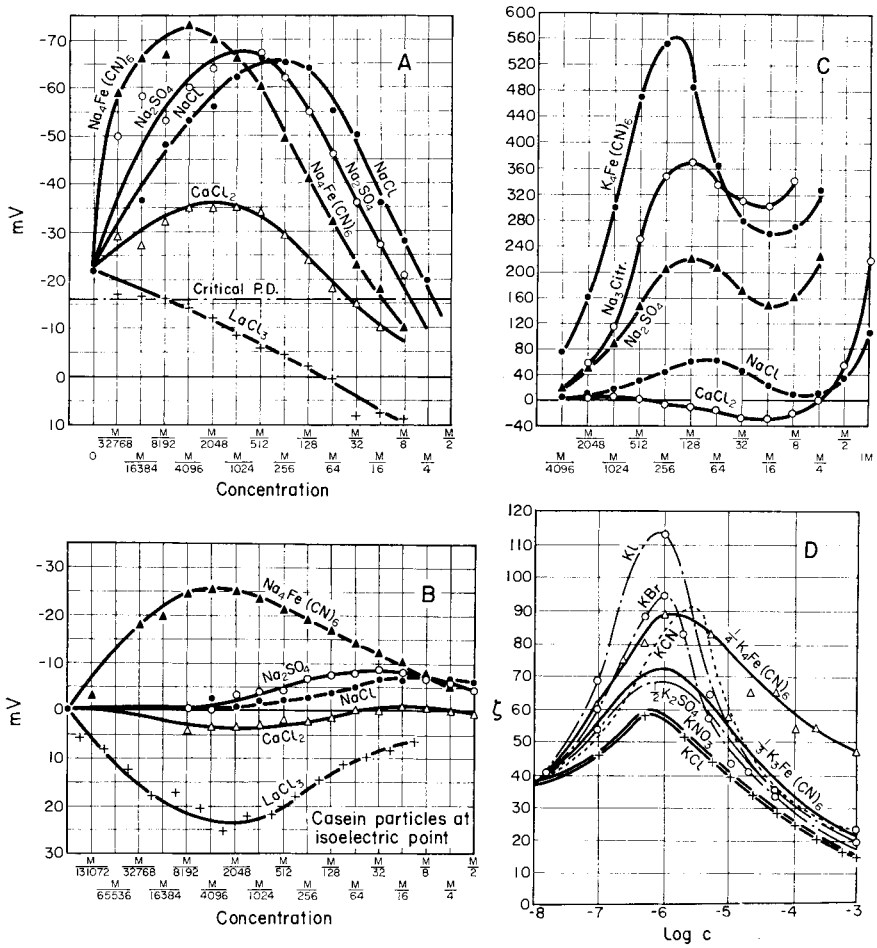


FIG. 6. Influences of electrolytes on the cataphoretic P. D. of collodium (A) (after Loeb, 1922–23 a) and protein particles (B) (after Loeb, 1922–23 b) and on the anomalous osmosis of collodium membrane (C) (after Kakiuchi, 1930) and on the ζ potential at a glass interface (D) (after Furutani, 1929).

rapid the higher the valency of the anion, until a maximum is reached, after which a further increase in the concentration of the salt results in a depression of the P.D. Polyvalent cation salts behave in an opposite manner to monovalent cation salts, and especially, a trivalent cation salt like $LaCl_3$

reverses the sign of the charge of the double layers surrounding the particles (Loeb, 1922–23 a). Similar opposite behavior of polyvalent cation salts was likewise observed in the results of the present experiments on the chemoreceptor stimulation. The analogy, between the data on the electrokinetic phenomena observed in other materials mentioned above and the data on the chemoreceptor activity, may provide a possible explanation for the mechanism of the chemoreceptor stimulation by dilute electrolyte solutions, suggesting that an electrokinetic process at the receptor membrane interfaces and certain accompanying physicochemical phenomena may well be involved in the chemoreceptor stimulation process. If such is the case, the depression caused by increasing salt concentrations may be accounted for by the concept of depression of an electrokinetic potential by increasing the ionic strength of the solution (Verwey, 1935; Watanabe *et al.*, 1961 a, b; Watanabe, 1964). The mechanism of the DWE initiation might also be explained by introducing the concept of the double layer at solid/liquid interfaces. The present results leave no doubt that at least one kind of chemoreceptor stimulation is due to the anion. If receptor surfaces or sites are positively charged, anions may be specifically adsorbed to the inner region of the double layer at the receptor surface; thus the adsorption of anions may determine the Stern potential (or ζ potential). If so, it will be fully expected that response magnitudes may largely depend on the valency of the respective anion. If stimulation of the chemoreceptors by dilute electrolytes depends on the specific adsorption of anions, it seems possible that the depression of the response caused by increase of ionic strength of the solution may be due to the screening effect of the cation. A similar depressing effect of the cation on the cataphoretic P.D. of collodium particles was demonstrated by Loeb (1924 b). The normality dependency of the peak of the response–concentration curve suggests that the cation may play an important role in the determination of the peak. The data to be described below may be useful as a basis for the support of the hypothesis thus far presented in this paper.

Studies on the effect of treatment of the receptors with acid and alkali previous to stimulation with dilute salt solutions demonstrated that reacting surfaces of the receptor which respond to dilute electrolyte solutions are positively charged. The responses to dilute $Na_4Fe(CN)_6$ solution were completely inhibited by previous treatment with alkali (pH 8·0), whereas no marked changes were observed for previous acid (pH 3·9) treatment (Fig. 7). The effects of treatments with alkali solutions in different pH ranges are shown in Fig. 8. A slight change of the pH on the alkaline side sharply influenced the receptor activity, showing almost complete depression of the response

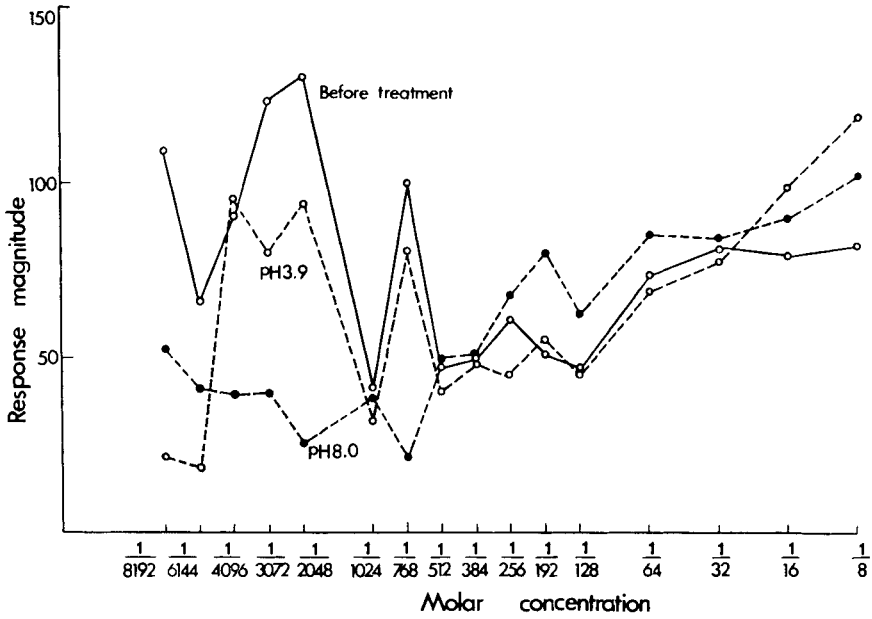


FIG. 7. Effect of previous treatments of chemoreceptors with acid (M/4096 HCl (pH 3·9)) and alkali (M/2048 NaOH (pH 8·0)) on the response to dilute solution of $Na_4Fe(CN)_6$. Abscissa: concentration of $Na_4Fe(CN)_6$ solution.

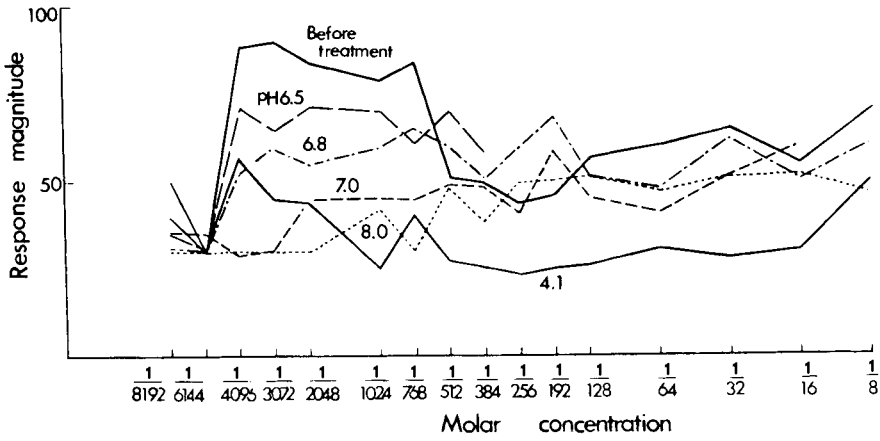


FIG. 8. Effects of previous treatments of chemoreceptors with alkaline solutions of different pH (NaOH, pH 6·5–8·0) on the response to dilute solution of $Na_4Fe(CN)_6$. Abscissa: concentration of $Na_4Fe(CN)_6$ solution.

even at neutrality. Final treatment with acid (HCl, pH 4·1) after a series partially of alkali treatments restored the receptors to be responsive to dilute $Na_4Fe(CN)_6$ solution. These results strongly suggest that the initial step

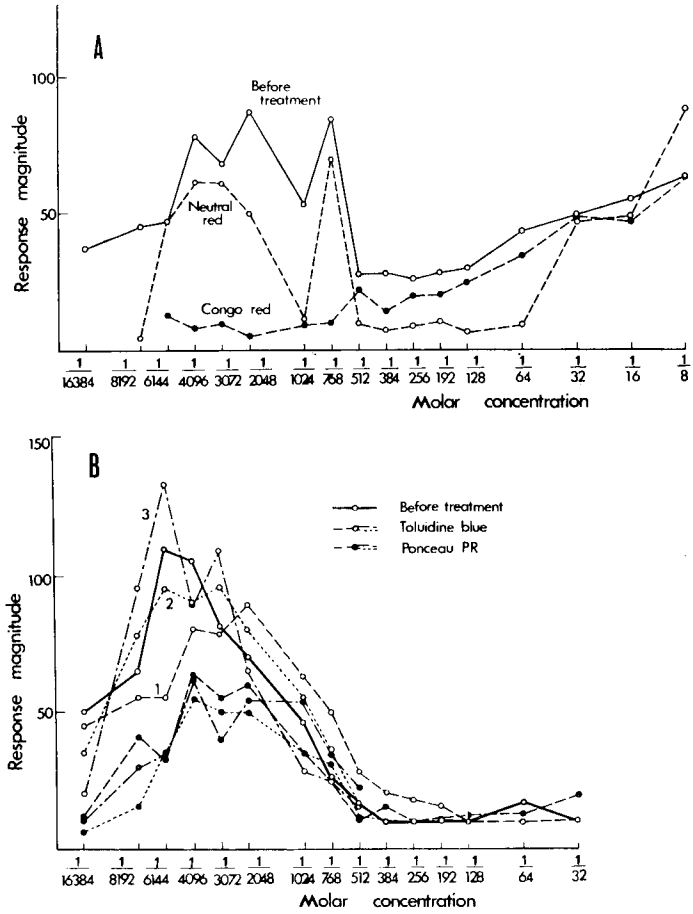


FIG. 9. Effects of previous treatments of chemoreceptors with dye salts on the response to dilute solution of $Na_4Fe(CN)_6$. Dye salt used in A: M/4096 neutral red (pH 5·9) and M/2048 congo red (pH 6·3). Dye salt used in B: 0·0025% toluidine blue (pH 3·8) and 0·005% ponceau PR (pH 5·1). Note enhancement of response by repetition of treatment indicated by numerals on the curves in B. Abscissa: concentration of $Na_4Fe(CN)_6$ solution.

leading to excitation of the chemoreceptors may be the counter ion binding of anions with positively charged radicals of the ampholytes of the receptor membrane. The depression of the responses due to previous alkali treat-

ment may be explained by the prevention of the adsorption of anions due to negative ionization of the receptor macromolecules by the treatment.

The treatment with dye salts led to a similar conclusion. As was demonstrated by Loeb (1924 a) on collodium particles, it would be expected that the film which is formed on the surface of the receptor membrane by previous treatment with dye ions charged oppositely to the membrane would influence the adsorption of other ions. Previous treatment with oppositely

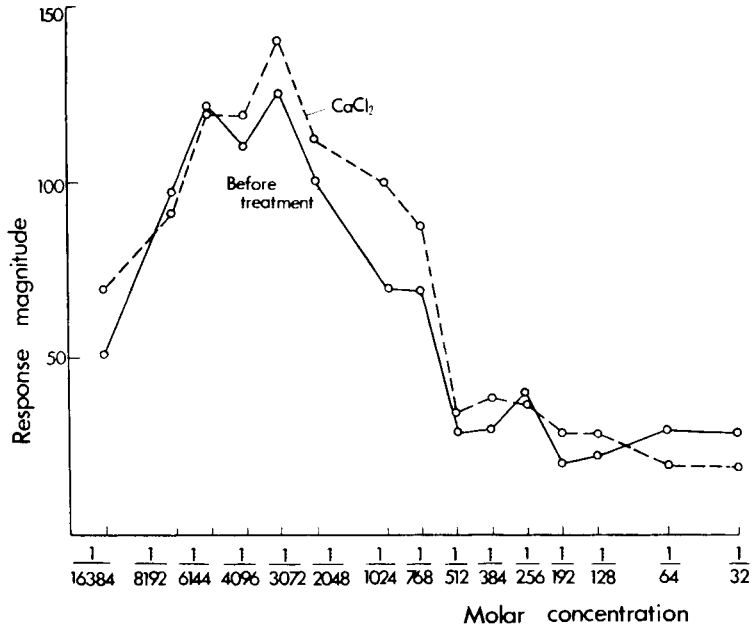


FIG. 10. Effects of previous treatments of chemoreceptors with salts with polyvalent cations (M/2048$CaCl_2$) on the response to dilute solution of $Na_4Fe(CN)_6$. Abscissa: concentration of $Na_4Fe(CN)_6$ solution.

charged dye ions led to the following results: Ponceau PR (dye anion) lowered the stimulation effectiveness of $Na_4Fe(CN)_6$, while toluidin blue (dye cation) had no marked influence though sometimes repeated treatment resulted in an enhancement of the response (Fig. 9). The enhancement of the response due to the repetition of dye cation treatments may be explained by assuming that the binding of dye cations with anion groups of the receptor surfaces promotes adsorption of the stimulus anion. The small depressing effect of the dye anion, as compared with the strong depressing effect of alkali, may be assigned to the partial adsorption of dye anions on to the receptor surfaces, as was suggested by Loeb (1924 a) on collodium

particles. The previous treatment of the receptors with $CaCl_2$ did not significantly influence the stimulation effectiveness of the dilute solution of $Na_4Fe(CN)_6$ and sometimes rather enhanced it (Fig. 10), as in the case of a dye cation. This may be further evidence for positively charged receptor sites.

If the magnitude of chemoreceptor responses to weak electrolyte solutions is assumed to be directly proportional to the amount of chemical stimulant adsorbed to receptor sites, according to the theories of taste stimulation with a high concentration stimulus based upon the assumption that the stimulation process is in thermodynamic equilibrium (Renqvist, 1919; Lasareff, 1922 and Beidler, 1954, 1961), saturation of response must take place with increasing stimulus concentration. Furthermore, it may be expected that a mixture of different electrolytes which stimulate the same receptor produces a larger response than that for an individual electrolyte alone, as was clearly demonstrated by Beidler (1953) on the rat tongue. However, the present experiments on the effects of the addition of supporting electrolytes to the stimulating solution gave different results. The responses of $Na_4Fe(CN)_6$ solutions dissolved in supporting electrolyte (NaCl) solutions in different concentrations were studied. The addition of M/1024 NaCl (NaCl in this concentration produced maximal response) depressed the response to higher concentrations of $Na_4Fe(CN)_6$ and a further increase of NaCl concentration caused progressive depression over a wide concentration range, and the response was completely depressed by the addition of M/128 NaCl (the response to NaCl alone was completely depressed at this concentration) (Fig. 11 A). Despite the fact that dilute solutions of both salts stimulated the same receptor neither an additive effect nor saturation of response by mixture of salts was found, except for the slight additive responses to extremely low concentration of $Na_4Fe(CN)_6$ solution (M/16384 and M/8192) dissolved in a dilute NaCl solution (M/1024). A less likely possibility to account for the depression by addition of a supporting electrolyte is the competitive inhibition (Beidler, 1961); less likely because of the complete depression of the response by addition of a supporting electrolyte of high concentration, and also because stimulation and depression of the response may be produced by oppositely charged ions, as stated later. Effects of a supporting electrolyte on the DWE for $Na_4Fe(CN)_6$ are given in Fig. 11 B. The addition of NaCl caused a shift of the peak concentration of the DWE towards the side of lower concentrations, the more so the higher the concentration of supporting electrolyte. The maximal DWE for NaCl alone at around M/128 is seen at the left in Fig. 11 B (cf. Fig. 5 A). It is apparent from the above results that the

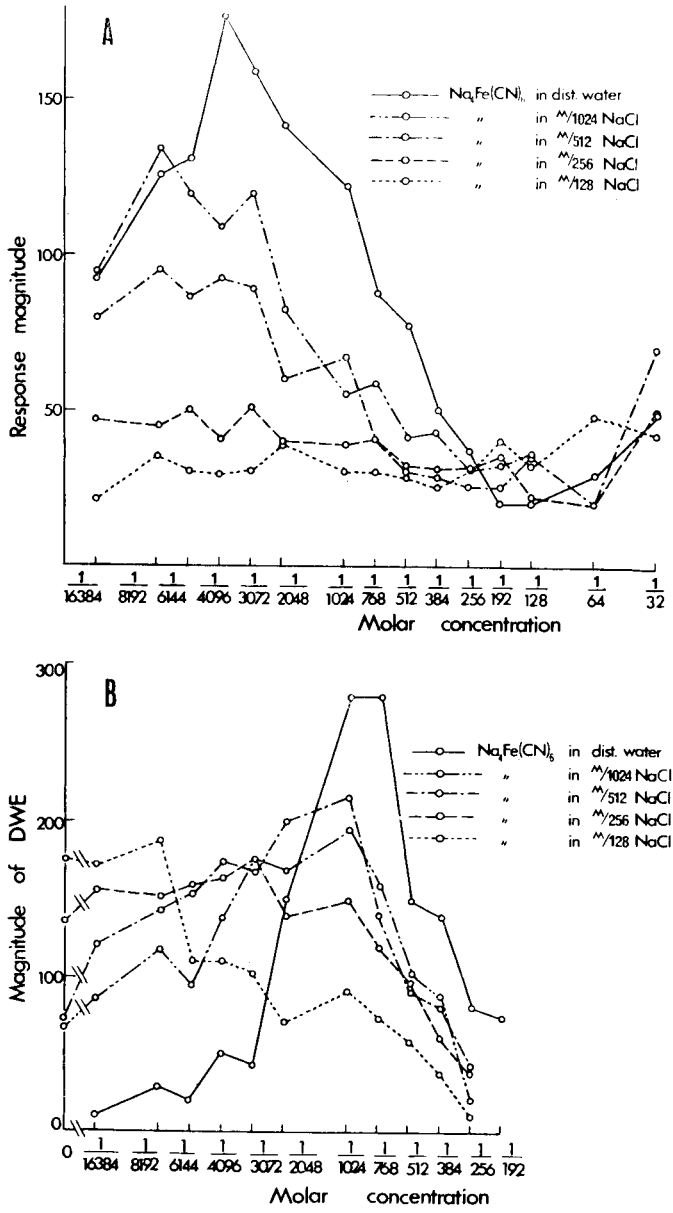


FIG. 11. Influence of uni-univalent supporting electrolyte (NaCl) on the response to dilute solution of $Na_4Fe(CN)_6$(A) and the distilled water effect (B). Abscissa: concentration of $Na_4Fe(CN)_6$ solution.

total ionic strength of the solution determines the chemoreceptor activity, a statement to be confirmed by the following experiment: NaCl of the same molar concentration as that of $Na_4Fe(CN)_6$ which was increased above M/4096, from where depression started to take place, was added to a M/4096 $Na_4Fe(CN)_6$ solution. The results illustrated in Fig. 12 clearly

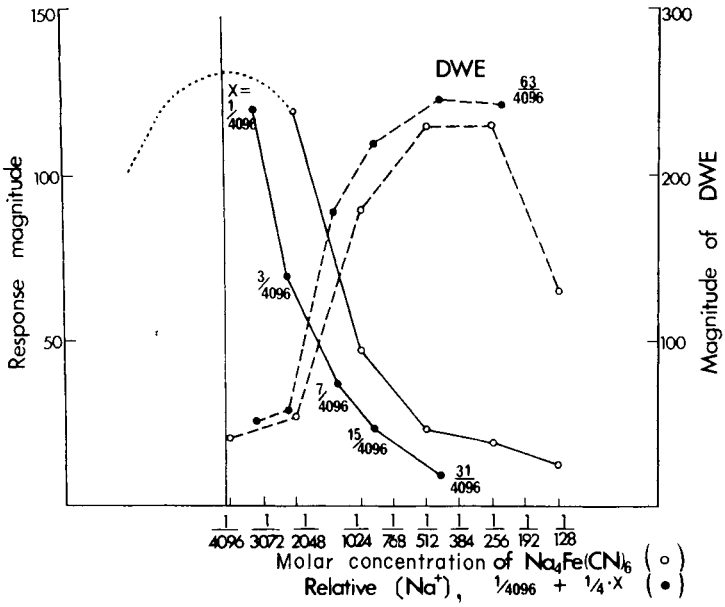


FIG. 12. Comparison of depressions of the response to $Na_4Fe(CN)_6$ (solid curves), produced by increasing concentration of the same salt (open circles) and by addition of NaCl to M/4096 $Na_4Fe(CN)_6$ solution which produces maximal response (closed circles). Broken curves: the distilled water effects. *X*: molar concentration of NaCl added.

demonstrated that an increase of the ionic strength of the solution, independent of the kind of salt, caused a depression of the response. The dependency of the initiation of the DWE on the ionic strength of solution was also demonstrated. Magnitudes of response as well as DWE to mixed solution are plotted in Fig. 12 as a function of relative cation concentration on the same abscissa as that which expresses molar concentrations of the $Na_4Fe(CN)_6$ solution.

Addition of $CaCl_2$ as a supporting electrolyte gave different results (Fig. 13). Addition of this salt in minute quantities markedly depressed the response, but the depression proceeded from the low concentration side with increasing concentrations of $CaCl_2$ oppositely to the effect of

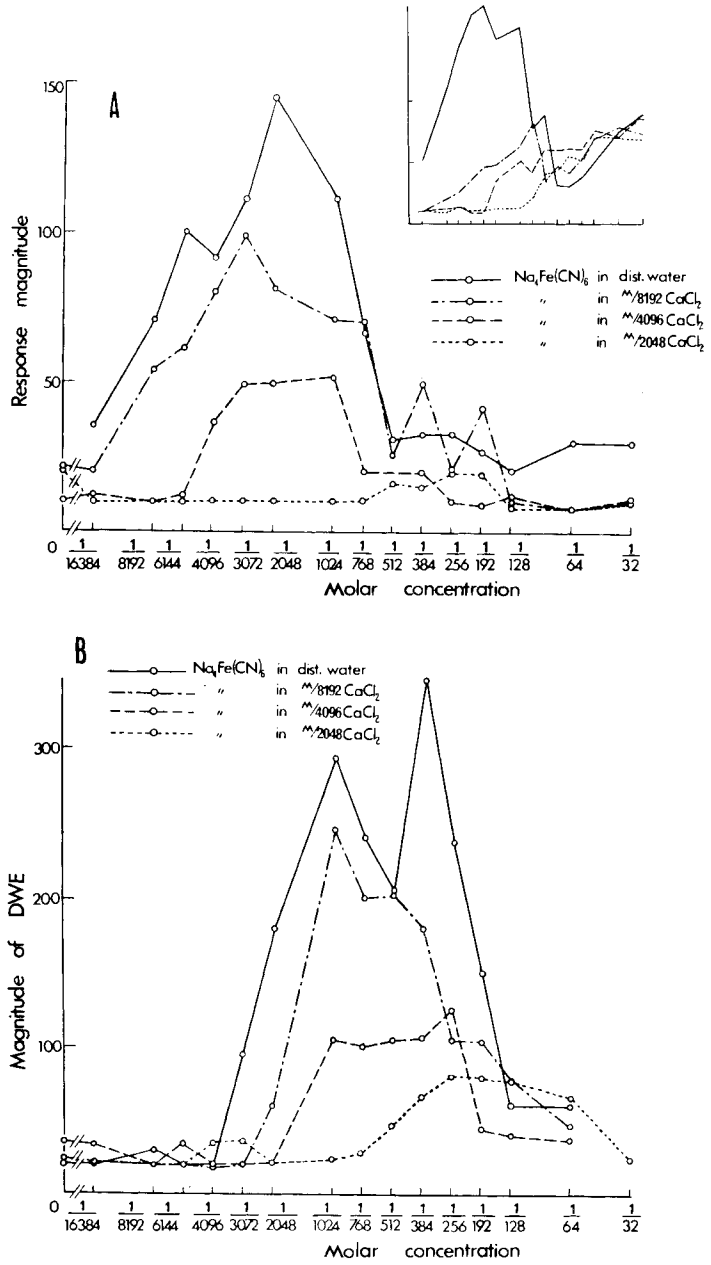


FIG. 13. Influences of supporting electrolyte with divalent cation ($CaCl_2$) on the response to dilute solution of $Na_4Fe(CN)_6$(A) and the distilled water effect (B). Abscissa: concentration of $Na_4Fe(CN)_6$ solution. Inserted figure in A is from another preparation.

the uni-univalent supporting electrolyte. Almost complete depression was observed by adding $CaCl_2$ of M/2048, or weaker.

The above results on the behavior of supporting electrolytes may be helpful in understanding the phenomena which occur at the receptor membrane interfaces in connection with excitation and inhibition of the chemoreceptors. The phenomenon that receptor activity is greatly influenced by the ionic strength of solution, besides the occurrence of adsorption of specific ions to the receptor surfaces, appears to support strongly the hypothesis proposed in the present paper as to the stimulation mechanism of the chemoreceptor by dilute electrolyte solution. It is well known, in the surface chemistry of colloids, that the increase of ionic strength in the bulk aqueous phase at the surface of colloidal particles diminishes the Stern potential (or ζ potential) produced by specific adsorption of a specific ion on to the surface. The diminution results either from the increase of the concentration of an electrolyte or from the addition of a large amount of indifferent electrolyte to the sol. By introducing a similar concept to that in the theory of the electrical double layer, established in surface electrochemistry, it seems possible to explain the above results. The results presented here will be considered assuming that the magnitude of response is dependent upon the ζ potential at the receptor surfaces. The additive responses to extremely low concentration of $Na_4Fe(CN)_6$ solutions dissolved by weak NaCl solution (see Fig. 11 A) may be explained by assuming that the adsorption of both $Fe(CN)_6^{4-}$ and Cl^- ions contributes to raiising the ζ potential. The depression of the response in the region of higher concentration caused by the addition of NaCl may be explained by the diminution of the ζ potential due to a further increase of the ionic strength of the solution compared to that which it had before addition of supporting electrolyte.

The strong depressing effect of a salt with a polyvalent cation (valency effect) observed when it is added as a supporting electrolyte strongly suggests that the depression of response with increasing ionic strength may be due to a screening effect caused by the approach of cations to the inner layer of the double layer of the receptor (diminution of the ζ potential due to a decrease in the double-layer thickness). Such a screening is effective because the cations have a charge of sign opposite to the specifically adsorbed ion, namely, anions. The data on the DWE, described later, strengthen the possibility presented above. If we suppose a strong depressing action of polyvalent cations, it is to be expected that the depression of the response to $Na_4Fe(CN)_6$ by $CaCl_2$ prominently takes place at the dilute concentration region of the former salt, in contrast to the case of

the uni-univalent supporting electrolyte. If the curve of the depression obtained by the addition of NaCl to the $Na_4Fe(CN)_6$ solution of the concentration at which maximal response is obtained is plotted against cation concentration, it should be expected to show almost agreement with the curve obtained when increasing the concentration of $Na_4Fe(CN)_6$ alone,

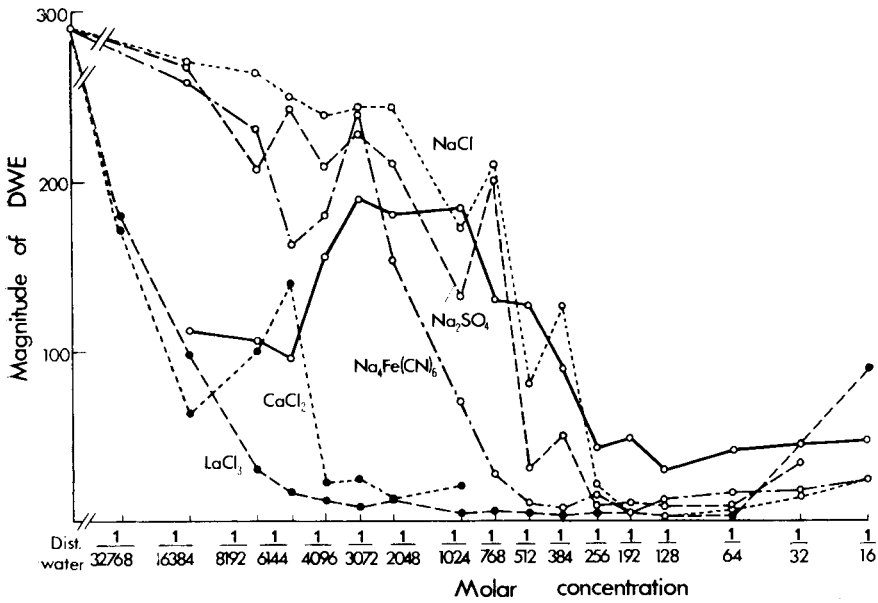


FIG. 14. Depression of the distilled water effect by various electrolytes. Each point on dotted, broken and chain curves represents magnitude of the distilled water effect produced by the application of an electrolyte solution in the concentration indicated on abscissa, instead of distilled water, after previous adaptation to M/256 $Na_4Fe(CN)_6$. Solid curve represents the response-concentration curve for $Na_4Fe(CN)_6$ plotted on the same abscissa.

if indeed the depression is determined by cation. Although we have not sufficient evidence to answer the question why the depressing effect by NaCl addition exceeds that obtained with $Na_4Fe(CN)_6$ itself, we may attempt to explain it as follows: The stimulating action of $Fe(CN)_6$ ion is stronger than that of Cl ion. If the actual depression is assumed to occur in consequence of a balance of the antagonistic actions of cation and anion, the above-mentioned phenomenon would indeed occur.

Further evidence for the support of the proposed hypothesis will be obtained by analyzing the DWE: The application of tap water instead of

distilled water produces only a small DWE, suggesting a depression of the DWE by minute amounts of electrolytes dissolved in it. The effects of varying concentration of different inorganic electrolytes on the depression of the DWE for previous adaptation to M/256 $Na_4Fe(CN)_6$ were investigated. As can be seen from Fig. 14, the strongest inhibition is produced by polyvalent cation salts. The DWE is completely depressed at M/6144 in $LaCl_3$

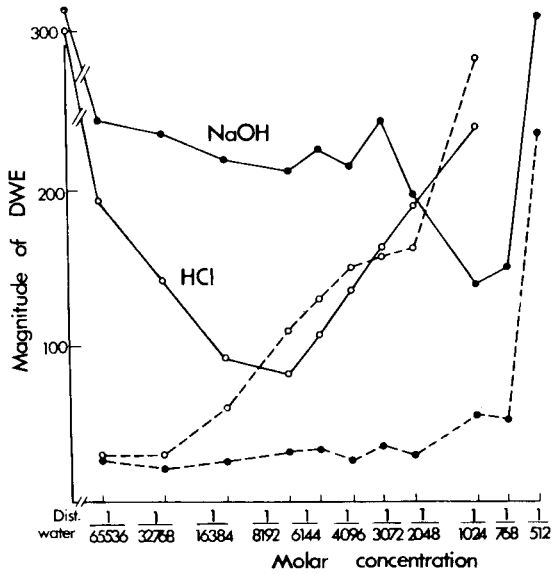


FIG. 15. Depressions of the distilled water effect by acid and alkali. Each point on solid curves represents magnitude of the distilled water effect produced by the application of solution of HCl or NaOH in the concentration indicated on abscissa, after previous adaptation to M/256$Na_4Fe(CN)_6$. Broken curves represent the response-concentration curves for HCl and NaOH.

and at M/4096 in $CaCl_2$. The three sodium salts can be arranged in increasing order of depressing effectiveness as follows: $Na_4Fe(CN)_6 > Na_2SO_4 > > NaCl$. Complete depression is found at M/512 in $Na_4Fe(CN)_6$, M/256 in Na_2SO_4 and at around M/192 in NaCl, demonstrating a normality relationship in this case too. It is of importance to note that these values coincide exactly with the respective concentrations at which the magnitude of the DWEs for these salts reach a maximum. The above results mean that the depressing action of three sodium salts is almost the same for the same concentration of cations, regardless of the anion, while the actions of $CaCl_2$ and $LaCl_3$ are 21 and 32 times as great respectively as that of NaCl.

This leaves no doubt that the depressing effect is due to cation. Furthermore, the results of the DWE depression by acid and alkali may support the view stated above, indicating stronger depressing action of H^+ than OH^- ions (Fig. 15). A similar depression was also observed with an organic electrolyte such as quinine-HCl, but a non-electrolyte like sucrose failed to depress the DWE; in other words, sucrose solution had the same effect as distilled water (Fig. 16) inasmuch as M/4 sucrose occasionally

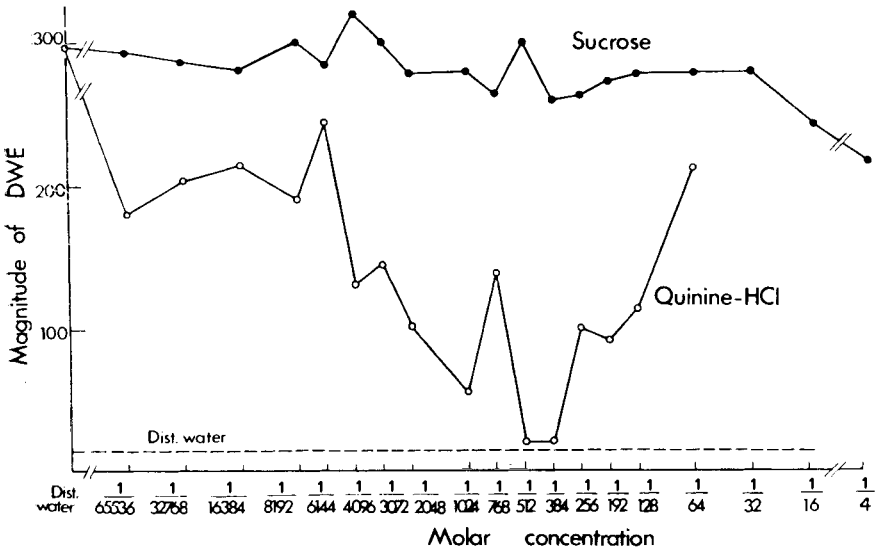


FIG. 16. Depressing effects of quinine-HCl and sucrose on the distilled water effect. Each point represents magnitude of the distilled water effect produced by the application of solution of quinine-HCl or sucrose in the concentration indicated on abscissa, after previous adaptation to M/256 $Na_4Fe(CN)_6$.

behaved like distilled water. As described before, the DWE has its origin in the activity of the same receptors as those stimulated by extremely dilute electrolyte solutions, and is elicited when the receptor activity to salt is depressed by increasing the ionic strength of the solution. These facts will lead one to assume that the reduction of ionic strength in the outer region of the double layer at the receptor surfaces due to washing with distilled water may produce the DWE. The ζ potential at the solid/liquid interface attains a maximum at a certain concentration which is determined by both factors, the charge density of the Stern plane determined by the specifically adsorbed ions and the ionic strength of the solution (Watanabe *et al.*, 1961 a, b; Watanabe, 1964). It also decreases again with

an increase of the latter. Under the conditions where the potential is depressed by high ionic strength, if the ionic strength suddenly falls on account of distilled water, the potential will naturally rise again. It seems not unreasonable to interpret a possible mechanism of the DWE by a similar concept to that which was advanced to explain the reaction to dilute electrolytes. In particular, the above experiments clearly showed that the de-

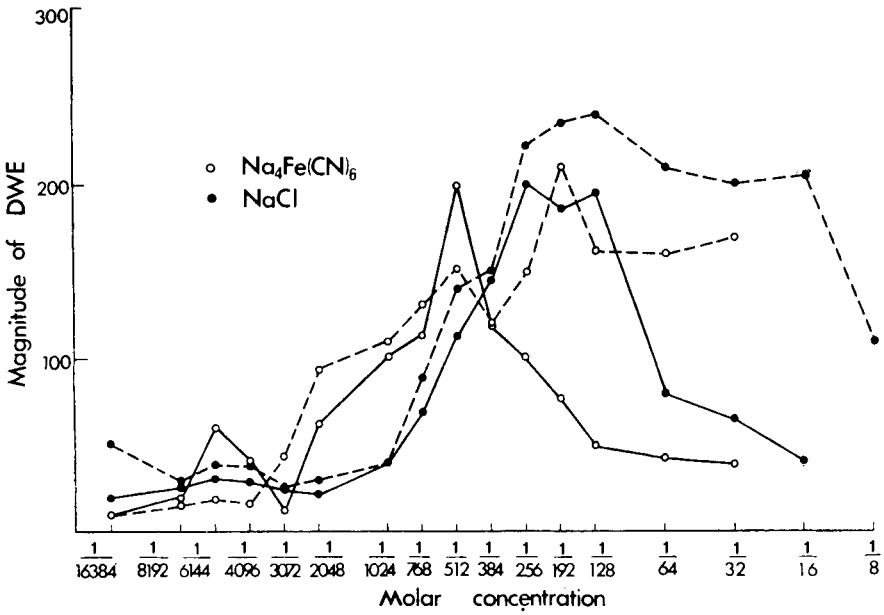


FIG. 17. Comparison of the distilled water effects produced by small amount (solid curves) and continuous (broken curves) flows of distilled water, after previous adaptations to $Na_4Fe(CN)_6$ and NaCl solutions of the concentrations indicated on abscissa.

pression of the DWE by salts was due to the cation. As has been stated, the depression of response caused by the increase of ionic strength of salt solutions might be also due to the screening effect of cations. These observations strongly suggest that the effective removal of cations from the receptor interfaces may lead to initiation of the DWE. It is usually considered that cations, owing to their strong hydration, adhere to solid/liquid interfaces without losing water of hydration. Therefore, it seems possible that cations are easily removed from the receptor surfaces by washing. The DWE usually starts to appear beginning with the concentration of the salt solution previously applied, where the maximal response to the same salt it obtained (M/4096 in $Na_4Fe(CN)_6$, for instance); (see Fig. 5).

In view of the above postulated mechanism of the DWE it may be presumed that the chemoreceptor responds with maximal magnitude under the inhibitory influence of cations. It should be recalled here that normality relationships were seen among the peak concentrations of the response–concentration curves for different sodium salts.

Rapid and efficacious removal of cations may be necessary to provoke a DWE. It is natural that there is a limit to the rate of their effective removal by washing, and also that the rate becomes smaller the higher the ionic

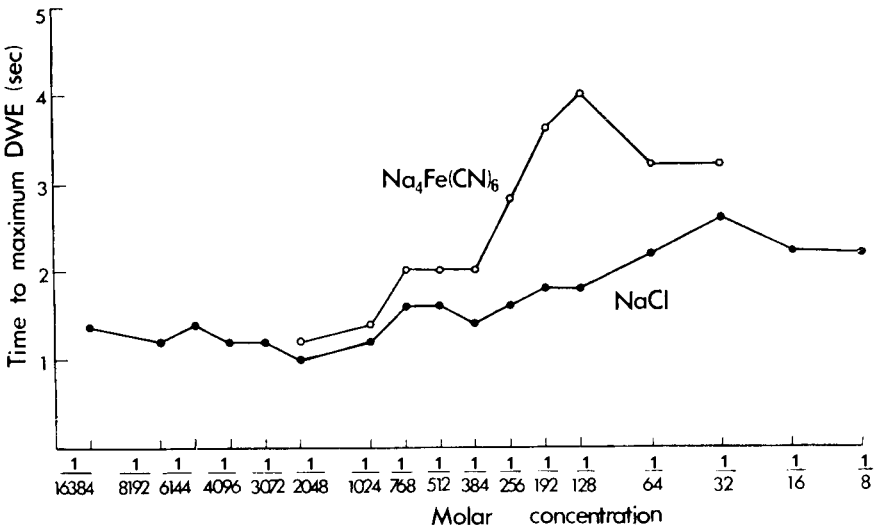


FIG. 18. Time to maximum magnitude of the integrated distilled water effect, produced by continuous flow of distilled water, after previous adaptation to salt solutions in the concentration indicated on abscissa.

strength of the solution previously applied. This may be a possible reason that the DWE vs. concentration curve produces a peak and the normality dependency of the peak of the DWE curve may be well explained by the above idea. No appreciable differences between the DWE curve obtained by applying small amounts of distilled water (1 ml) and by continuous application were seen at least until the DWE curve reached a maximum (Fig. 17). However, despite diminution of the DWE by small amounts of water after previous adaptation to the salt solution of higher concentration, continuous application of distilled water still produced a large DWE. However, when the salt concentration was further raised, the DWE hardly occurred even on continuous application, independent of flow rate. The time interval between onset and maximum of the DWE integrat-

ed record obtained by persistent flow of distilled water lengthens above about M/512 with $Na_4Fe(CN)_6$ and above about M/128 with NaCl (Fig. 18), indicating that the application of large quantities of distilled water is necessary to produce the DWE when the concentration of the salt solution is raised above the concentration where a maximal DWE is obtained by applying small amounts of water. This further suggests that the above values for the two salts are the critical concentrations which elicit the DWE most efficiently.

Opposite effects on the chemoreceptor activities of a polarizing current with the same polarity at opposite regions of salt concentration range were demonstrated. As shown in Fig. 19, the application of an anodal current (the polarizing electrode on the palatal organ is the anode) markedly lowers the threshold for the initiation of the fast type response, which is usually produced by hypertonic $Na_4Fe(CN)_6$ solution, and greatly enhances the response magnitude with raising of the concentration of the solution, while it depresses the slow type responses in the low concentration region. Thus, two response components are seen in the integrated records at moderate stimulus concentrations of $Na_4Fe(CN)_6$ solution. Yet, the fast type response in the high concentration region is completely depressed under cathodal current (the polarizing electrode on the palatal organ is the cathode), and no appreciable change or occasional enhancement in magnitude of the slow type response to low stimulus concentration below M/512 is observed. These findings give positive proof of two distinct receptor systems which respond in opposite sensitive regions. Under anodal current, the magnitude of the response to $CaCl_2$ which always produces only the fast type response in the high stimulus region is markedly augmented. This is just similar to the situation observed on the $Na_4Fe(CN)_6$ fast type response. In view of these findings, it seems most likely that cations may be chiefly concerned in the response which appeared in the high stimulus region of the response–concentration curve. This appears to be consistent with Beidler's findings (1954).

A hypothesis postulated in this paper on the mechanisms of stimulation of the chemoreceptors found in freshwater fish, which respond specifically to dilute inorganic electrolytes is summarized as follows: The reacting surfaces of the receptors are positively charged. The specific adsorption of anions on to the receptor surfaces advances with increasing concentration of a salt solution and thus renders the electrokinetic potential, which will appear at the receptor surfaces, more and more negative. This effect would be the larger the higher the valency of the anion. The occurrence of such a potential may trigger some secondary ionic changes at the receptor

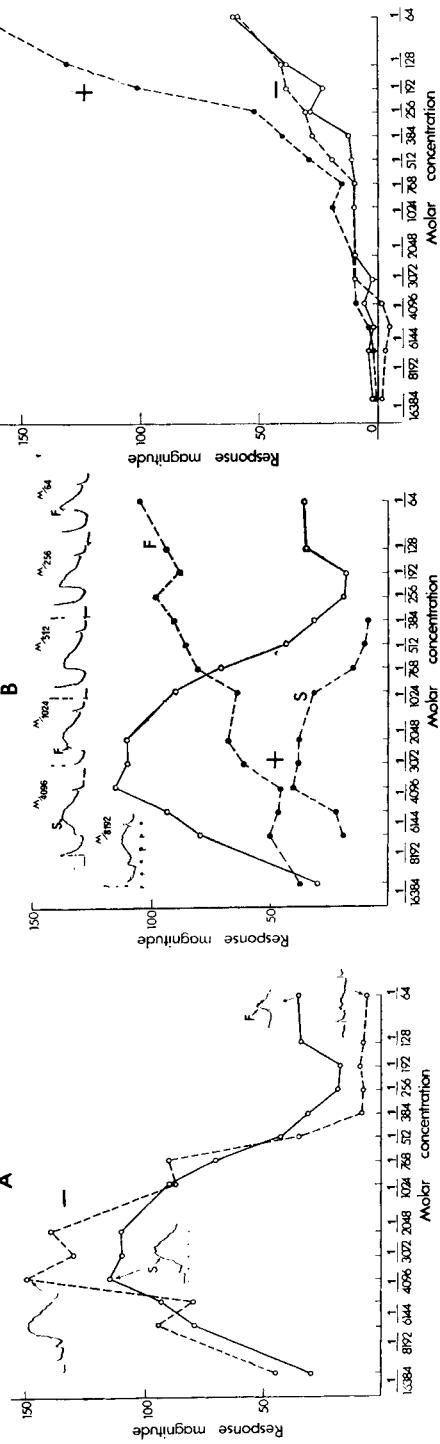


FIG. 19. Effect of polarizing current on chemoreceptor response. (A), effect of cathodal current (25 μA) on the response to $Na_4Fe(CN)_6$. (B), effect of anodal current (25 μA) on the response to $Na_4Fe(CN)_6$. (C), effects of cathodal and anodal currents (15 μA) on the response to $CaCl_2$. Solid curves: before polarization. *S*: slow type response. *F*: fast type response. Time mark one second.

membrane which lead to the excitation of the receptors. If the concentration of the salt solution is increased, the diminution of the potential due to the screening effect of the cation may take place and result in a depression of the response. We have no direct evidence, but it seems possible that ionic movements brought about by certain electrokinetic driving force may be involved in the stimulation of the chemoreceptors by extremely dilute electrolyte solution. In this connection, Teorell's theory (1959 a, b, 1962) is very attractive because it helped to analyze the behavior of excitable tissues in terms of electrokinetic properties.

ACKNOWLEDGEMENTS

The author gratefully acknowledges the much valued advice of Dr. A. Watanabe (Kyoto University) and Dr. M. Sato (Kumamoto University) and the kind criticism of Prof. Y. Zotterman (Veterinärhögskolan, Stockholm) and Dr. J. E. Bardach (University of Michigan).

This research was supported by a research grant from the National Institutes of Health, U.S. Public Health Service (5 R01 NB04727-02).

REFERENCES

BEIDLER, L. M. 1953. Properties of chemoreceptors of tongue of rat. *J. Neurophysiol.* **16,** 595–607.

BEIDLER, L. M. 1954. A theory of taste stimulation. *J. gen. Physiol.* **38,** 133–139.

BEIDLER, L. M. 1961. Taste receptor stimulation. *Progress in Biophysics and Biophysical Chemistry.* **12,** 107–151.

COHEN, M. J., HAGIWARA, S. and ZOTTERMAN, Y. 1955. The response spectrum of taste fibres in the cat: A single fibre analysis. *Acta Physiol. Scand.* **33,** 316–332.

FURUTANI, N. 1929. Measurements of electrokinetic potential and thermodynamic potential. *Butsuri-Kagaku No Shimpo.* **3,** 17–41.

KAKIUCHI, S. 1930. *Cellular Biochemistry* (in Japanese). Tokyo. Iwanami.

KONISHI, J. and ZOTTERMAN, Y. 1963. Taste functions in fish. In *Olfaction and Taste* ed. Y. Zotterman, 215–233, Oxford. Pergamon Press.

KONISHI, J. and NIWA, H. 1964. Some properties of taste receptors in freshwater fish: Responses to weak solutions. *Jap. J. Physiol.* **14,** 328–343.

KRINNER, M. 1935. Über die Geschmacksempfindlichkeit der Elritzen. *Z. vergl. Physiol.* **21,** 317–342.

LASAREFF, P. 1922. Untersuchungen über die Ionentheorie der Reizung. III. Mitteilung. Ionentheorie der Geschmacksreizung. *Pflügers Arch. f. d. ges. Physiol.* **194,** 293–297.

LOEB, J. 1922–23 a. The influence of electrolytes on the cataphoretic charge of colloidal particles and the stability of their suspensions. I. Experiments with collodion particles. *J. gen. Physiol.* **5,** 109–126.

LOEB, J. 1922–23 b. The influence of electrolytes on the cataphoretic charge of colloidal particles and the stability of their suspensions. II. Experiments with particles of gelatin, casein, and denatured egg albumin. *J. gen. Physiol.* **5,** 395–413.

LOEB, J. 1924 a. On the location of the forces which determine the electrical double layer between collodion particles and water. J. gen. Physiol. **6,** 105–129.

LOEB, J. 1924 b. Proteins and the theory of colloidal behavior. New York. McGraw-Hill.

RENQVIST, Y. 1919. Über den Geschmack. *Skand. Arch. Physiol.* **38,** 97–201.

TEORELL, T. 1959 a. Electrokinetic membrane processes in relation to properties of excitable tissues. I. Experiments on oscillatory transport phenomena in artificial membranes. *J. gen. Physiol.* **42,** 831–845.

TEORELL, T. 1959 b. Electrokinetic membrane processes in relation to properties of excitable tissues. II. Some theoretical considerations. *J. gen. Physiol.* **42,** 847–863.

TEORELL, T. 1962. Excitability phenomena in artificial membranes. *Biophysical J.* **2,** No. 2, 27.

VERWEY, E. J. W. 1935. The electrical double layer and the stability of lyophobic colloids. *Chemical Rev.* **16,** 363–415.

WATANABE, A., TSUJI, F. and UEDA, S. 1961 a. On the influence of surface active agents on ζ-potentials. Denki-Kagaku. **29,** 777–782.

WATANABE, A., TSUJI, F. and UEDA, S. 1961 b. Study on surface electricity. VIII. On the influence of surface active agents on ζ-potentials. *J. Electrochem. Soc. Japan.* **29,** No. 4, 236–240.

WATANABE, A. 1964. The coagulation of colloidal powders. *J. Japan Soc. Powder Metallurgy.* **11,** No. 2, 73–96.

OBSERVATIONS ON THE TASTEBUDS IN SUNCUS MURINUS

L. W. CHACKO, K. J. R. NAIDU and B. K. MUKHOPADHYAYA

Department of Anatomy,
All India Institute of Medical Sciences,
Ansari Nagar, New Delhi,
India

OBSERVATIONS ON TASTEBUDS IN THE COMMON SHREW (SUNCUS MURINUS)

Information on tastebuds in Insectivores has been meagre in the earlier accounts of their tongue (Wyss, 1869; Tuckermann, 1890; Boeke, 1913). Suncus murinus, being a primitive mammal, lends itself for a profitable study of the morphology of tastebuds, their relation to the surrounding epithelium, their cellular composition and differentiation, their behaviour after nerve lesion and colchicine injection and the phylogeny of tastebuds in vertebrates.

The circumvallate papillae in this animal are two in number situated at the basal angles of the vallate triangle and rarely a third at its apex. The papilla has a characteristic contour of an hour glass with a constriction in the middle between non-gustatory area above and a gustatory bulge below where the tastebuds are arranged in a radial manner in three to five tiers with a total of 80–150 on one papilla (Fig. 1). Occasionally a solitary tastebud of a diminutive type appears on its dorsal surface. The outer trench wall is devoid of tastebuds except for an occasional one. Ducts of mucous glands open on the general lingual surface. About 15–18 ducts of serous glands open on the floor of the trench. The fungiform papillae appear in three rows along each lateral border of the tongue and are also irregularly scattered on the dorsum. They number about 60 in total carrying one tastebud each on its summit.

Each tastebud differentiates in particular relation to its surrounding epithelium. Figure 2 shows an epithelial ridge differentiated into a tastebud with a capsule of peripheral cells around it. Another alternative site of differentiation suggested from Fig. 3 is the epithelium on the summit

of a dermal papilla, as also in fungiform papillae, from where the tastebuds grow down to invaginate the connective tissue below. In both types a connective tissue space carrying networks of capillaries surrounds the lower

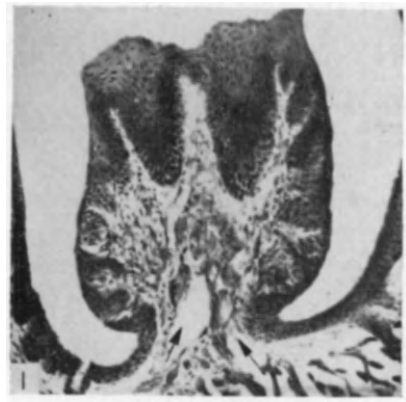


FIG. 1. Circumvallate papilla of the tongue of Suncus murinus. 150 ×.
Note the dorsal nongustatory and ventral gustatory epithelium containing tastebuds, the ganglion cells in the core and nerve bundles passing through the pedicle and arborizing among them. To the left are nerves proceeding towards the gustatory and non-gustatory epithelium. Note the arrows in the large blood vessels at the pedicle and others intimately related to the ganglion; still other large vessels are seen proceeding in all direction inside the papilla. A duct of serous gland is seen to open into the floor of the trench.

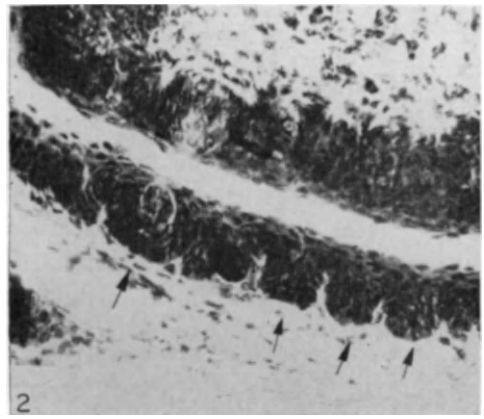


FIG. 2. Gustatory epithelium of the circumvallate papilla above and ridged epithelium of the outer trench wall below. 360 ×. In one of the ridges (the extreme left arrow) a tastebud has developed.

part of the tastebud, the distal part being set in the epithelium itself. Tracing the cells of tastebuds in serial sections, three morphological types were detected: firstly the centrally placed cells with large round or oval, lightly stained vesicular nuclei, differentiated from germinal cells of the base of the epithelial ridge; secondly, the peripherally placed cells with rod shaped, more deeply stained nuclei, derived from germinal cells of the sides of the ridge and lastly the cover cells which are undifferentiated germinal cells forming a capsule around.

The connective tissue core of the circumvallate papillae is a very vascular structure with large thin walled vessels passing through the pedicle terminating in intercommunicating sinusoidal channels of about 15 μ

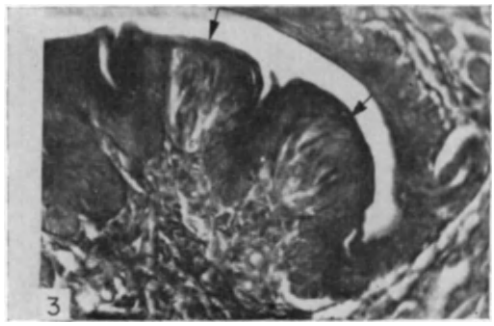


FIG. 3. Gustatory epithelium. 360 $\times$. Tastebuds seen between epithelial ridges at the arrows.

in diameter besides capillary network. Intimately related to the wide sinusoidal network are the ganglion cells 23–77 in number and 20–25 μ in diameter located in the centre of the papilla. The eccentric position of the nucleus with single or double nucleolus and fine Nissl granules characterize them. Satellite cells are few and irregularly placed around each cell. Nerve bundles pass through the pedicle of the papillae and each divides into two main branches, one arborizing among the ganglion cells and then proceeding mainly to supply the walls of the blood vessels and the other turns laterally towards the gustatory epithelium. The basement membrane on the deep surface of the tastebud is interrupted for the passage of gemmal nerves. A fine network and branching of the nerves are noted at the base of the tastebud, fine nerves are seen among the cells and others proceeding in the direction of the tastepore.

EFFECTS OF GLOSSOPHARYNGEAL NERVE LESION ON THE CIRCUMVALLATE PAPILLAE AND TASTEBUDS

Besides overall reduction in size of the papillae and apparent thinning of non-gustatory epithelium after the nerve lesion, there was a reduction in the number of tastebuds to about two-thirds of the original during the first three days and till the 6th day the degeneration of tastebuds proceeded with little further reduction in number. On the 7th day about 50% remained. On the 8th day all of them disappeared except rarely one or two unhealthy looking ones persisting for a time.

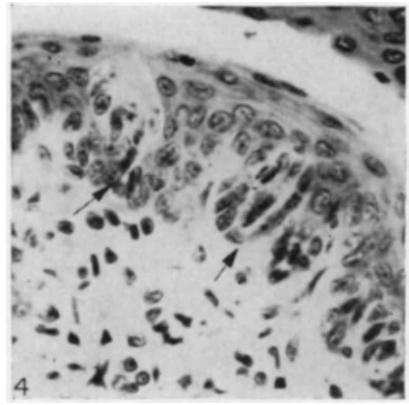


FIG. 4. Gustatory area of circumvallate papilla 3 days after glossopharyngeal nerve lesion showing early stage of cell nest formation (arrows). 600×. New cell-formation at the base of tastebud (left arrow).

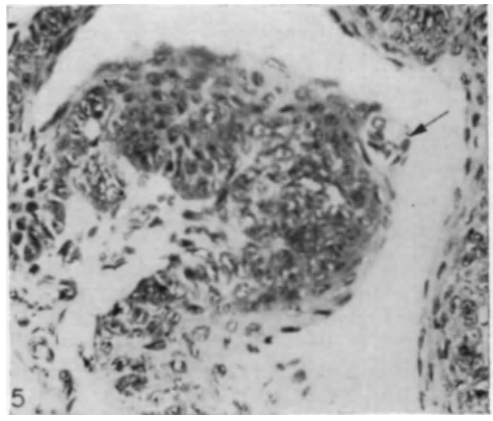


FIG. 5. A degenerated cell nest of tastebud being extruded (arrow). 360×. New formation of cells at the base of tastebud pushes it to the free surface.

Cells showing pyknosis and fragmentation of the nuclei formed cell nests which became gradually reduced in size (Fig. 4). Mitotic activity and accumulation of new cells were evident under the basal region of the tastebuds and these pushed the degenerating tastebuds towards the free surface of the epithelium (Fig. 5). The shrinkage of tastebuds was most marked on the 7th day. No leucocytic infiltration was observed. After the tastebuds are disposed off on the free surface, the remaining epithelial cells get organized. By the 8th to 10th day the gustatory epithelium is converted into the non-gustatory type with dermal papillae and epithelial ridges (Fig. 6).

The ganglion cells showed very slow response of degeneration to nerve lesion during the period of active degeneration of tastebuds when they

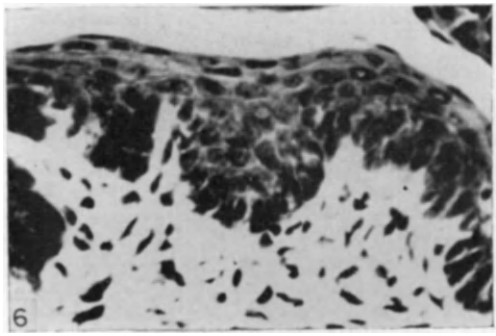


FIG. 6. Remains of gustatory epithelium organized into non-gustatory epithelium 28 days after the nerve lesion.

appeared healthy with slight reduction in their size.

The glands showed definite degenerative reaction during the period of tastebud degeneration.

COLCHICINE TECHNIQUE IN THE STUDY OF CELL DIFFERENTIATION IN TASTEBUDS

In our study attention was directed to the cell reaction in tastebuds both during the period of mitotic block and after colchicine effect has worn out leading to regeneration of tastebuds so that a clue to the origin of different types of cells in tastebuds might be looked for. The general single dose of 1 mg per kg body weight was given intraperitoneally to adult

shrews (ages of which were not determined) and also in young rats weighing 25–30 mg. The preliminary results are briefly given below.

In young rats, metaphase figures appeared in one hour (1–2 numbers) and increased in 2 hours to 7–10 followed by a short period of decrease;

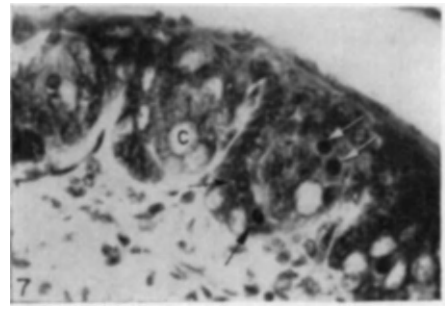


FIG. 7. Gustatory epithelium in Suncus murinus 5 hours after colchicine injection. The characteristic large unstained degenerated cells of the tastebuds marked *C*. Four metaphase figures related to one tastebud (arrows). Tastebud cells swollen and disrupted.

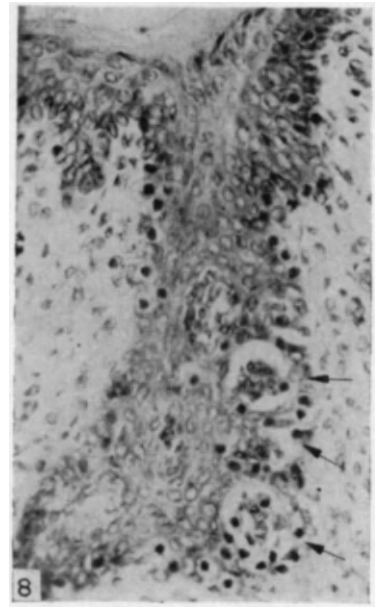


FIG. 8. The gustatory epithelium 18 hours after colchicine injection in young rat. Tastebuds showing circle of peripheral cells enclosing 5–10 cells; maximum number of metaphase figures seen at this stage 360 ×.

from the 5th hour onwards a steady increase of those figures were noted reaching a number of 50 on one-half of the gustatory epithelium in one section at 18 hours. From 24 hours onwards there was marked reduction in these figures. With regard to the cell behaviour, at 5 hours there was loss of cohesion and compactness of the cells. Unhealthy cells with large unstainable nuclei appeared (Fig. 7). Between 5 and 12 hours the tastebud cells were quite dispersed and the outline of the organ less definable. There was a general reduction in cell density in and around the tastebuds; from 12 to 18 hours this reduction was marked with maximum increase in metaphase figures (Fig. 8); at 24 hours the cells were arranged into a single layered peripheral circle enclosing about 5–10 cells in the centre. The nuclei of

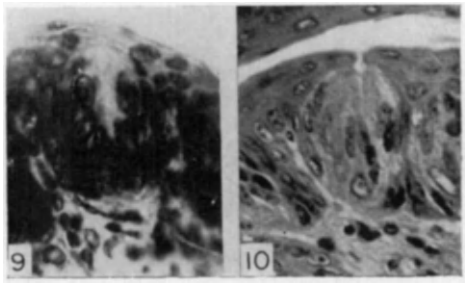


FIG. 9. A regenerated tastebud in shrew 72 hours after colchicine injection. The cells are younger and healthier, formed in relation to the taste canal and pore 600 ×.

FIG. 10. Normal tastebud in shrew-longitudinal section 500 ×.

these cells were round or oval and appeared healthier. During the 2nd day the tastebuds began to resume their position and identity. From the third to the fifth day the normal contour of the tastebuds was restored and the nuclei of the differentiated cells were of the round or oval variety. It appeared that the tastepore was not destroyed and the new tastebud was formed in relation to it. In the newly formed tastebud the pore had very few cell nuclei around it (Figs. 9 and 10).

Silver staining of circumvallate papillae at 1, 5 and 10 hour periods during colchicine reaction showed that nerves leading to the tastebuds have been intact in contrast to the complete disappearance of nerves in nerve lesion.

The glandular ducts and acini displayed a high degree of proliferative activity, destructive reaction followed by regenerative changes.

In shrews the period of rise in metaphase figures extended to 30 hours

and at 48 hours the number decreased markedly. Anaphase figures appeared again in 54 hours implying that mitosis block was removed and process of division could proceed to anaphase from about 24 to 54 hours.

PHYLOGENETIC DIFFERENTIATION OF TASTEBUDS AS OBSERVED IN FROG TADPOLES, FROGS, LIZARDS AND MAMMALS

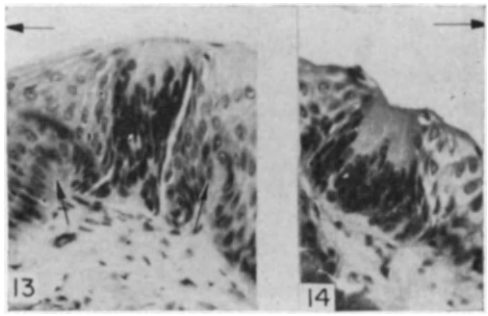
The typical mammalian tastebuds are not seen in lizards and frogs. Frog tadpoles which are discriminative in the choice of food, do not show any tastebud on the tongue or mouth epithelium. The membrane guarding the gill chamber from the mouth cavity, however, is studded with epithelial

FIG. 11. Tastebuds in the mucous membrane of the gill chamber of frog tadpole. The bulbous contour of the organ; its well demarcated free surface is a circular depressed surface (arrow) 150 ×.

FIG. 12. The "taste disk" (arrow) in the glandular epithelium of the dorsum of the frog tongue. Note stratified columnar epithelium with a striated border. 120 ×.

organs resembling undifferentiated tastebuds (Fig. 11). This and the well developed olfactory epithelium would subserve their descriminative abilities. The frogs have the so-called "taste disks" irregularly distributed over the glandular epithelium on the dorsum of the tongue. They are made up of stratified columner epithelium with striated border. There is no taste pore but a circular area of the free surface of the constituent cells. The shape and cellular character of these organs are illustrated in Fig. 12. In lizards (Gecko) the tastebuds are seen on the floor of the mouth. These are conical or cylindrical organs. Two types of cells are distinct in this organ besides the germinal cells; cells with vesicular round nuclei are centrally and basally placed and those with characteristic deeply stained, long, rod-shaped nuclei are situated more peripherally. Distinct coarse cilia or

striated border probably due to presence of microvilli were present. The superficial surface of the organs formed a circular shallow depression. In one instance, a very large pore was seen.



FIGS. 13 and 14. Tastebuds in lizard (Gecko) of pyramidal shape with truncated apex (300 ×). The deeply stained rod-shaped nuclei of cells are peripherally situated and cells with round or oval, less deeply stained nuclei are basally and centrally placed. Connective tissue dip into the epithelium around the lower part of the organ (arrows). The cover cells are distinguishable in relation to it.

COMMENTS

An account of the distribution of the circumvallate and fungiform papillae of the tongue and morphology of their tastebuds in shrew is presented here. As the tastebuds are more differentiated in mammals (Neal and Rand, 1936) a primitive mammal like Suncus murinus reveals certain fundamental features of the morphology of tastebuds. Two circumvallate papillae observed in shrew are also seen in Gymnura Rafflessii. The rare occurrence of a third brings this animal in line with the majority of insectivores and lower orders of primates. The occasional presence of tastebuds in a diminutive form on the dorsal surface of the circumvallate papillae in the shrew is a passing phase as these disappear when tastebuds develop on the gustatory area. The distribution of fungiform papillae in three definite lateral rows is characteristic of the shrew. These papillae and those scattered on the dorsum of the tongue, carrying one tastebud each, lends a wide surface area for taste discrimination.

The connective tissue core of the circumvallate papillae deserves special mention in view of its richly innervated vasculature in very intimate relation to the ganglion cells and the distinct nerve bundles, one arborizing around the ganglion cells and the others supplying gustatory epithelium. The cells of the ganglion have the features of the autonomic type. Similar groups

of ganglion cells scattered among glandular acini are most proablyb parasympathetic secretomotor and vasodilator in function whereas the group carried well into the core of the circumvallate papilla with no glands around suggests its role in vasal control in the mechanism of taste. The slow degeneration of these after nerve lesion suggests that these can exist for a longer period without the impact through the glossopharyngeal nerve than the tastebuds can.

The tongue of shrew is well provided with mucous and serous glands. The mucous secretion flushes the trench wall from above; the serous secretion flushes it from below. These glands undergo degenerative changes during the period of tastebud degeneration and are dependent on their nerve supply for their maintenance. In colchicine reaction these glands display a high degree of proliferative activity as seen from the large number of metaphase figures. After the mitotic block is removed the gland quickly begins its regenerative phase.

Morphologically three types of cells are present in the astebuds in shrew. Although they are all developed from the germinal cells of the tastebud, the differentiation and eventual placements of the cells depend on the orientation of the basement membrane on which these germinal cells are situated. In Fig. 15 three parts *A*, *B* and *C* of the basement membrane of an epithelial ridge from which germinal cells differentiate are shown. *A* represents the horizontally oriented segment, *B* the vertical segment and '*C*' the upper fold of the basement membrane. The cells differentiating from *A* have round or oval vesicular nuclei as in ordinary straight stratified epithelium of skin and they have to occupy a more central position in the tastebud. But those arising from *B* being restricted in space between *B* and cells proceeding from *A*, are compressed and elongated with deeply staining rod-shaped nuclei and these would only occupy a position peripheral to those developed from *A*. The cells from *C* proceed to fill up the interval between the distal parts of adjacent tastebuds and would reinforce the walls of the tastepore and the taste canal. Similar differentiation and orientation of cells have been observed in epithelial ridges related to another variety of epithelial organ, the filiform papillae of the tongue of shrew. The rationale of the mechanics of differentiation and orientation of cells of an epithelial organ gives a clue to what types of cells may be expected to occur in tastebuds and their orientation. Accordingly three types of cells are feasible. The morphology of the tastebuds cells in lizard and shrew support this mode of differentiation. Besides the two types of cells differentiated from *A* and *B*, the basement membrane retains germinal cells which form a capsule of undifferentiated cover cells to the tastebud.

The taste pore is a later formation in phylogeny as is evident from the circular, flat or depressed free surface of tastebuds in lizards and frog tadpoles where the columnar cells display very coarse cilia or striated border probably made up of microvilli. This free surface becomes concave and even close in to form a saccular taste canal and tastepore as displayed in mammalian tastebud.

The shape of the tastebud gets modified in phylogeny. In lizard and frog tadpole it is pyramidal with a broad flat base and a truncated apex. In this stage the original epithelial ridge in which it is developed, is not pronounced and the connective space merely shows a dip into the epithelium

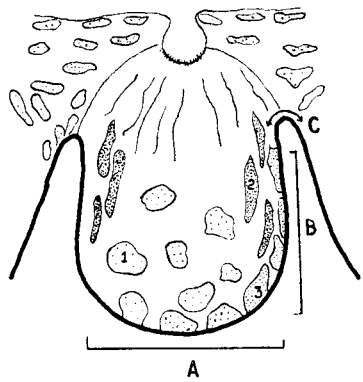


FIG. 15.

to demarcate the base of the tastebud all around. On the other hand, in mammals the barrel shaped tastebud develops in a pronounced epithelial ridge (Fig. 2). The basement membranes *A*, *B* and *C* of the ridge (Fig. 15) reach the mid height of the organ and are surrounded by connective tissue space. The cells differentiated from *C* mould the distal part of the organ and the wide free circular surface of amphibian and reptilian tastebuds close in to form the taste canal and pore of mammals. However, the taste canal and pore are first shown in reptiles as is apparent in one such observed in Gecko among the large number of primitive tastebuds without taste pore.

In regard to the effects of glossopharyngeal nerve lesion on tastebud the time of complete disappearance of tastebuds in shrew is the eight day, which is close to that found in dog by Vintschgau (1880) and in rat by Guth (1957), i.e. the seventh day.

Several suggestions have been made in the past regarding the disposal of degenerating tastebuds. Vintschgau (1880) suggested that the cover cells

of tastebuds metamorphosed into the surrounding epithelium. Ranvier (1882) thought that they were disposed off by wandering leucocytes and that the protective cover cells were expelled through the tastepore. Others were of the opinion that tastebuds differentiated into epithelial cells by a process of de differentiation. Guth (1957) found that the process was one of desquamation.The day-to-day changes in tastebuds found in our study showed that they underwent a gradual reduction in size forming cell nests of degenerating cells. The shrinkage was most marked on the 7th day. Mitotic activity and accumulation of new cells were evident under the basal region of the tastebuds and these pushed the degenerating tastebud towards the free surface of the epithelium (Fig. 5) and expelled it. No leucocytic infiltration was observed. After the tastebuds are disposed off on the free surface, the general epithelial cells get organized into non-gustatory type with dermal papillae and epithelial ridges.

In rats metaphase figures increased steadily till by 24 hours the mitotic block was removed, the anaphase stage was reached at 54 hours. Between 5 and 18 hours there was disruption of cell arrangement and marked reduction in cell density of tastebuds and also steady increase in metaphase figures. The regeneration proceeded to full formation of tastebuds by 72 hours. Colchicine technique in both rats and shrews showed regeneration of tastebuds after the disruptive reaction period was over. In the early stages of regeneration the cover cells and central cells were distinguishable. The cells with round or oval nuclei were seen and were sparsely arranged. This may be due to the low cell content of the differentiating tastebud having ample space to develop freely. However, in the later stages cells with oval and elongated nuclei began to appear.

In shrews these stages were extended. The difference in the period of metaphase block in shrews and rats may be due to the fact that the young rats would begin regenerative activity earlier. There was never complete disappearance of tastebud in both circumvallate and fungiform papillae. The nerves to tastebud were intact throughout colchicine reaction and in the presence of which regeneration took its normal course.

REFERENCES

BOEKE, J. (1913). Die Regenerationverscheinungen bei der Verheilung von motorischen und rezeptorischen Nervenfasern. *Archiv. f. ges. Physiol.* **151,** 8,57–60. (Quoted by MAY, R. M. 1925).

GUTH, L. (1957). The effects of glossopharyngeal nerve transaction on the circumvallate papilla of the rat. *Anat. Rec.* **128,** 715–731.

MAY, R. M. (1925). The relation of nerves to degenerating and regenerating taste buds. *J. Exp. Zool.* **42,** 371–410.

METTLER, F. A. (1947). *History of Medicine*. The Blakiston Co., Philadelphia.
NEAL, H. V. and RAND. (1936). *Comparative Anatomy*. The Blakiston Co., Philadelphia.
RANVIER, L. (1882). *Traite technique d'histologie*, Paris.
TUCKERMANN, F. (1890). On the gustatory organs of some of the mammalia. *J. Morph.*, IV, 152–193.
VON VINTSCHGAU, M. (1880). Beobachtungen über die Veränderungen der Schmeckbecher nach Durchschneidung des N. glossopharyngeous. *Arch. f. d. gesam. Physiol Bd.*, **23**, S. 1–13.
WYSS, V. (1869). Quoted by Mettler, 1947. *History of Medicine*. The Blackiston Co., Philadelphia.

OCCURRENCE OF A VISCOUS SUBSTANCE AT THE TIP OF THE LABELLAR TASTE HAIR OF THE BLOWFLY

BRUNHILD STÜRCKOW

Entomology Research Division, Agr. Res. Serv., U.S.D.A., Beltsville, Maryland†

INTRODUCTION

Since 1895 the labellar hairs of the blowfly have been assumed to function as taste hairs because of their location and sensory supply (Lowne, 1893–1895). In 1926 Minnich stimulated a single hair with a sugar solution. The fly responded by extending its proboscis and spreading the labella. Many behavioral tests have subsequently been made with taste hairs of the blowfly and other insects, either with single hairs or with appendages provided with many hairs.

Additional features of insect taste hairs have become known since Hodgson, Lettvin and Roeder (1955) employed electrophysiological methods, and Adams (1961) used the electronmicroscope. Nevertheless, we are still far from understanding the structure and function of the taste sensillum as a whole, and may occasionally be confused by the abundance of new details.

The latest work necessary for an understanding of the fly taste hair has been made:
Behaviorally by Dethier (1955), and Evans (1963); *electrophysiologically* by Hodgson and Roeder (1956), Wolbarsht and Dethier (1958), Morita (1959), Morita and Yamashita (1959), Evans and Mellon (1962 a, b), Barton Browne and Hodgson (1962), Steinhardt, Morita and Hodgson (1963), Stürckow (1963, 1964), Wolbarsht and Hanson (1965), Hanson (1965), den

* In memory of Dr. David R. Evans, John Hopkins University, Baltimore, Maryland.

† The experiments were done at the I. Institut für Physiologie der Universität des Saarlandes, Homburg/Saar, Germany.

Otter and von der Poel (1965), and Wolbarsht (1965); *morphologically* by Adams (1961), Stürckow (1962), Larsen (1962), and Peters (1965). Although each author used one or two of these methods, each revealed new characteristics of the sense organ and added to the picture as a whole.

Electrophysiological and electronmicroscopical investigations have been made with flies of the genera *Phormia*, *Calliphora*, *Lucilia* and *Stomoxys*. Although results derived from different genera should not be considered

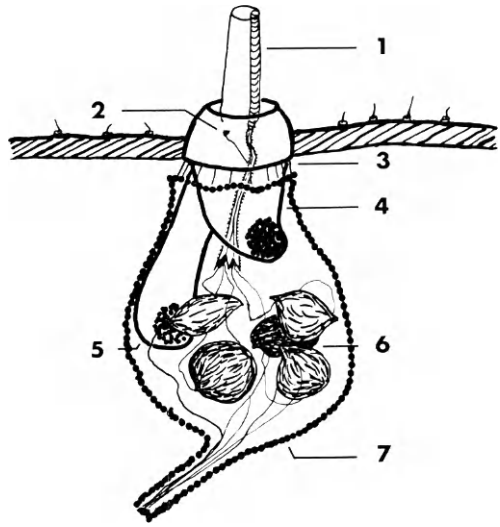


FIG. 1. Schematic of a labellar taste hair of the blowfly (for details see Stürckow, 1962).
1 channel containing the chemosensitive dendrites; *2* termination of the mechanosensitive dendrite; *3* at this location a fluid may enter the sensillum; *4*, *5* tormogen (socket-forming) and trichogen (hair-forming) cells; *6* five receptor cells; *7* heavily pigmented layer around the sensillum and nerve

homologous without proof, a cautious comparison is permissible. The results obtained with *Phormia* and *Calliphora* have shown no basic differences.

The labellar taste hairs of the blowflies *Phormia* and *Calliphora* are generally supplied with 5 bipolar sense cells embedded in a sac below the shaft of the hair (Fig. 1). One sense cell may function as a mechanoreceptor. The dendrite of this cell ends at the base of the hair, whereas each chemosensitive cell sends its dendrite to the tip of the hair. On the way to the tip the dendrites are enclosed in a tube eccentrically positioned throughout the trichogen cell. The tube ends distad at the tip of the hair. Thus, the hair shaft is divided into a dendrite-free channel and a channel that contains the distal nerve endings.

Loewenstein (1959) has shown for the Pacinian corpuscle that the site of the mechanoelectric transducer, or at least its final stage, is located in the membrane of the dendrite and not in adventitious tissue. This principle of reception is probably the same in sense cells of insects. In the taste hair of *Stomoxys* the dendrites end at the tip of the hair beneath a pore in the cuticle (Fig. 2). This construction of the tip is undoubtedly similar in taste hairs of other flies. In the labellar hair of *Lucilia* the pore is so effective

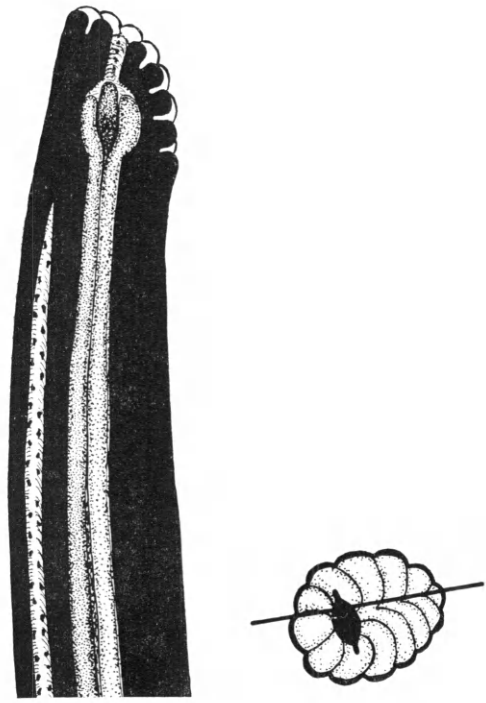


FIG. 2. Schematic longitudinal section of a tarsal taste hair of *Stomoxys* (drawn from electronmicrographs after Adams, 1961).
Left: Longitudinal section showing the blind-ending, dendrite-free channel, and three dendrites in the other channel ending below a pore in the cuticle.
Right: Hypothetical view proximad upon the tip of the hair showing the pore and the cuticular ridges of the hair shaft fused in a manner that would explain the teeth seen in longitudinal sections. The line indicates the location of the section on the left.

that latencies of 1 and 5 msec occurred for the first spike response of the salt receptor and sugar receptor when 250-mM solutions were used (Barton, Browne and Hodgson, 1962).

The plasma surrounding the dendrites may fill the pore at the tip of the hair and may occasionally extrude from the pore, since Morita and Takeda (1957) and Stürckow (1959) found a sticky viscous droplet at the tip of chemosensitive hairs in *Vanessa* and *Leptinotarsa*.

This presentation is a discussion of experiments showing in *Calliphora* the existence of a viscous substance at the tip of the hair and its probable effect on the reception of stimuli.

RESULTS AND DISCUSSION

I. Behavioral Responses to Stimulation of single Hairs

Single labellar hairs of intact flies were stimulated with a multichanneled flow capillary* (Stürckow, 1963) until extension of the proboscis occurred. The tip capillary of this flow system served as a mixing space during the change of solutions. The flow speed was determined by the flexibility of the hair and allowed a complete change between two solutions within

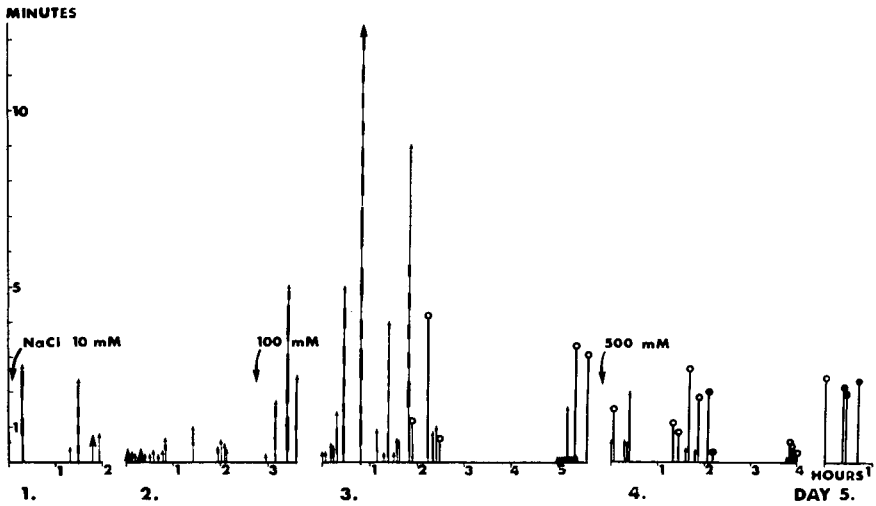


FIG. 3. Latency of behavioral responses to stimulation of the same hair over 5 days.
Ordinate: Time of stimuli in min; abscissas: length of experimentation in hours. The hair was always stimulated first with a flowing NaCl solution (thin line), concentration change indicated. After two or more seconds the salt solution was changed to 500 mM sucrose (thick line). Response to sucrose (small arrows); response to salt (4 large arrows). Stimulus removed without response (open circle); viscous substance noticed (filled circle). Although the fly had indicated its readiness to respond quickly to sugar prompt responses of 1·63 sec ±0·03 SE alternated irregularly with slow responses ranging from 7 to 110 sec. In a few cases no response was obtained during stimulation of 20 sec to 4 min.

about 1 sec. The interval between the change from a salt to a sugar solution and the extension of the proboscis was taken as the latency of the response. A primary record of all data throughout the experiments was made with a tape recorder.

* The capillary was developed at the suggestion of Professor R. Stämpfli, Homburg/Saar, Germany.

Figure 3 shows that prompt responses alternated irregularly with slow responses. The interval between trials did not influence the response latency. The intensity and rinse time of the salt solution also did not noticeably

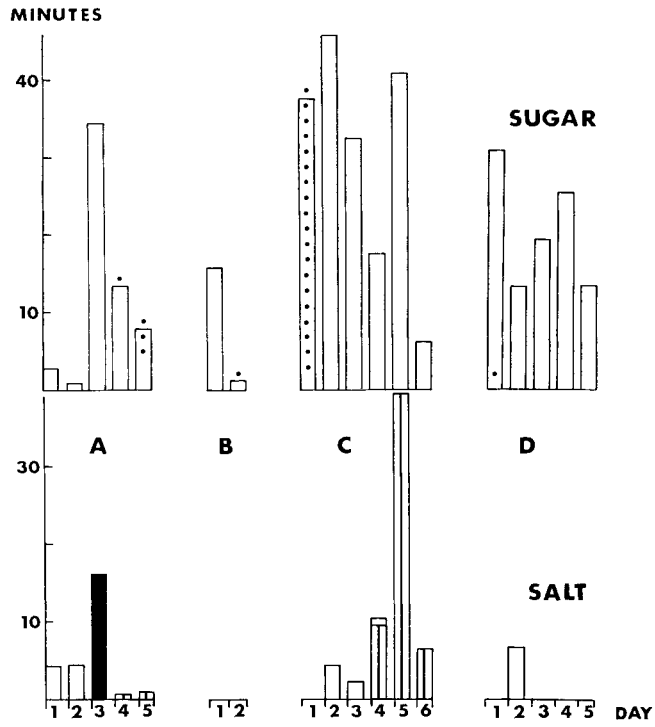


FIG. 4. Type and duration of the stimuli applied to four main hairs (A – D) of four flies.
Ordinates: length of stimulation in min; abscissas: successive days of experimentation. The duration of the daily stimuli, as shown in Fig. 3 for hair A, is summarized. NaCl 10 mM (white), 100 mM (black), 1M (vertical stripe), sucrose (500 mM). Viscous substance noticed at the tip of the hair (dot). Hair C is the main hair in Fig. 5.

affect the reaction. Therefore, a factor other than regular adaptation processes of the involved nervous tissue probably caused the great variation in response latency.

Experimentation with the same fly was repeated daily over two to six days. Each trial included a main hair and one to three alternate hairs all selected from the two longest hairs at the most distal part of the labellum (rear, Peters, 1965, Fig. 1). If the fly failed to respond for several minutes to stimulation of the main hair, the alternate hairs were used to determine

the readiness of the fly to respond at all. An interval of 3–5 min between tests with different hairs made probable that the response was not due to a central summation of inputs from different sugar receptors. For the same reason, the first alternate hair was taken from the labellum other than that bearing the main hair.

During assays with seven flies a substance was noted at the tip of the hair in four of seven main hairs and in five out of ten alternate hairs. The

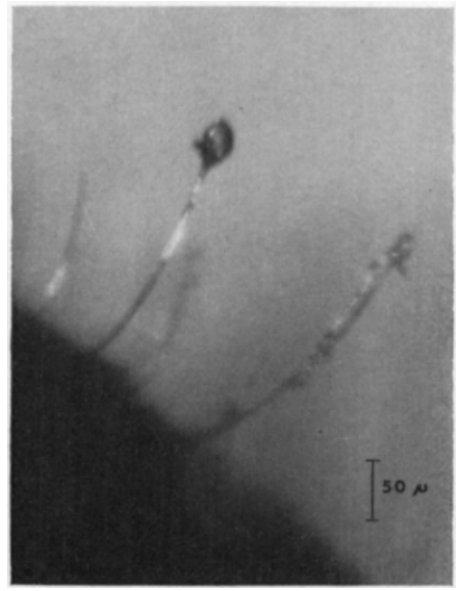


FIG. 5. Two hairs at the most distal portion of the right labellum with extruded viscous substance.
This material has formed a ball on the main hair. The alternate hair bears a freshly extruded coating with a curled tip.

occurrence of this substance showed no dependence upon the length and quality of the stimuli (Fig. 4). The first evidence of this material was a thin thread appearing during the removal of the stimulus between the tip of the hair and the capillary. The thread parted with further elongation. The portion remaining on the hair contracted to form a coating at the tip as seen on the alternate hair in Fig. 5. During repeated stimulation this sticky, slightly viscous coating could be pulled into a thread with each removal of the capillary.

In one instance (the main hair in Fig. 5) the coating on the hair increased considerably during one hour of experimentation. With the increase, the

substance contracted into a ball after the removal of the capillary. The thread pulled from the ball shortened in length and increased in thickness. A small part of the material formed a long thin filament at the end of the thread. This filament appeared to consist of a freshly extruded substance.

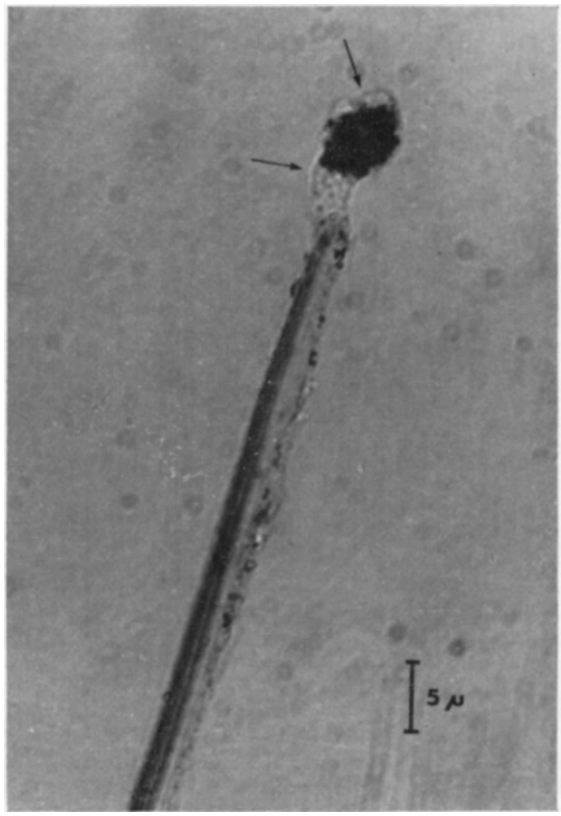


FIG. 6. Outer layer of the viscous substance (see text).

The viscosity of the material apparently became slightly greater within the first hour of exposure to air. For the next 7 hr the viscosity and stickiness of the ball remained the same. Finally, after 7 hr the ball slipped from the hair when an attempt was made to pull it into a thread. Within the next 5 days no viscous substance was noticed and no response occurred (Fig. 4, hair C). The sensillum was apparently damaged.

The viscous substance of another hair was photographed three weeks after the experiment (Fig. 6). During this time the hair was stored in air.

Microorganisms had grown on the viscous substrate and had partly consumed it with the exception of an outer layer. The attachment of this layer to the hair and the location of the droplet indicate that the material originated from the tip of the hair. An electronmicrograph of a taste hair of *Stomoxys* confirms this finding (Fig. 7). The figure shows a substance with a definite outer layer extruded from the pore of the channel that contains the dendrites. This layer ranges in thickness from about 150 to 1000 Å.

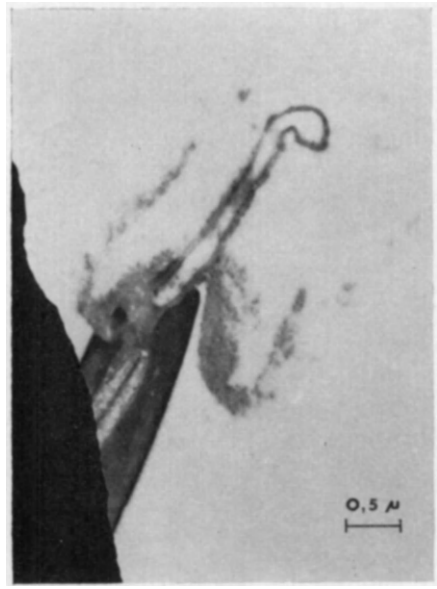


FIG. 7. Electronmicroscopical longitudinal section through the tip of a tarsal taste hair of *Stomoxys* (from Adams, 1961).
The pore above the dendrites measures 0·25 μ in diameter. A substance covered on the outside with an irregular layer of a granular material has been extruded through the pore.

Its structure suggests a membrane, the outside of which is covered with an irregular layer of a granular material. These fine granules can also be recognized in the end of the channel, the pore and the area surrounding the tip of the hair. These granules have probably been extruded from the pore together with the viscous substance.

The main hair in Fig. 5 was rinsed with a sugar solution for a total of 37 min during 1 hr of experimentation. The amount of viscous substance extruded within this time was greater than the volume of the channel that contains the dendrites. The copious flow of viscous material from this channel suggests a source of supply from the immediate vicinity. Since the

viscous substance has never been observed during numerous electrophysiological tests with a severed proboscis, the extrusion of this material probably depends upon the pressure and circulation of the haemolymph. The heavily pigmented layer around the sensillum is impermeable to water solutions (Stürckow, 1962). Therefore, a fluid may enter the sensillum only at the distal portion of the sac where the pigmented layer is lacking (Fig. 1). Within the sensillum a fluid may diffuse into the channel that contains the dendrites either through its proximal nonsclerotized portion (Peters, 1965) or through the wall from the dendrite-free channel.

A–C impedance measurements of single hairs suggest an electrolytically conductive path either between the two channels or between the dendrite-free channel and the outside of the hair at the tip (Stürckow and Weymann, 1965). Adams' longitudinal sections (1961) through tips of 11 taste hairs of *Stomoxys* do not indicate a pore between the dendrite-free channel and the outside of the hair. An electrolytically conductive path between the channels may exist either as an ionic bridge throughout the wall or a limited portion of it or in the form of one or several pores in the wall.

No behavioral response occurred when the viscous substance was noticed at the tip of the hair. Therefore, the extrusion of the substance in submicroscopical amounts may also have caused the irregular behavioral responses (Fig. 3). The substance had already been extruded in considerable amounts when it became visible at a stereoscopical magnification of 160 times.

Since prompt responses alternated irregularly with slow responses the viscous substance apparently varied in its capability to block the reception. Either the flowing solution may have partially loosened the substance, or intrinsic processes may have changed its characteristics.

The application of sugar solution to hairs noticeably covered at the tip with the viscous substance occasionally resulted in a slight trembling of the retracted proboscis. At times this trembling also occurred when no substance was seen. In a few instances a partial extension of the proboscis accompanied this trembling. Therefore, it was interpreted as a response to a stimulus received below the threshold that is required for the full extension of the proboscis.

II. Electrophysiologically recorded Responses of single Hairs

The impulse activity of single hairs was recorded over 30–45 min during stimulation with water and solutions of NaCl and sucrose. The stimuli were applied with the flow capillary. One of the electrodes was inserted

into the severed proboscis whereas the other was slipped over the tip of the hair (for details see Stürckow, 1963). A moist chamber was used. All hairs were first stimulated by bending. Only hairs without the characteristic response of the mechano receptor were used. In the most common type of response pattern these hairs showed three different impulses that proved to be from a water, a salt, and a sugar receptor as evidenced by either amplitude or frequency or both. A fourth receptor of unknown function or the sugar receptor responded to 10 mM NaCl. The water receptor reacting to water and all solutions mainly determined the course of the response.

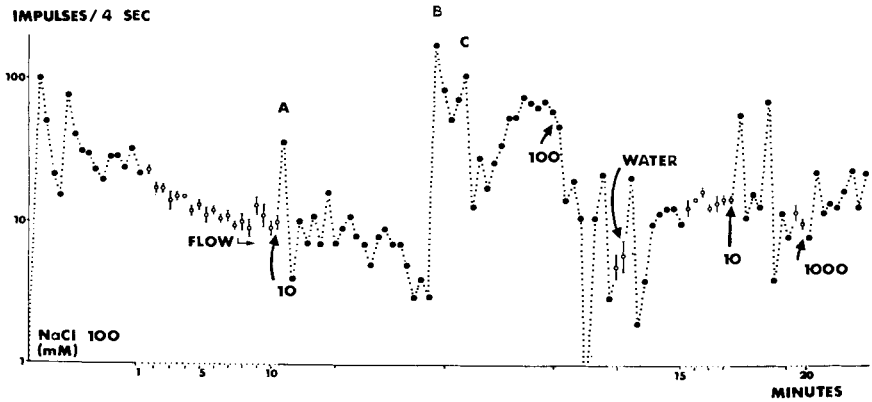


FIG. 8. A response pattern of the most common type.
Ordinate: Total impulse discharge per 4 sec at successive 4-sec intervals; log scale variation. Abscissa: Time in min; mean ±SE of the total impulse discharge per 4 sec over 30-sec periods (dotted line). After $7^1/_2$ min the standing solution was changed to a flowing one. Stimulation with NaCl (in mM) and water is marked. The arrows indicate the change of the stimulus. For an explanation of *A*, *B*, and *C* see Fig. 9 and text.

The activity of this most common type of response pattern was recorded from one short and two long hairs of three flies. Figure 8 shows the total impulse discharge during the first 20 min of the experiment with the short hair:

(a) The sensillum responded to a change in stimulus with either a peak or depression of excitation (depending upon the preceding stimulus), which finally reached a steady level. The sensillum as a whole reacted as a phasic-tonic receptor. Since each portion of the response was composed of impulses specific to both solute and water, each sense cell also reacted as a phasic-tonic receptor. However, the response pattern was modulated by oscillations.

(b) A sudden increase in spike frequency without change of stimulus was observed during stimulation with 10 mM NaCl (Figs. 8 and 9 peaks *B* and *C*). [A similar increase in excitation occurred in the experiment with one long hair, once during stimulation with water and a second time during stimulation with 1 M NaCl. The initial high response dropped within 1–2 min, while oscillating, to the original level.] These sudden increases in activity showed no evident dependence upon the quality of the stimulus or the duration of the experiment. Since no additional impulse occurred during the increase in spike frequency, such increases were probably caused by a sudden change in, or injury to a structure common to two or more receptors and located within the sensillum. Only the viscous substance surrounding the dendrites appears to meet these requirements.

SUMMARY

Behavioral Experiments

Single hairs of the intact fly were stimulated with flowing solutions. The interval between the change from a salt to a sugar solution and the extension of the proboscis was taken as the latency of the response. This latency ranged irregularly from prompt responses of about 1·6 sec to slow responses of 7 sec to 4 min or longer.

A sticky viscous substance was noticed at the tip of several hairs during the removal of the capillary. This substance was probably extruded from the channel that contains the dendrites. Although stimulation of other hairs indicated the readiness of the fly to respond, no extension of the proboscis occurred when a hair tip, noticeably covered with this substance, was stimulated with sugar. Therefore, the slow responses to sugar were probably also caused by extrusion of this substance in submicroscopical amounts.

Electrophysiological Experiments

The spike activity of single hairs was recorded over 30–45 min during stimulation with flowing water and solutions of salt and sugar. A sudden increase in excitation without change of stimulus occurred in two experiments. This increase in spike frequency was probably caused by a sudden change in the viscous substance at the tip of the hair.

This viscous sticky substance represents a material that is highly resistant to drying. It may fill the pore at the tip of the hair and facilitate reception by providing at least a moist surface. At times this substance extrudes from the pore and inhibits the reception of sucrose.

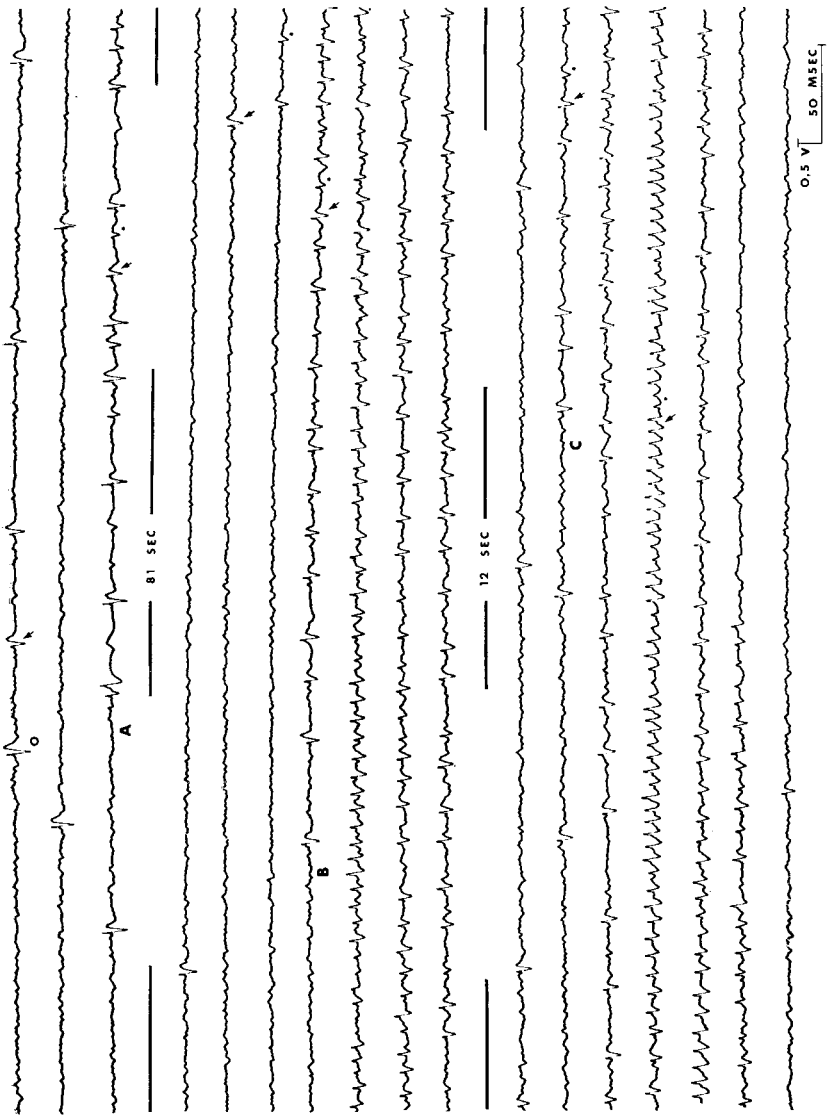


FIG. 9. Sudden increase in spike frequency without change of stimulus.
A, *B* and *C* indicate the beginning of the peaks of excitation *A*, *B*, and *C* in Fig. 8. The trace begins with the response to 100 mM NaCl, spike specific to salt (circle), spike specific to water (arrow). At *A* the change to 10 mM NaCl occurred. The spikes specific to salt disappeared and another impulse (dot) different from that probably specific to water (arrow) appeared. Both spikes remained throughout the stimulation with 10 mM NaCl.

REFERENCES

ADAMS, J. R. 1961. The location and histology of the contact chemoreceptors of the stable fly, *Stomoxys calcitrans* L. Doctoral Dissertation, Rutgers University, New Brunswick, New Jersey.

BARTON BROWNE, L. and HODGSON, E. S. 1962. Electrophysiological studies of arthropod chemoreception. IV. Latency, independence, and specificity of labellar chemoreceptors of the blowfly, *Lucilia*. *J. cell. comp. Physiol.* **59**, 187–202.

DETHIER, V. G. 1955. The physiology and histology of the contact chemoreceptors of the blowfly. *Quart. Rev. Biol.* **30**, 348–371.

EVANS, D. R. 1963. Chemical structure and stimulation by carbohydrates. Proceed. I. intern. Symposium *Olfaction and Taste* (Stockholm), **1**, 165–176.

EVANS, D. R. and MELLON, D. 1962 a. Electrophysiological studies of a water receptor associated with the taste sensilla of the blowfly. *J. gen. Physiol.* **45**, 487–500.

EVANS, D. R. and MELLON, D. 1962 b. Stimulation of a primary taste receptor by salts. *J. gen. Physiol.* **45**, 651–661.

HANSON, F. E. 1965. Electrophysiological studies on chemoreceptors of the blowfly, *Phormia regina*. Doctoral Dissertation, University of Pennsylvania, Philadelphia, Pa.

HODGSON, E. S., LETTVIN, J. Y. and ROEDER, K. D. 1955. The physiology of a primary chemoreceptor unit. *Science*, **122**, 417–418.

HODGSON, E. S. and ROEDER, K. D. 1956. Electrophysiological studies of arthropod chemoreception. I. General properties of the labellar chemoreceptors of diptera. *J. cell. comp. Physiol.* **48**, 51–75.

LARSEN, J. R. 1962. The fine structure of the labellar chemosensory hairs of the blowfly, *Phormia regina* Meig. *J. Ins. Physiol.* **8**, 683–691.

LOEWENSTEIN, W. R. 1959. The generation of electric activity in a nerve ending. *New York Acad. Sci.* **81**, 367–387.

LOWNE, B. T. 1893/95. The anatomy, physiology, morphology and development of the blowfly *(Calliphora erythrocephala)*. London, Vol. 2.

MINNICH, D. E. 1926. The organs of taste on the proboscis of the blowfly, *Phormia regina* Meig. *Anat. Rec.* **34**, 126.

MORITA, H. 1959. Initiation of spike potentials in contact chemosensory hairs of insects. III. D. C. Stimulation and generator potential of labellar chemoreceptor of *Calliphora*. *J. cell. comp. Physiol.* **54**, 189–204.

MORITA, H. and TAKEDA, K. 1957. The electrical resistance of the tarsal chemosensory hair of the butterfly, *Vanessa indica*. *J. Fac. Sci.*, Hokkaido Univ., Series VI, *Zool.*, **13**, 465–469.

MORITA, H. and YAMASHITA, S. 1959. The back-firing of impulses in a labellar chemosensory hair of the fly. Memoirs Fac. Sci., Kyushu Univ., Series E *(Biol.)*, **3**, 81–87.

OTTER, C. J. den and POEL, A. M. von der. 1965. Stimulation of three receptors in labellar chemosensory hairs of *Calliphora erythrocephala* M. by monovalent salts. *Nature* **206**, 31–32.

PETERS, W. 1965. Die Sinnesorgane an den Labellen von *Calliphora erythrocephala* MG. (Diptera). *Z. Morph. Ökol. Tiere* **55**, 259–320.

STEINHARDT, R., MORITA, H. and HODGSON, E. S. 1963. Electrophysiological analysis of inhibition and specificity in labellar chemoreceptors of the blowfly. Proceed. XVI. *intern. Congress Zool.* (Washington, D. C.) **3**, 99–101.

STÜRCKOW, B. 1959. Über den Geschmackssinn und den Tastsinn von *Leptinotarsa decemlineata* SAY (Chrysomelidae). *Z. vergl. Physiol.* **42**, 255–302.

STÜRCKOW, B. 1962. Ein Beitrag zur Morphologie der labellaren Marginalborsten der Fliegen *Calliphora* und *Phormia*. *Z. Zellforsch.* **57**, 627–647.

STÜRCKOW, B. 1963. Electrophysiological studies of a single taste hair of the fly during

stimulation by a flowing system. *Proced.* XVI. *intern. Congress Zool.* (Washington, D.C.) **3,** 102–104.

STÜRCKOW, B. 1964. Some common features of three types of insect sensilla. *Experientia* **20,** 509–510.

STÜRCKOW, B. and WEYMANN, D. 1965. To be published.

WOLBARSHT, M. L. 1965. Receptor sites in insect chemoreceptors. *Cold Spring Harbor Symposium* (to be published).

WOLBARSHT, M. L. and DETHIER, V. G. 1958. Electrical activity in the chemoreceptors of the blowfly. I. Responses to chemical and mechanical stimulation. *J. gen. Physiol.* **42,** 393–412.

WOLBARSHT, M. L. and HANSON, F. E. 1965. Electrical activity in the chemoreceptors of the blowfly. III. Dendritic action potentials. *J. gen. Physiol.* **48,** 673–683.

INHIBITION AND EXCITATION OF SINGLE INSECT OLFACTORY RECEPTORS, AND THEIR ROLE AS A PRIMARY SENSORY CODE

J. BOECKH

Max-Planck-Institut für Verhaltensphysiologie
8131 Seewiesen, Germany

INTRODUCTION

Receptor cell reactions are known to be influenced not only by excitatory but also by inhibitory processes. Such an inhibition can on the one hand be of nervous nature caused synaptically, as in the case of crustacean stretch receptors (Kuffler and Eyzaguirre, 1955) and in the case of the lateral inhibitory effect as it was found in the arthropod complex eye by Hartline (1940) and Hartline *et al.* (1956). We are in these situations dealing with an inhibitory "control" of receptor cells which are themselves only responding in an excitatory manner when stimulated individually. Some types of individual receptor cells seem, on the other hand, also to show either an excitatory or an inhibitory response depending upon stimulus characteristics.

Such a characteristic may be due to the composition of the chemical stimulating a chemoreceptor or to the direction of the stimulus as in mechanoreceptors. In a number of cases it is difficult to decide whether the inhibition of the cell which is under observation actually is the direct response to the stimulus applied or it is caused by other neurons as described above. This possibility, however, is excluded, if only the cell under study and none of its neighbors is stimulated as it was shown recently by Nicklaus (1965) with cercal hair mechanoreceptors of cockroaches. Bending of the hair in one direction increased the response and bending in the opposite direction blocked it. There is also good evidence that some chemoreceptor cells do respond with either an excitation or an inhibition to different stimuli (Hodgson 1957, Boeckh 1962, Gesteland 1963, Lacher 1964, Schneider, Lacher and Kaissling 1964, Boeckh, Kaissling and Schneider 1965,

Tucker and Shibuya 1965, Lettvin and Gesteland 1965). Here, isolated stimulation of only one receptor cell was not yet possible. Although "lateral" inhibitory effects of some kind can not be fully excluded in some cases, it is safe to state that they are very probably not the result of inhibitory synaptic influence. In insect taste hairs or vertebrate as well as insect olfactory cells, there is morphologically neither a central efferent control nor any lateral connection known so far. This, of course, does not exclude the theoretical possibility of ephaptic effects of sensory nerve cells adjacent to one another, if one would find a way to explain how excitation of one cell depresses the reaction of its neighbor. Even this remote explanation of receptor cell inhibition is fully excluded, if all three neighboring cells of one olfactory hair of a moth or even a whole field of many hundreds of singly innervated olfactory hairs of a carrion beetle are inhibited simultaneously by certain odors. (Boeckh, 1962, Schneider, Lacher and Kaissling 1964, Boeckh, Kaissling and Schneider 1965.) Here, one can therefore not escape the conclusion that the receptor cell itself is capable of responding either by excitation or by inhibition, depending upon the qualitative nature of the stimulus. It seems to be sure by now that Bullock's (1959) earlier suggestion of the presence of primary excitatory *and* inhibitory receptor reactions is correct. His opinion was based upon opposite electroantennogram polarities elicited by different olfactory stimuli (Schneider 1957 a and b). In the meantime, the electroantennogram was proved to be the sum of many partially synchronized olfactory receptor potentials. If a given odor excites most of the cells, the antennal periphery is showing a relative electrical negativity as a response; if one part of the cells is excited and another inhibited, the antennogram is usually complex; if most cells are inhibited, the antennal periphery is showing a relative electrical positivity.

In principle, receptor cells responding with either excitation or inhibition to different stimuli is nothing new in the nervous system, because these two antagonistic processes may—similarly to many neurons with polysynaptic contact—occur at different receptor membrane loci. Thus, sensory cells with their receptive dendritic membranes are not only selective as input channels which are specifically tuned to the adequate stimulus modality giving either a "plus" or no response, but also antagonistically selective to qualitatively different stimuli of one modality by means of a "plus" or a "minus" response, if they respond at all. This paper deals with the most recent observations of this nature.

METHODS

The olfactory receptors studied in the course of this investigation belong to antennal hair sensilla of insects. Figure 1 shows such a unicellularly innervated organ of a carrion beetle in semidiagrammatic manner. Recording is done extracellularly with a glass capillary electrode at the hair base,

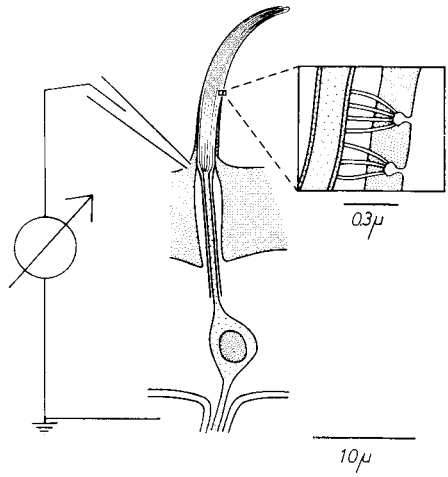


FIG. 1. *Necrophorus vespillio* (Carrion beetle). Diagrammatic drawing of an olfactory basiconic sensory hair on the antenna. The bipolar sensory cell sends its proximal process to the CNS, the distal process to the hair socket where the latter branches into several dendrites. An extra magnified part of the hair wall region (inset) shows a dendrite together with tube-like processes with a diameter of about 100 Å running towards the bottom of small pores, which penetrate the hair wall. The recording system is sketched at the left-hand side of the drawing.

while the indifferent electrode is placed in the hemolymph space of the antenna. This method permits simultaneous recordings of receptor potentials and nerve impulses (see Boeckh, 1962, Lacher, 1964). Stimulation and recording are critically evaluated by Boeckh, Kaissling and Schneider (1965).

RESULTS

1. Polarities of the Receptor Potential

Figure 2 demonstrates the reaction of a single antennal olfactory receptor cell from a carrion beetle's antenna to different odors. Carrion extract (a) is a very potent excitatory stimulus and it is assumed that this reaction is biologically of vital importance for the animal. The lower DC-trace (Fig.

2a) of the recording shows a few msec. after the onset of the stimulus a sharp downward deflection. This indicates that the recording electrode site—the hair base—is becoming negative, relative to the ground potential of the blood electrode beyond the basement membrane. Nerve impulses appear simultaneously with an additional latency (see the upper beam with higher gain). The impulse frequency course is phasic-tonic. Impulse

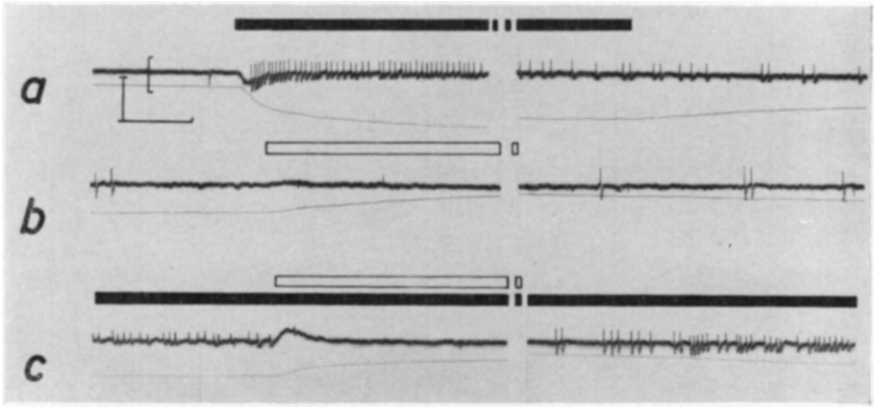


FIG. 2. *Thanatophilus rugosus* (Carrion beetle). Sensillum basiconicum. Original registration of the activity of a carrion odor receptor. Upper beam AC (calibration bar 1 mV), lower beam DC (calibration 20 mV), time bar 200 msec. Downward deflection represents a negative potential at the recording electrode. (a) carrion odor (black bar); the registration is interrupted for 1 sec (b) propionic acid (white bar); the registration is interrupted for 700 msec (c) continuous carrion odor stimulation (black bar) superimposed by propionic acid (white bar); the registration is interrupted for 500 msec.

amplitudes are not constant, but decrease with the receptor potential increase.

Propionic acid (Fig. 2b) as an olfactory stimulus drives the DC-potential in the opposite (and hair base positive) direction. Nerve impulses do not appear before the end of the stimulus and slow backswing of the positive potential. This supposed inhibition of the receptor is fully apparent when both stimuli are superimposed upon one another (Fig. 2c). The figure begins with the plateau of a carrion response. Impulses are blocked by the introduction of the propionic acid, while the DC receptor potential is driven into positive direction. Impulses reappear after the end of propionic acid odor influx, while the DC-potential reverses its trend very slowly. It is interesting to note again that the nerve impulses in Fig. 2c are small under the influence of strong stimulus, several times larger soon

after the end of the inhibitory odor and become small again later during the excitation.

The good temporal correlation of the slow negative (DC) potential with the impulses indicates that we are actually dealing here with a membrane depolarization of the distal area of the dendrites which works as the generating stimulus for the nerve impulses. It is generally agreed that these extracellularly recorded DC potentials from insect hair sensilla represent the generator potentials of the sensory cell. (Morita 1959, Boeckh, 1962, Schneider and Boeckh, 1962, Schneider, 1963 a and b, Thurm, 1963, Wolbarsht and Hanson, 1965, Boeckh, Kaissling and Schneider, 1965.)

Consequently, the extracellularly recorded positive DC-potential deflection which is elicited by some odors is understood as membrane potential increase and impulse threshold increase, i. e. an inhibition.

We like to call these slow potentials for simplicity reasons "excitatory" (= impulse eliciting) and "inhibitory" (= impulse suppressing) potentials.

2. Time Course of the Excitatory and Inhibitory Receptor Potential

Besides the opposite polarities the most remarkable difference between excitatory and inhibitory potentials can be found in the time courses. This

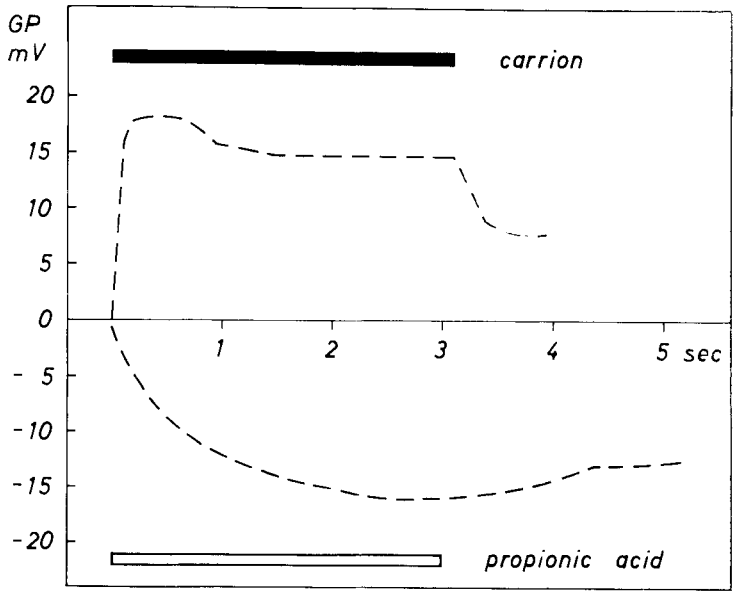


FIG. 3. *Thanatophilus rugosus*. Carrion receptor. Receptor potentials under stimulation with carrion (black bar) and propionic acid (white bar). The excitatory (depolarizing) potential is drawn upwards, the inhibitory potential downwards. The 0-line marks the extracellular potential of the unstimulated *p* preparation.

is obvious already in Fig. 2 but even more so with compressed time axis (Fig. 3). The typical excitatory potential rises in this recording from the carrion beetle cell in 80–100 msec to 75 per cent of its maximal amplitude. It then goes through a maximum (dynamic peak), turns and adjusts to a steady state (plateau). The inhibitory potential rises much more slowly. Its time constant is approximately 10 times that of the excitatory potential. There is no dynamic peak in the inhibitory potential. It also recovers more slowly after the end of the stimulus. This situation was in general similar wherever we recorded excitatory and inhibitory potentials in insect olfaction (carrion receptors in *Calliphora*, *Necrophorus* and *Thanatophilus)*. Excitatory potentials may also develop without the dynamic peak as it is apparent during a heptanol stimulation (see Fig. 7).

3. The Effectiveness of the Inhibiting and Exciting Substances

When we studied the qualitative range of insect olfactory receptors, we mostly found that identically reacting cells which are present in multitude on the antenna, are especially sensitive to one or a few compounds. These compounds are known in some cases (e.g. some sexual pheromones)

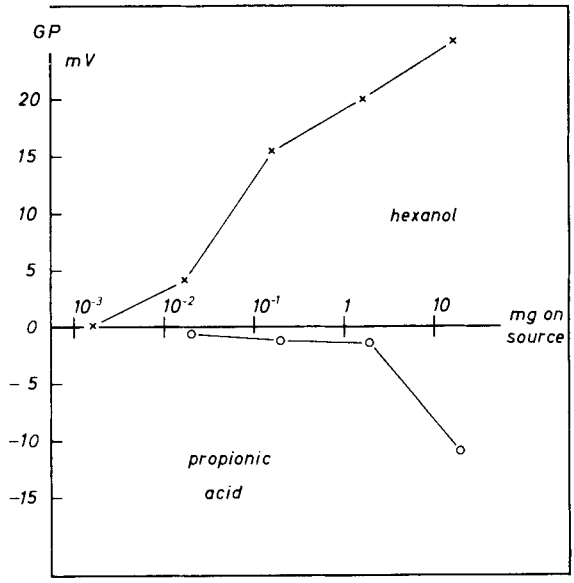


FIG. 4. *Thanatophilus rugosus*. Carrion receptor. Receptor potential amplitudes of a carrion odor receptor stimulated with various concentrations of an exciting (hexanol) and an inhibiting (propionic acid) substance. Polarities as in Fig. 3.

or not known as in the case of decaying meat, but seem to be of vital importance for the animal (Boeckh, Kaissling and Schneider 1965; Schneider 1965/66). No inhibitory compound was so far found to be even remotely as effective a stimulus as the "vital" excitatory odor. Figure 4 gives a good example for this. Hexanol (which may not even be the biologically important compound) elicits an excitatory receptor potential at a 10^4 times higher dilution than the strongest inhibitory substance, proprionic acid.

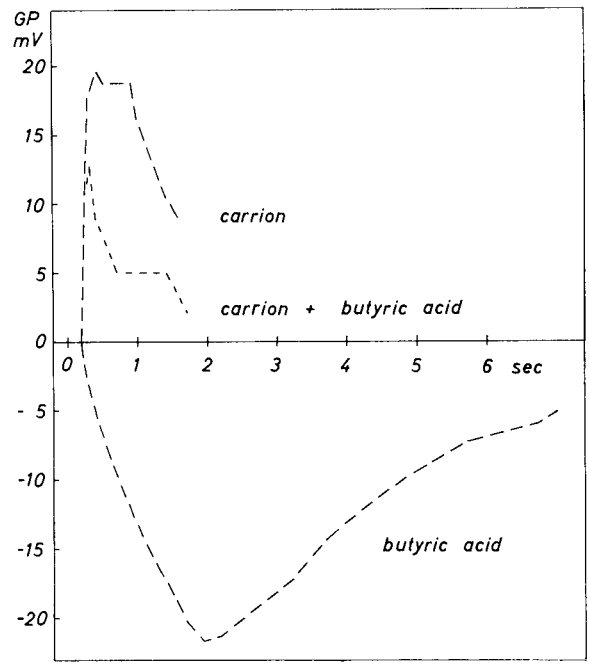


FIG. 5. *Thanatophilus rugosus*. Carrion receptor. Receptor potentials under stimulation with carrion odor, propionic acid, and with a mixture of both substances.

The time course differences of excitatory and inhibitory reactions are obvious also when superimposing the two types of stimuli (Fig. 5). A mixture of the two stimuli still elicits a clear excitatory peak before reaching a steady state much below the normal exitatory one. The first rise of the "mixed" receptor potential is as steep as with excitation alone. Consequently, the dynamic peak of the impulse frequency is identical in carrion and carrion + butyric acid stimulation. The difference in the impulse output is restricted to the steady state (Fig. 6).

Replacement of the strong (e. g. carrion) excitatory stimulus by a somewhat weaker one (heptanol) in the mixture does not yet cancel the dynamic peak of the impulse frequency. There is, however, no steady state left. The impulses appear only as a brief volley, but after that short period the receptor potential goes positive during the continuation of the mixed stimulation (Fig. 7).

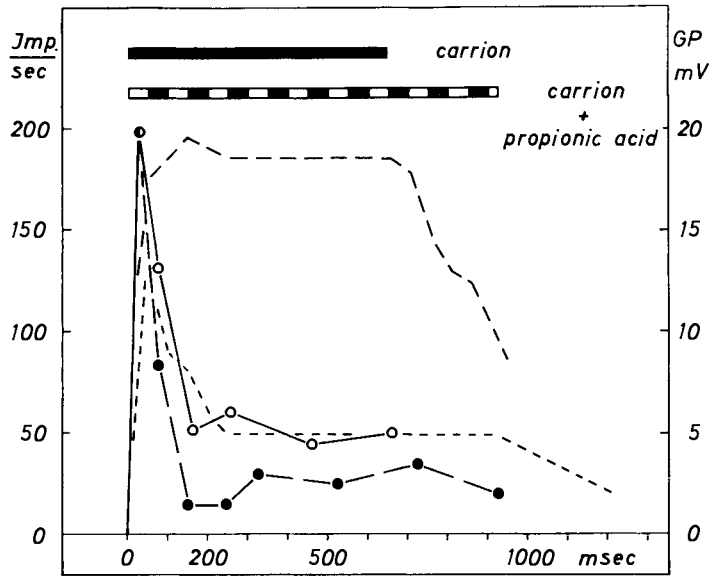


FIG. 6. *Thanatophilus rugosus.* Carrion receptor. Receptor potentials and nerve impulse frequencies under stimulation with carrion (black bar) and propionic acid (black and white bar). Dashed lines without dots: receptor potentials (right-hand ordinate, GP). Solid and slightly dashed lines with circles: impulse frequency curves (left-hand ordinate). High-amplitude receptor potential together with frequency curve with open circles: carrion odor response. Low level receptor potential together with the frequency curve with filled circles: mixed odor stimulation.

4. Excitation and Inhibition in Relation to the Olfactory Cells' Reaction Spectrum

Individual insect olfactory receptors do have fixed and reproduceable "spectra" of compounds they react to. The response to a given substance is either excitation or inhibition. Polarities of the receptor potentials are independent of the concentration: if a compound is effective at all, it either excites or inhibits the cell. No polarity change was ever observed, not even with extremely high or low stimulus intensities.

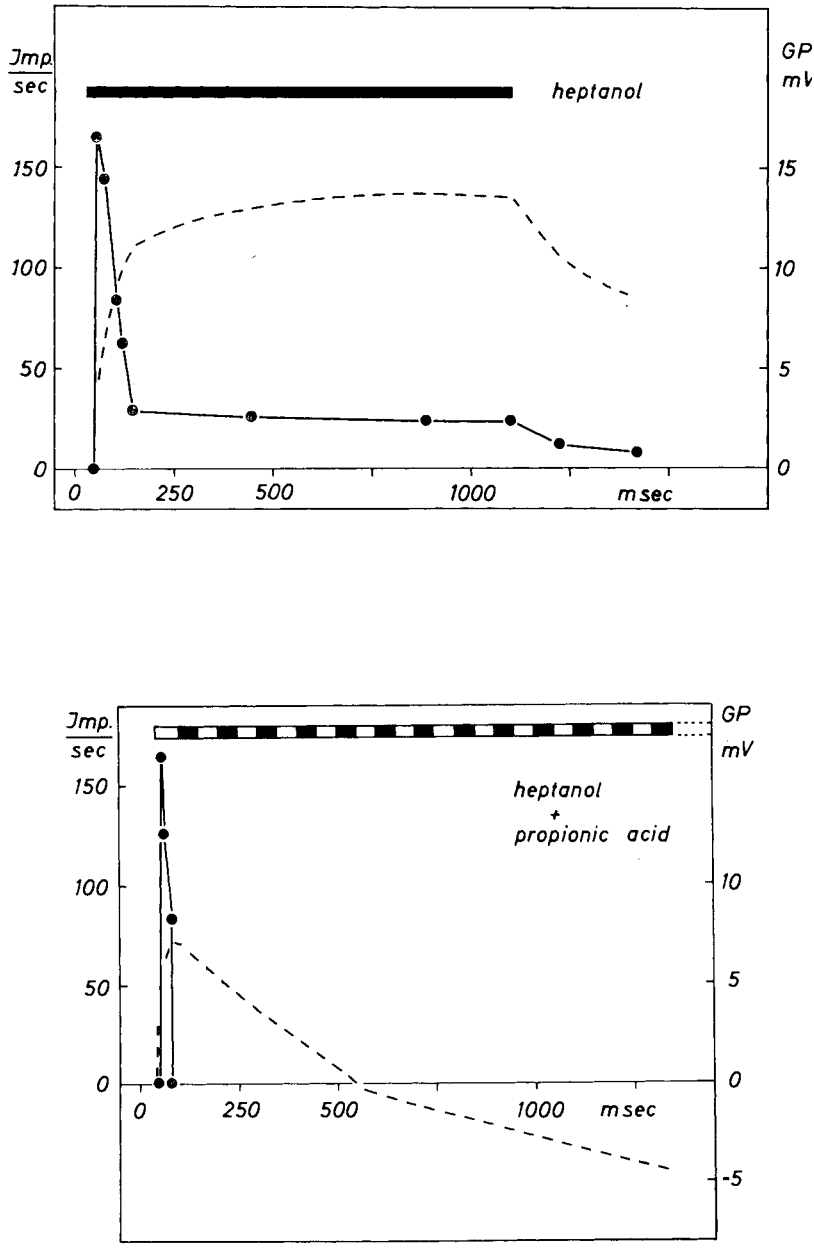


FIG. 7. *Thanatophilus rugosus.* Carrion odor receptor. (A) reaction to heptanol (black bar). (B) reaction to a mixture of heptanol with concentrated propionic acid (black and white bar). Dashed lines: receptor potentials (right-hand ordinates, GP) solid lines with filled circles: impulse frequencies (left-hand ordinates).

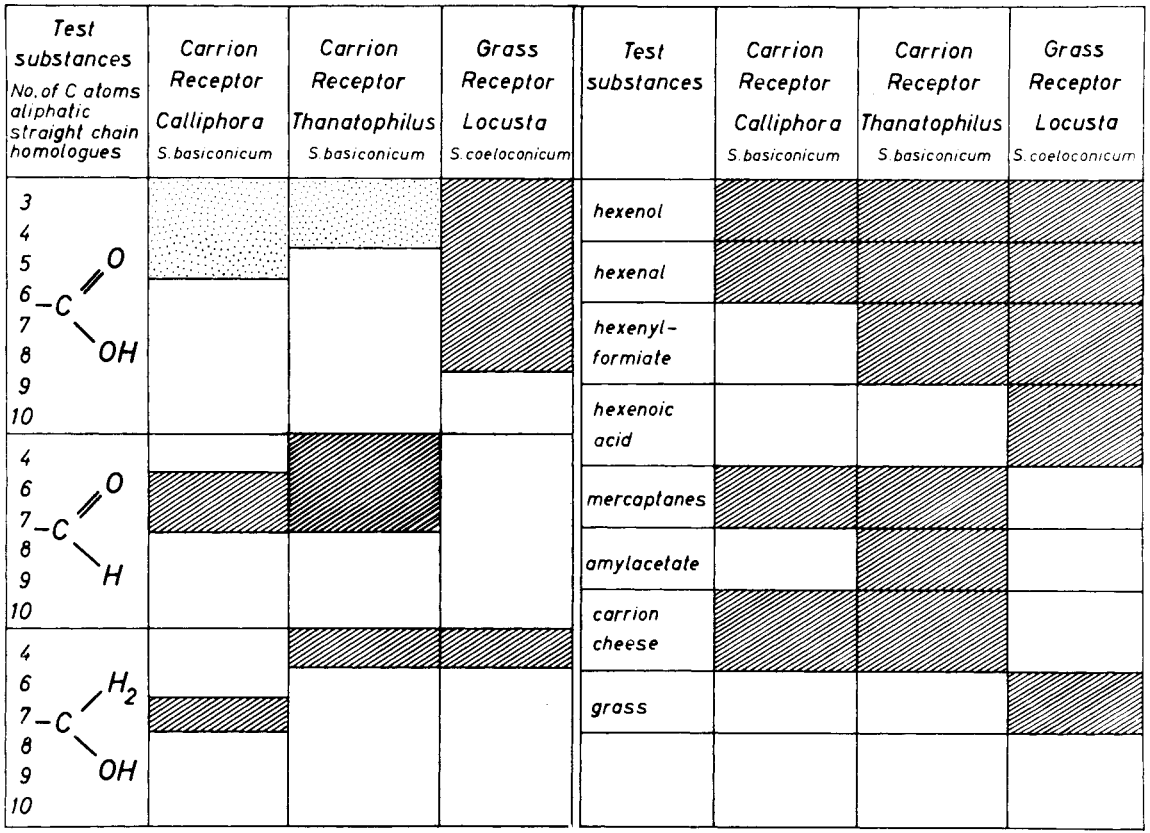


FIG. 8. *Calliphora erythrocephala* (Blowfly), *Thanatophilus rugosus*, *Locusta migratoria*. Reaction spectra of specialized food odor receptors. Left-hand part shows stimuli with fatty acids $\left(\begin{matrix}C=O\\ \diagdown OH\end{matrix}\right)$, aldehydes $\left(\begin{matrix}C\diagup O\\ \diagdown H\end{matrix}\right)$, and alcohols $\left(\begin{matrix}C=OH\\ \diagdown H_2\end{matrix}\right)$, all out of a homologous series of saturated, unbranched hydrocarbon-derivatives. The right-hand part shows-reactions to the "vital" odors and other compounds. Hatched areas excitation, dotted areas inhibition, white areas no response.

The patterning of excitation and inhibition as shown in Fig. 8 relates to olfactory cells specialized to food odor. Besides the receptor response to the vital odor (grass, decaying meat), there is a certain number of compounds it responds to in addition. All the insects shown in the figure do have thousands of receptors of this type on their antennae, all responding uniformly to the set of stimuli as in Fig. 8. Receptors of that kind we called odor specialists (Boeckh, Kaissling and Schneider, 1965).

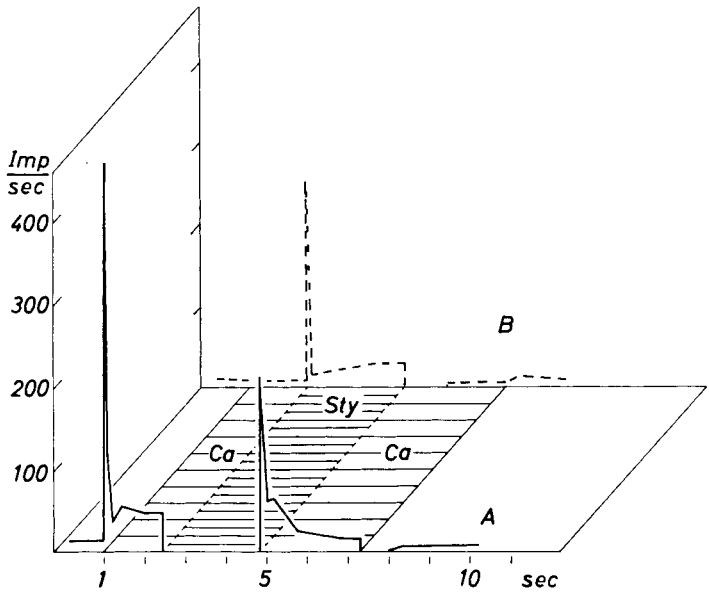


FIG. 9. *Calliphora erythrocephala.* Impulse frequency of two odor specialists (A) carrion odor specialist, (B) styrolylacetate-specialist. (Ca) continuous stimulus with carrion, (Sty) additional stimulus with styrolylacetate.

We also found other odor specialized receptor cells on the antennae of the three insects described so far. The *Calliphora*-antenna has cells responding well to styrolylacetate, a substance which inhibits carrion receptor cells. The reversed effect (carrion as a blocker of the styrolyl acetate receptor) is only weak. Figure 9 shows a superposition of styrolyl acetate upon the response of the carrion receptor cell and simultaneously the response of a styrolylacetate receptor cell. The meaning of this seems to be that a compound does not only excite its specialized cell but simultaneously inhibits another specialist.

On the antenna of the carrion beatle *Thanatophilus*, we found two types of odor specialists with overlapping reaction spectra. Both cell types are

abundant. One is the carrion receptor which also responds with excitation to some other compounds (see Fig. 8) but is inhibited by the ketone cycloheptanone. The other receptor of this pair is—as well as the carrion receptor—responding to amylacetate, but is excited by cycloheptanone. This is exemplified on Fig. 10 with a simultaneous recording from these two neighboring cells which may perhaps even belong to one and the same sensillum.

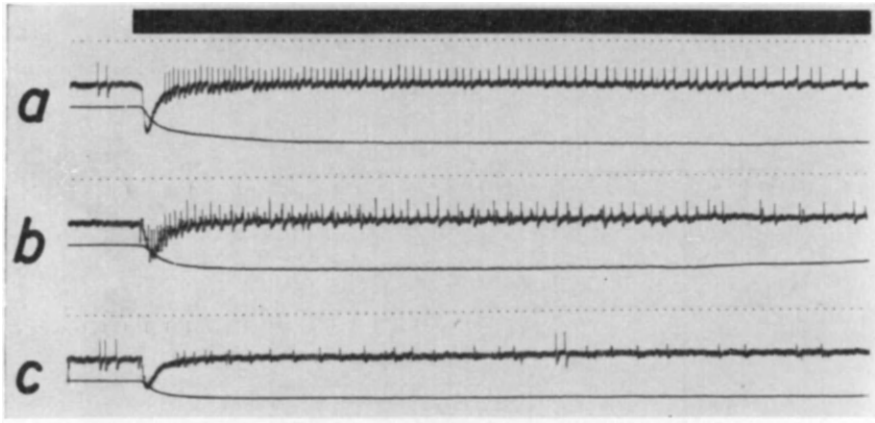


FIG. 10. *Thanatophilus rugosus*. Recordings from two neighboring odor receptors. The cell with the big spike type is a carrion receptor. (a) hexenylformiate, only the big spike appears. (b) amylacetate, both cells respond. (c) cycloheptanone, only the small impulse type responds. Upper beam AC, lower beam DC. Amplitude of the big spike = 0·9 mV. Time mark 20 msec. Stimulus duration is marked by the black bar.

DISCUSSION

Excitation and inhibition in insect olfactory cells show a striking similarity to the depolarizing EPSP's and hyperpolarizing IPSP's of central neurons where one finds corresponding differences in the time course as well as additive effects if the two potentials appear at the same time (Eccles, 1964). Such a similarity, however, is only a formal one as long as the extracellular recording does not give us any information on the real situation of the receptormem brane. It is interesting to note in this context that giant ganglion cells of the snail *Aplysia*, where intracellular recording is easily performed, are depolarized by the extracellular application of some odorants and hyperpolarized by other odors (Chalazonitis, 1965). The time courses are again similar to electrically stimulated synapses and insect odor receptors: fast rising depolarization and slow rising hyperpo-

larization. Chemically and electrically induced de- and hyperpolarizations are additive in the snail ganglion cells. There is—as stated in the introductory chapter—good reason to assume that insect olfactory cells do really show direct (primary) excitatory *and* inhibitory responses to odor stimuli.

Finally, it has to be considered whether inhibitory olfactory response of the kind described here is only the effect of inadequate "irritating" stimuli. This, however, is also very improbable for the following reasons: (1) Substances which inhibit carrion beetle cells very strongly (e.g. fatty acids) are exciting locust receptors (cf. Fig. 8). (2) With the "odor generalist" type of olfactory receptors Schneider, Lacher and Kaissling, 1964 found such a variation in response patterning (+/−/0) that simple irritation can be excluded as an explanation of the phenomenon of inhibitation. (3) With all the many insect olfactory receptors tested so far, no single substance was an effective inhibitor to all of them. Even "caustic" substances as ammonia and chlorine do effect only very few types of receptor cells. All this leads to the final conclusion that those olfactory responses which induce an inhibition are adequate and physiological.

It is challenging to assume that inhibitory responses are important as a means of peripheral coding in olfactory systems (Schneidner, 1963 b; Schneider, Lacher and Kaissling 1964; Boeckh, Kaissling and Schneider, 1965). A coding system is immediately improved, if one has not only a+/0 but a+/0/− system available. In this way such a +/0/− system in a receptor population with overlapping reaction spectra provides the basis for a peripheral coding of many different odors (Schneider, 1963; Schneider, Lacher and Kaissling, 1964; Boeckh, Kaissling and Schneider, 1965).

Unfortunately, this attractive hypothesis has not been tested as yet with crucial behavior experiments using excitatory and inhibitory stimuli. Such a research program, however, depends upon a detailed knowledge of the receptor in question. Here, the electrophysiological study of the cells' properties has to be done first before the behavior test can begin.

SUMMARY

1. Olfactory receptor cells of insect antennae respond to qualitatively different odor stimuli with either excitation (increase of impulse frequency) or inhibition (depression of impulse frequency). No odorous compound is only excitatory or inhibitory.

2. Receptor potentials and nerve impulses are recorded simultaneously with the same extracellular electrode. The resting potential is decreased

(depolarization) during excitation and increased during inhibition (hyperpolarization?).

3. The time courses of excitatory and inhibitory receptor potential are different.

4. The electrophysiological reaction threshold of the inhibitory stimulus is higher than of the excitatory one.

5. Excitatory and inhibitory effects of different odors applied simultaneously to a cell are additive according to the time course differences.

6. Excitation and inhibition are components of the odor specific response pattern of a receptor.

7. The inhibitory odor stimulus is assumed (supposed) to be as adequate a stimulus for the receptor cell as the excitatory stimulus.

ACKNOWLEDGEMENT

The author is indebted to Dr. D. Schneider for preparing the English manuscript and also for constructive discussions, and to Dr. W. Steiner (Drom Company, Munich) for providing us with many test substances.

REFERENCES

BOECKH, J. 1962. Elektrophysiologische Untersuchungen an einzelnen Geruchsrezeptoren auf den Antennen des Totengräbers (Necrophorus, Coleoptera). *Z. vgl. Physiol.* **46,** 212–248.

BOECKH, J., K. E. KAISSLING and D. SCHNEIDER. 1965. Insect olfactory receptors. Cold Spring Harbor Sympos. *Quant. Biol.* **30,** 263–280.

BULLOCK, T. H. 1959. Initiation of nerve impulses in receptors and central neurons. *Rev. Modern Physics*, **31,** 504–514.

CHALAZONITIS, A. and N. CHALAZONITIS 1965. Molecular control of the excitability of giant neuron in relation to olfactory perception mechanisms. Proc. second interntl. Sympos. on *Olfaction and Taste* (T. HAYASHI, Ed.) (in press).

ECCLES, J. C. 1964. *The Physiology of Synapses.* Springer-Verlag, Berlin.

GESTELAND, R. C. 1963. *Initial events of the electroolfactogram.* Manuskript.

HARTLINE, H. K. 1940. The receptive fields of optic nerve fibres. *Am. J. Physiol.* **130,** 690–699.

HARTLINE, H. K., H. G. WAGNER and F. RATLIFF 1956. Inhibition in the eye of Limulus. *J. gen. Physiol.* **39,** 651–673.

HODGSON, E. S. 1957. Electrophysiological studies of Arthropod chemoreception. II. Responses of labellar chemoreceptors of the Blowfly to stimulation with carbohydrates. *J. Insect Physiol.* **1,** 240–247.

KUFFLER, S. W. and C. EYZAGUIRRE. 1955. Synapse inhibition in an isolated nerve cell. *J. gen. Physiol.* **39,** 155–184.

LACHER, V. 1964. Elektrophysiologische Untersuchungen an einzelnen Rezeptoren für Geruch, Kohlendioxyd, Luftfeuchtigkeit und Temperatur auf den Antennen der Arbeitsbiene und der Drohne (*Apis mellifica* L.). *Z. vgl. Physiol.* **48,** 587–623.

LETTVIN, J. Y. and R. C. GESTELAND. 1965. Speculations on smell. Cold Spring Harbor Sympos. *Quant. Biol.* **30,** 217–225.

MORITA, H. 1959. Initiation of spike potentials in contact chemosensory hair of insects. III. DC stimulation and generator potential of labellar chemoreceptors of *Calliphora*. *J. cell. comp. Physiol.* **54,** 189–204.

NICKLAUS, R. 1965. Die Erregung einzelner Fadenhaare von *Periplaneta americana* in Abhängigkeit der Größe und Richtung der Auslenkung. *Z. vgl. Physiol.* **50,** 331–362.

SCHNEIDER, D. 1957(*a*). Elektrophysiologische Untersuchungen von Chemo- und Mechanorezeptoren der Antenne des Seidenspinners *Bombyx mori* L. *Z. vgl. Physiol.* **40,** 8–41.

SCHNEIDER, D. 1957(*b*). Electrophysiological investigation on the receptors of the silk moth during chemical and mechanical stimulation. *Experientia* (Basel) **13,** 89–91.

SCHNEIDER, D. 1963(a). Electrophysiological investigation of insect olfaction. Proc. first interntl. Sympos. on *Olfaction and Taste* (Y. ZOTTERMAN, Ed.) Pergamon Press, Oxford.

SCHNEIDER, D. 1963(*b*). *Vergleichende Rezeptorphysiologie am Beispiel der Riechorgane von Insekten*. Jahrbuch 1963 Max-Planck-Geselschaft.

SCHNEIDER, D. 1965/66. Chemical sense communication in insects. *Soc. Exptl. Biol. Sympos.* N°. 20 (in press).

SCHNEIDER, D. and BOECKH, J. 1962. Rezeptorpotential und Nervenimpulse einzelner olfaktorischer Sensillen der Insektenantenne. *Z. vgl. Physiol.* **45,** (405–412).

SCHNEIDER, D., V. LACHER and K. E. KAISSLING. 1964. Die Reaktionsweise und das Reaktionsspektrum von Riechzellen bei *Antheraea pernyi* (Lepidoptera, Saturniidae). *Z. vgl. Physiol.* **48,** 632–662.

THURM, U. 1963. Die Beziehungen zwischen mechanischen Reizgrößen und stationären Erregungszuständen bei Borstenfeld-Sensillen von Bienen. *Z. vgl. Physiol.* **46,** 351–382.

TUCKER, D. and T. SHIBUYA 1965. Unit response of olfactory receptors in vultures. Proc. of the second interntl. Sympos. *Olfaction and Taste*, Tokyo 1965. (T. HAYASHI, Ed.) This volume, p. 219.

WOLBARSHT, M. L. and F. E. HANSON 1965. Electric activity in the chemoreceptors of the Blowfly. III. Dendritic action potentials. *J. gen. Physiol.* **48,** 673–683.

HYDROCARBON INHIBITION OF PRIMARY CHEMORECEPTOR CELLS

E. S. HODGSON and R. A. STEINHARDT

Columbia University, New York, N.Y., USA

PRACTICALLY all studies on the chemical senses have dealt with increased activities of the receptor cells. Consequently, a reaction to a taste or odor stimulus is viewed as the eventual result triggered by an increased frequency of afferent receptor impulses. Even when it is technically impossible to monitor the outputs of the primary chemoreceptor cells involved, this assumption is usually automatic.

Not all cases, however, may properly fit this conception. It is the purpose of this communication to analyze a few examples of such exceptions, in the hope that they may provide ideas of some general interest.

DEVELOPMENT OF PREPARATION AND PROBLEM

It is an interesting coincidence for this symposium that these problems grew out of early electrophysiological studies on insects. Just a decade ago, chemoreceptor cells on the mouthparts of flies became the first single-cell primary chemoreceptors, in any animal, to yield to direct electrophysiological analysis—a development which came independently, and almost simultaneously, in the United States and Japan (Hodgson, Lettvin and Roeder, 1955; Morita, 1956). This approach has fostered a continuing series of stimulating and cooperative exchanges with Japanese laboratories, including some contributions to the present experiments, as acknowledged below.

Within a few years after development of electrical techniques amenable to the fly's labellar chemoreceptors, the general actions of cations, sugars, and water upon these sense organs were discovered (c.f. the reviews of Dethier, 1963; Hodgson, 1964). The modes of actions of other compounds, which had previously been subjected to extensive behavioral studies, raised new problems. Alcohols and other aliphatic hydrocarbons were outstanding examples of this type.

Alcohols were among the first group of compounds in which correlations between molecular structure and stimulating effectiveness were sought, using behavioral tests on blowflies. Typically, these compounds elicited patterns of "rejection" behavior, even in anosmic flies. Alcohols applied to receptors on one leg of a fly also inhibited responses to sugars applied to the contralateral leg (Dethier, 1963). It was assumed that, in order to do this, the alcohols must stimulate a "rejection" fiber, such as the one sensitive to electrolytes. This interpretation seemed to be supported by the early electrical recordings from taste receptors on the mouthparts of flies (Hodgson and Roeder, 1956). While this was a possible explanation for the observations made up until that time, it posed the problem of how chemicals as different as cations and hydrocarbons could activate the same receptor cell.

Recent experiments, using the improved recording technique of Morita and Yamashita (1959), suggested that hydrocarbons could modulate receptor activities in more than one way. A more detailed investigation was, accordingly, initiated by Steinhardt, Morita, and Hodgson (1963).

Labellar chemoreceptors of blowflies *(Phormia regina)* were chosen for testing because impulses from three chemoreceptor cells, of the four sometimes present, could be identified in favorable electrophysiological records. Standard side-wall recording techniques were used, except that precise controls for stimulus duration were added. The freshly-filled stimulating electrode was mounted on the movable cone of a small loudspeaker, energized by a Grass stimulator. Thus, the exact time that the chemical solution touched the hair tip could be adjusted from the stimulator—a control that proved quite crucial for reproducibility of results.

Reproducible responses were obtained at 5 min test intervals, as long as the stimulus durations were below 500 msec. Steady-level responses were obtained by counting impulses during 200–300 msec periods, starting 50–100 msec from the moment of stimulus application. Normal alcohols, methanol through pentanol, were tested because these cover the most significant points on the behavioral data curves (Dethier and Chadwick, 1947).

RESULTS WITH ALCOHOLS

A typical result is shown in Fig. 1. Both distilled water and NaCl applications to the hair elicit predominantly single fiber responses—the action potentials coming from the water and the cation receptors. Ethanol, mixed with the salt solution, inhibits the salt response. No stimulation was observed to preceed this inhibition. After stimulation with a higher (0·3 M) con-

centration of alcohol, there is an injury discharge from both water and salt receptors. The diagnosis of injury is made because of the non-specificity of the effect, and its persistence after withdrawal of the stimulating solution.

In no case did any of the tested alcohols directly stimulate either electrolyte, sugar, or water receptors. On the contrary, all receptor types were

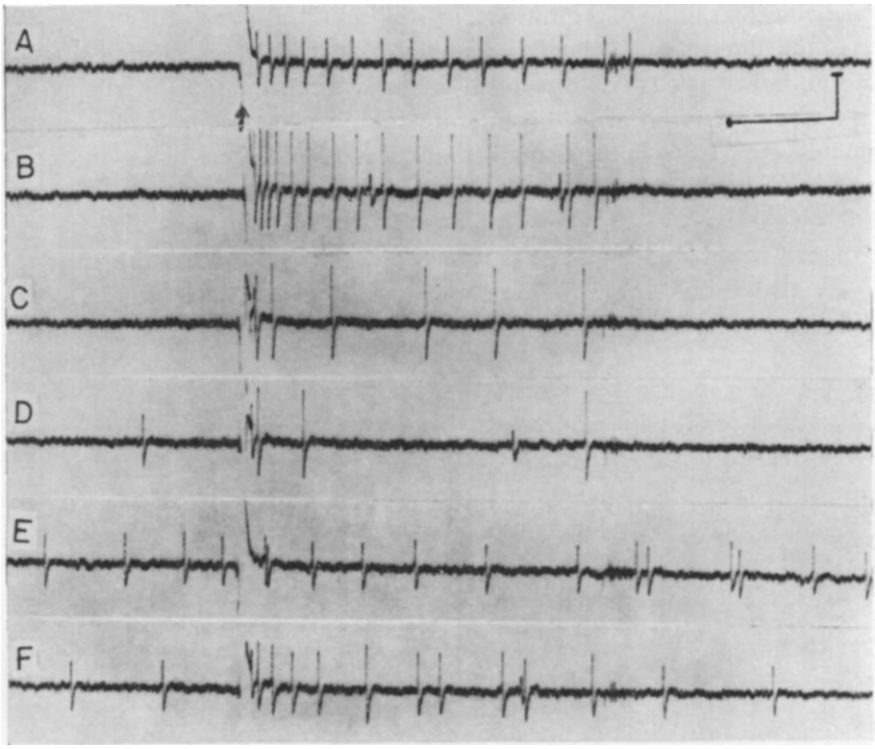


FIG. 1. Inhibition and injury effects of ethanol. *A*—Stimulation with distilled water, showing water response. *B*—0·2 M NaCl, showing salt fiber response. *C*—0·2 M NaCl plus 1 M ethanol, showing inhibition of salt response. *D*—Further inhibition of response, second test, with first spontaneous spike appearing before stimulation. *E*, *F*—Control stimulations with distilled water after prolonged exposure to 6 M ethanol in 0·2 M NaCl, showing injury effect. Horizontal bar, 100 msec in all records; vertical bar 500 μV, except as otherwise noted.

inhibited to some degree. This conclusion is based upon results of multiple tests of each alcohol on at least 9 different preparations. Because of the large variability between different receptor cells of the same type, even on the same labellar preparation, the inhibitory effectiveness of alcohols is

best compared at the 50% behavioral rejection threshold concentrations. At those concentrations, all the alcohols produced 30–100% inhibition of the steady-state response to 0·1 M sucrose or 0·2 NaCl. As carbon chain length increased above propanol, the salt receptors were uniformly more sensitive to alcohols than the sugar receptors. There was complete inhibition of steady-state responses to 0·2 M NaCl at concentrations of propanol, butanol, and pentanol which were one-third to one-sixth of the behavioral rejection threshold concentrations.

In the absence of any increased spike frequencies during alcohol stimulation, how is it possible to explain the behavioral rejections, particularly in the two leg experiment? It's interpretation would seem to require some impulses to pass from the alcohol-stimulated leg to the central nervous system, in order to inhibit effects of afferent impulses from sugar fibres in the contralateral leg.

Three hypotheses might be advanced: (1) the tarsal chemoreceptors, stimulated in the behavioral tests, might react entirely differently to alcohols than the labellar receptors studied electrophysiologically; if not, then

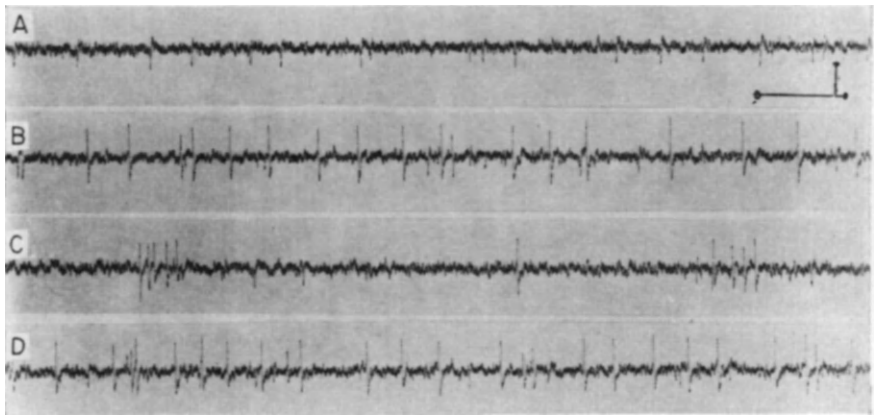


FIG. 2. Reversible hydrocarbon inhibition of tarsal sugar receptors. *A*—Control application of 0·1 M choline chloride. *B*—Sugar receptor impulses during stimulation with 0·1 M sucrose in 0·1 M choline chloride. *C*—The inhibited sugar response to 0·1 M sucrose, 0·1 M choline chloride plus 0·16 M pentanol. *D*—Recovery of sugar response to 0·1 M sucrose. Vertical calibration, 300 μV. Recordings made with capacity-coupled amplifier.

(2) mere lack of afferent impulses from one leg may inhibit normal coordination of feeding reflexes; or (3) injury discharges following prolonged alcohol stimulation may inhibit feeding. The tarsal receptors cannot be

tested with the side-wall technique because of their toughness. However, tip stimulation can be used to test hypothesis (1).

A typical result of tarsal stimulation is shown in Fig. 2. Choline chloride (0·2 M) was used as a non-stimulating electrolyte in the tip electrode, and sugar responses were obtained from 0·1 M sucrose. These were reversibly inhibited by alcohol, as were the salt responses in other records. Higher concentrations of alcohols produced irreversible injury effects. The tarsal and labellar receptors, therefore, exhibit similar responses to alcohols. More information will be needed for a clear choice between the two hypotheses concerning mechanisms of contralateral integration in the CNS.

One apparent exception to the alcohol effects was observed during the tests on inhibition of labellar salt receptors. At low concentrations of NaCl, which inhibit the water response about 50%, both salt and water receptor discharges are easily discerned in the records. When inhibiting concentrations of alcohols are added to this mixture, the frequency of water discharge increases. Since this occurs only when there is some salt inhibition of the

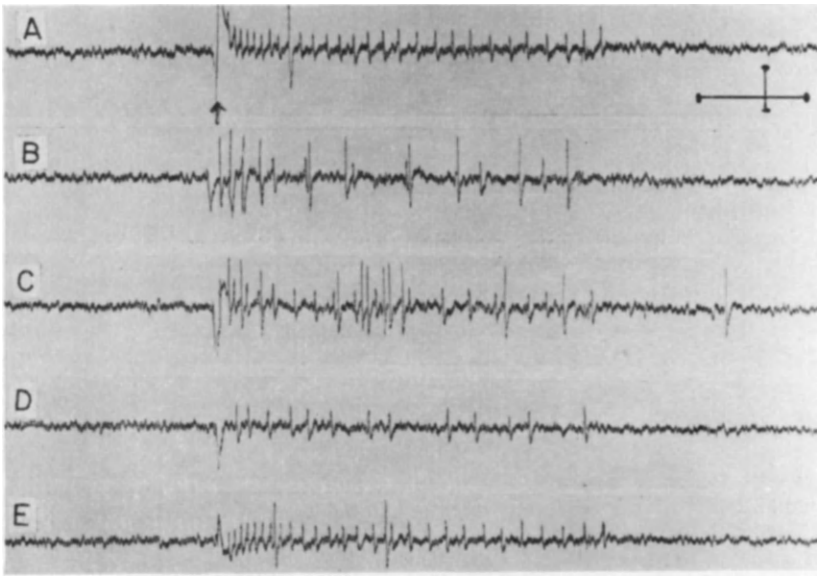


FIG. 3. Indirect stimulation of water receptor by addition of alcohol to salt solution. *A*—Distilled water, showing small spikes of water receptor discharge, and single large salt spike. *B*—0·15 M NaCl, showing increased salt spikes and inhibited water response. *C* and *D*—First and second applications of 0·15 M NaCl plus 0·55 M propanol, showing increase of water response above the inhibited levels observed with salt alone. *E*—Repeat of distilled water alone, with two salt spikes possibly resulting from some residual cations.

water response, it is interpreted as an indirect effect on the water fiber, rather than direct stimulation. Such a case is illustrated in Fig. 3. This type of effect could easily have been misinterpreted prior to the discovery of the water fiber, and it might explain early reports of increased receptor firing elicited by alcohols.

AMINES

For quantitative studies on effects of long-chain hydrocarbons on chemoreceptors, the alcohols are inconvenient, due to evaporation problems and the relatively small range between concentrations producing inhibition and those producing injury. Preliminary tests showed that hydrocarbon amines produced effects similar to the alcohols, but without the disadvantages of the alcohols. Octylamine, in particular, exhibits a large gap between inhibitory and injurious concentrations.

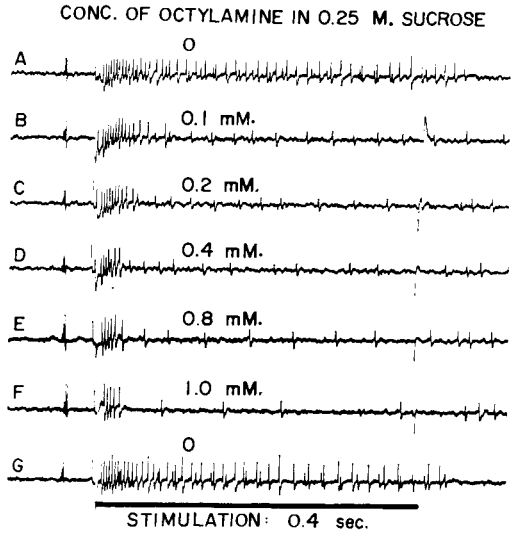


FIG. 4. Inhibition of sucrose and water receptor responses by octylamine. The small spikes are from the water receptor and the large spikes are from the sugar receptor. Note the higher concentrations of OA necessary to inhibit the water response. (After Steinhardt, Morita, and Hodgson, 1963.)

The relative sensitivity of different receptor types to octylamine inhibition is the same as found in the higher alcohols: the electrolyte receptor is most sensitive, then the sugar receptor, and finally the water receptor. Progressive inhibition of the sugar receptor, with relative immunity of the water receptor, is shown in Fig. 4. Note also the reversibility of the effect.

The exact amount of inhibition depends upon the amount of stimulant applied. In the cases of salt and water receptors, the relationship between hydrocarbon concentrations and the degree of inhibition is linear, although

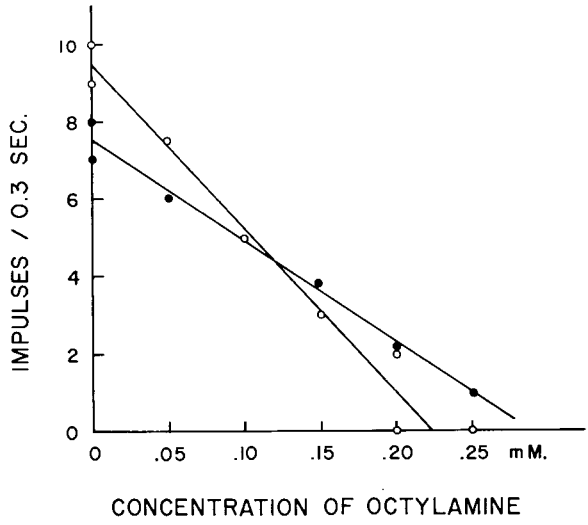


FIG. 5. Octylamine inhibition of two different salt receptors. Note linear relationship between concentration of hydrocarbon and degree of inhibition.

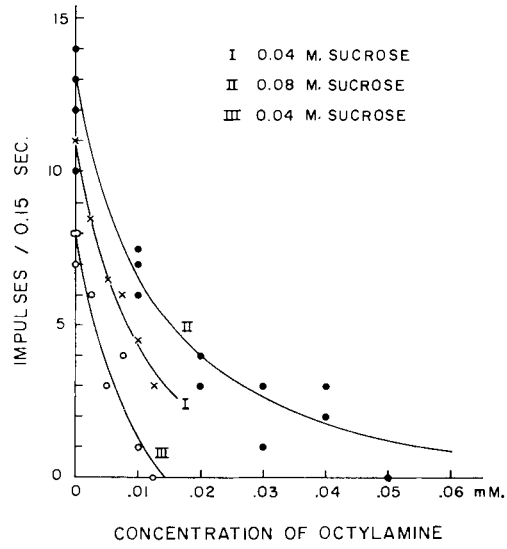


FIG. 6. Octylamine inhibition of a sugar receptor, non-linear at 3 different concentrations of sucrose.

the slope of the line expressing this relationship varies from receptor to receptor. Four cases have been analyzed in this manner for salt and water receptors. Figure 5 shows a typical result.

When similar plots are made of the relationship between octylamine concentration and inhibition of sugar receptors, no straight line relationship is found (Fig. 6). Consequently, it appears that there must be at least two different mechanisms by which hydrocarbon inhibition occurs. These are considered below.

COMPARISON WITH LOBSTER CHEMORECEPTORS

There remained a possibility that inhibition, rather than stimulation, might be a peculiarity of alcohol and amine effects on blowfly chemoreceptors alone. To check this possibility, similar tests were run on dactyl chemoreceptors of the spiny lobster *Panulirus argus*. These chemoreceptors are chiefly sensitive to amino acids. Essentially, the procedure involves dissection of nerve bundles down to a few fibers, which are contacted with external electrodes. The technique is an adaptation of methods previously used in recording from crab chemoreceptors (Case, 1964; Levandowsky and Hodgson, 1965).

In 7 lobster preparations, it was possible to record fairly steady firing rates from a number of fibers while the dactyls were bathed in a continuously flowing stream of sea water. 10^{-4} M glutamic acid was added to the sea

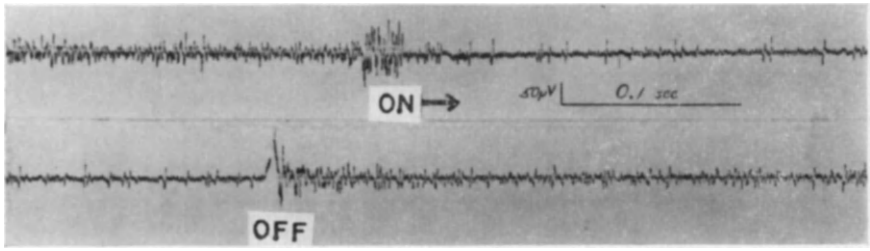


FIG. 7. Pentanol inhibition of glutamic acid response by dactyl chemoreceptors of *Panulirus*. 0·1 M pentanol introduced into flowing stream of 10^{-4} M glutamic acid in sea water at point designated; pentanol removed during lower section of this continuous trace. Note that recovery is almost complete. Calibrations as indicated.

water stream as a stimulant which increased the frequency of receptor firings. Introduction of 0·1 M pentanol, or 0·1–25 mM octylamine, quickly reduced the frequency of impulses, although identifications of single unit

responses to inhibition were only possible in 3 cases. The effects of octylamine were reversible, but those of pentanol only partially so. Higher concentrations evoked long-lasting injury discharges. Figure 7 shows the most fully reversible case of pentanol inhibition. Figure 8 illustrates octylamine

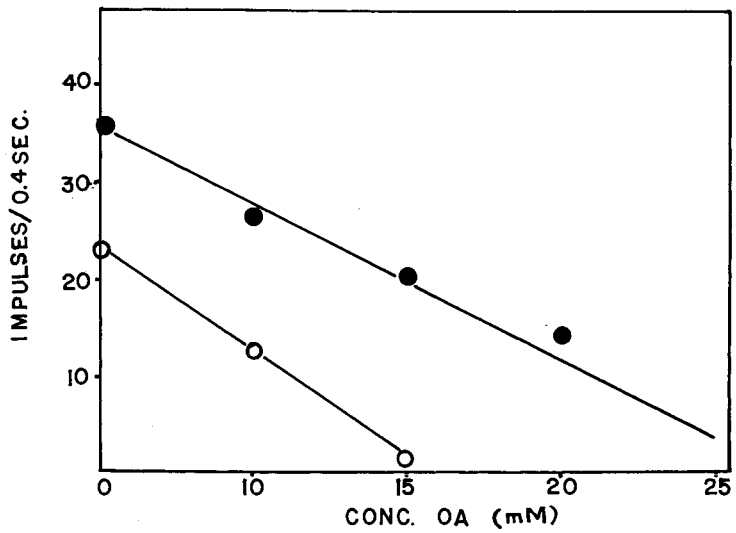


FIG. 8. Relationship between octylamine concentration and inhibition in two different dactyl receptors of *Panulirus*, responding to 10^{-4} M glutamic acid.

inhibition on two different fibers of another preparation, where single fiber responses could be identified. The similarity of these alcohol and amine effects to those observed with labellar receptors argues that they are probably of quite general occurrence.

MECHANISMS OF INHIBITION

A linear relationship between hydrocarbon concentrations and their effects is characteristic of ideal oil–water partition phenomena. It is also characteristic of the classical hypothesis of narcosis (Meyer, 1899; Overton, 1901), and a narcotic effect might explain the inhibition of salt and water receptors. It is noteworthy that the two stages of effects, inhibition and injury, correspond to the results obtained with alcohol narcosis of nerve (Laget *et al.*, 1951; Mullins and Gafferey, 1954). However, inactivation by denaturation of the receptor molecules is another alternative, and this is especially so for the non-linear inhibition of the sugar responses.

The different characteristics of the sugar inhibition might, of course, result from multiple mechanisms, each of which did involve a linear relationship between inhibitor concentration and amount of inhibition. Together, these mechanisms might sum as a curve. As another alternative, the hydrocarbons might bind to receptor molecules and inactivate them, not unlike enzymatic inhibition. Kinetic analyses, to be reported in detail elsewhere (Steinhardt, Morita, and Hodgson, 1963) suggest that, while hydrocarbon effects on sugar receptors superficially resemble competitive inhibition, other less specific effects on sugar receptor sites are more likely.

A non-competitive denaturation of protein in the sugar receptor membrane is suggested, also, by another observation. Glycerol, which does not stimulate but does stabilize all kinds of proteins, blocks the inhibitory action of octylamine. The glycerol concentrations needed to do this are comparable to concentrations used in stabilizing proteins against denaturation (Klotz, 1965; Chilson *et al.*, 1965).

DISCUSSION AND CONCLUSIONS

In assessing the significance of hydrocarbon inhibition, two considerations are paramount. Are such stimuli likely to be encountered in nature? Do their effects on chemoreceptors have any positive role in behavioral patterns, beyond simply anesthetizing parts of the nervous system, thereby eliminating part of the capacity to respond?

A positive answer to the first question is encountered more frequently than is commonly realized. Aliphatic alcohols are prominent components of bacterial breakdown products, and they are among the more important secondary synthetic products of green plants. They are encountered, at least by insects, as both taste and olfactory stimuli, and have been used as insect attractants (Dethier, 1947). Amines are common products of protein breakdown, and are found in great diversity in such materials as fungi and sea water (Dethier, 1947; Levandowsky and Hodgson, 1965). Preliminary tests indicate that some naturally occurring aliphatic acids have similar inhibitory effects on these preparations, so the effects are probably not uncommon among hydrocarbons.

Concerning the second question, it should be noted that the "rejection" behavior of a fly is not simply a lack of response. If, for example, the proboscis is partially extended when stimulated, it is withdrawn. If the animal is allowed to move freely, it can be seen that a coordinated pattern of avoidance behavior is triggered. A lobster, after exposure of one foreleg dactyl to octylamine, begins "sweeping" movements with its legs which, under

ordinary conditions, would rake food toward the mouth. These can hardly be haphazard results of nerve anesthesia!

The different characteristics of sugar receptor cell inhibition would explain why it appeared, from earlier behavioral studies, that hydrocarbons were not narcotizing blowfly chemoreceptors. It is clear from the present electrophysiological data that, at the concentrations of alcohols used in the behavioral tests, the main effect on the receptors would be inhibition of the sugar receptor, rather than stimulation of another cell. Either inhibition of the water receptor, or injury to all receptor types, might explain the experiments in which an alcohol applied to one tarsus inhibits the acceptance of a sucrose stimulus on the contralateral tarsus.

The results suggest that in some cases, particularly those dealing with such mixtures of chemicals as are found in natural situations, information may be conveyed by modulation of frequency of chemoreceptor firings, with some stimulus components tending to increase the afferent impulse flow, while others tend to decrease it. Little novelty can be claimed for this general viewpoint (cf. Hodgson, 1965), but the hydrocarbon effects illustrate some of the possibilities quite conveniently. The present results can be summarized as follows:

1. The fundamental actions of straight-chain hydrocarbons on primary chemoreceptor cells of arthropods were studied quantitatively. Lower alcohols and long-chain amines act in two stages: first a reversible inhibition, then injury of salt, water, and sugar receptors in labellar sensilla of flies; similar effects on amino acid receptors in lobsters were noted.

2. The primary effects on salt and water receptors resemble hydrocarbon narcosis of nerve. Effects of hydrocarbons on sugar receptors do not fit this interpretation, but several alternative explanations remain.

3. Electrophysiological results from both labellar and tarsal chemoreceptors of flies indicate that previously reported hydrocarbon rejection thresholds are best explained by the inhibition of sugar receptors.

ACKNOWLEDGMENTS

This investigation was aided by U.S. Public Health Research Grant AI 02271. Dr. H. Morita collaborated in some of the experiments at Columbia University, and he has helpfully discussed all of them. The studies on lobster chemoreceptors were made at the Lerner Marine Laboratory of the American Museum of Natural History.

REFERENCES

CASE, J. 1964. Properties of the dactyl chemoreceptors of *Cancer antennarius* Stimpson and *C. productus* Randall. *Biol. Bull.* **127,** 428–446.

CHILSON, O. P., L. A. COSTELLO and N. O. KAPLAN. 1965. Effects of freezing on enzymes. *Fed. Proc.*, **24,** Suppl. 15, 55–65.

DETHIER, V. G 1947. *Chemical Insect Attractants and Repellents.* Phila., Blakiston. 289 pp.

DETHIER, V. G. 1963. *The Physiology of Insect Senses.* New York, Wiley. 266 pp.

DETHIER, V. G. and L. E. CHADWICK. 1947. Rejection thresholds of the blowfly for a series of aliphatic alcohols. *J. Gen. Physiol.* **30,** 247–253.

HODGSON, E. S. 1964. Chemoreception. Chap. 9 in *Physiology of Insecta,* Vol. 1. M. ROCKSTEIN, ed. New York, Academic Press.

HODGSON, E. S. 1965. The chemical senses and changing viewpoints in sensory physiology. *Viewpoints in Biology* **4,** 81–122.

HODGSON, E. S., J. Y. LETTVIN and K. D. ROEDER. 1955. The physiology of a primary chemoreceptor unit. *Science,* **122,** 417–418.

HODGSON, E. S. and K. D. ROEDER. 1956. Electrophysiological studies of arthropod chemoreception. I. General properties of the labellar chemoreceptors of diptera. *J. Cell. Comp. Physiol.* **48,** 51–76.

KLOTZ, I. M. 1965. Role of water structure in macromolecules. *Fed. Proc.* **24,** Suppl. 15, 24–33.

LAGET, P., J. M. POSTERNAK and R. MANGOLD. 1951. Données electrophysiologiques sur action narcotique des alcohols aliphatiques. In *Mechanism de la Narcose,* C.N.R.S., Paris, pp. 189–194.

LEVANDOWSKY, N. and E. S. HODGSON. 1965. Amino acid and amine receptors of lobsters. *Comp. Biochem. Physiol.* **15,** 111–112.

MEYER, H. 1899. Zur Theorie der Alkoholnarkose. *Archiv. Exp. Path. Pharm.* **42,** 109–118.

MORITA, H. 1956. Personal communication.

MORITA, H. and S. YAMASHITA. 1959. Generator potential of insect chemoreceptor. *Science,* **130,** 922.

MULLINS, L. J. and C. T. GAFFERY. 1954. Depolarization of nerve by organic compounds. *Proc. Soc. Exp. Biol. Med.* **85,** 144–149.

OVERTON, E. 1901. *Studien über die Narkose.* Jena, G. FISHER.

STEINHARDT, R., H. MORITA and E. S. HODGSON. 1963. Electrophysiological analysis of inhibition and specificity of blowfly chemoreceptors. *Proc. 16th Internat. Cong. Zool.* **3,** 99–101.

ELECTRICAL AND BEHAVIORAL RESPONSES TO AMINO ACID STIMULATION IN THE BLOWFLY*

MYRON L. WOLBARSHT and FRANK E. HANSON†

Biophysics Division
U.S. Naval Medical Research Institute
Bethesda, Maryland 20014, U.S.A.

I. INTRODUCTION

The function of an animal's external chemoreceptor must be such that it responds meaningfully to the classes of compounds which are required for nutrition. It must also indicate those compounds which are deleterious. Yet some animals accomplish this with very simple chemoreceptors. For example, the chemoreceptive hairs of the blowfly contain at most 4 neurons which could be chemoreceptors (Wolbarsht, 1965). Of these, two are responsive to nutrients, sugar and water, and one responds to monovalent cations at concentrations high enough to perhaps be deleterious. If a fourth chemoreceptor exists, its identification has eluded definitive physiological demonstration. However, a behavioral study by Dethier (1961) suggests that the blowfly, *Phormia regina* Meigen, may detect some of the non-carbohydrate solutes in a brain–heart infusion. Using behavioral tests, Robbins *et al.* (1965) have shown that the housefly, *Musca*, can taste phosphate salts of certain amino acids but not the free forms. Since several investigators have shown that other arthropods (crabs) can taste free amino acids (Case and Guilliam, 1963; Laverack, 1963), we have tested the possibility that the blowfly's chemoreceptors can detect free amino acids and cause the positive behavioral responses to protein infusions reported by Dethier (1961).

* The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Navy Department or the Naval service at large.

† Dr. Hanson's present address is: Laboratory of Physical Biology, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland.

Our presentation is in two parts: a discussion of the behavioral and electrophysiological responses to a variety of amino acid and protein solutions, and an analysis of the basic electrophysiology of the receptors, with an emphasis on the receptor potentials resulting from amino acid stimulation.

Two types of electrical changes, impulses and slow potentials, can be recorded when chemical stimuli are applied. The action potential type of response is by far the best known effect. There are at least three impulse types, which have been identified as responses to water, sugar, and the monovalent cations, respectively. The other event occurring upon application of most chemicals is a receptor potential, a slow DC shift in the baseline, which Morita (1959) has classified as a "generator potential". In this paper we have used two definitions for "receptor" and "generator" potentials given by Wolbarsht (1960). "Receptor potential" is applied to any nonpropagated potential recorded near a receptor, be it intra- or extracellular and hyper- or depolarizing in nature. "Generator potential" is reserved for those potentials existing intracellularly at the site of impulse initiation.

To demonstrate that amino acids can be specifically detected by chemoreceptors one would have to show a unique electrical response to stimulation by a solution containing an amino acid. In general, our electrophysiological studies have failed to show a specific response of this type to amino acids or proteins. In agreement with these findings our behavioral studies have shown that the blowfly acts as if it cannot taste those solutions.

II. METHODS

The behavioral work was done using a modified method of Dethier and Chadwick (1947) whereby the tarsi and then the retracted proboscis were dipped into the experimental solutions to produce proboscis extension. Laboratory reared virgin imago *Phormia regina* Meigen blowflies were attached to waxed sticks 1–2 days after eclosion and given 2–8 trials per day for 3 days. The experimental animals were 1–4 days old and so should have been in the period of peak protein intake according to Dethier (1961).

All amino acids were reagent grade and the proteins (keratin, casein, gelatin, bovine albumin) were purified forms suitable for molecular weight determinations. The electrophysiological experiments used the sidewall recording technique (see Fig. 1) of Evans and Mellon (1962) which was modified from that of Morita (1959). The equipment has been described in detail previously by Wolbarsht and Hanson (1965).

III. RESULTS AND DISCUSSION

A. Behavioral Tests

Since Dethier's study (1961) indicated that virgin female blowflies showed their highest protein intake during their first 5 days after eclosion, 1- and 2-day-old virgin flies were tested for 3 days in succession. The results of behavioral tests of responses to 18 common amino acids are largely negative, as shown in Table 1. A similar study showed solutions or slurries of

TABLE 1. BEHAVIORAL TEST OF AMINO ACIDS

Amino acid	Conc.	Responses			Control responses to 0·1 M sucrose		
		+	±	0	+	±	0
DL glutamic acid	5×10^{-2}	1	3	28	30	0	2
DL glutamic acid	5×10^{-3}	0	0	28	27	0	1
L glutamic acid	10^{-2}	0	0	26	24	0	2
DL aspartic acid	10^{-2}	0	1	35	30	0	6
L-histidine	10^{-1}	1	0	23	23	0	1
glycine	10^{-1}	1	1	14	14	0	2
DL-serine	10^{-1}	1	0	29	29	0	1
L-tyrosine	10^{-3}	0	0	16	15	0	1
DL-tryptophan	10^{-2}	0	0	16	14	0	2
DL-threonine	10^{-1}	0	1	15	16	0	0
DL-methionine	10^{-1}	0	0	26	22	0	4
DL-alanine	10^{-1}	0	0	26	23	0	3
L-arginine	10^{-1}	0	0	16	12	0	4
DL-valine	10^{-1}	0	0	16	15	0	1
L-proline	10^{-1}	0	0	26	24	0	2
L-cysteine	10^{-1}	0	0	40	40	0	0
DL-leucine	10^{-2}	0	1	27	26	0	2
DL-phenylalanine	10^{-2}	0	0	34	29	0	5
DL-isoleucine	10^{-1}	0	0	30	30	0	0
DL-lysine	10^{-1}	0	0	48	44	0	4
		4	7	519	487	0	43

Responses: + = proboscis extension and sucking initiated.
± = proboscis extended and retracted without sucking.
0 = no response.

certain pure proteins (gelatin, casein, keratin, bovine albumin) to be equally ineffective as stimuli (5 positive responses out of 560 trials). In view of the above, it is probable that the positive responses to brain–heart infusion (a mixture of proteins, carbohydrates, and salts) reported by Dethier (1961)

were not from a protein degradation product. However, a more positive statement to this effect can only be made after a more rigorous series of experiments, perhaps one using Dethier's (1961) techniques with chemically defined peptide reagents.

B. Electrophysiological Responses to Amino Acids

In general, the electrophysiological records corroborate the behavioral results: no action potentials could be associated with the presence of any of the amino acids in solution. This is evident when the responses of a single

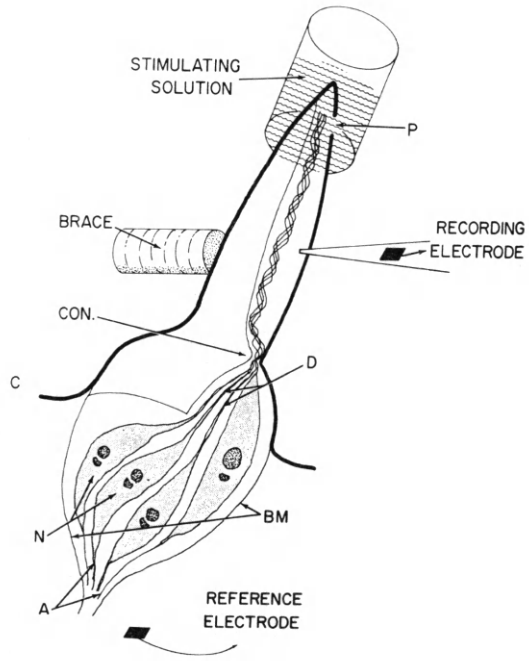


FIG. 1. Schematic drawing of anatomy and recording conditions from a labellar chemosensory hair of the blowfly. The recording electrode is at a hole in the side of the hair (Morita, 1959; Wolbarsht, 1965). Abbreviations are as follows: P, pore or pores at tip of hair; D, dendrites of chemosensory neurons; N, chemosensory neuron; A, axon; C, cuticle; BM, basement membrane; CON, constriction of extracellular space at base of hair. The tormogen and trichogen cells and a mechanoreceptor neuron have been omitted.

chemoreceptor hair to several solutions are compared as in Fig. 2. Here the impulses recorded upon application of the amino acids (traces D–F) resemble quite closely the response to water (trace C) but not at all the re-

sponses to sugar (trace B) or salt (trace A). This similarity of the appearance of the records of water and amino acids is a general one for all the monocarboxylic amino acids. The only consistent difference is in the size of the

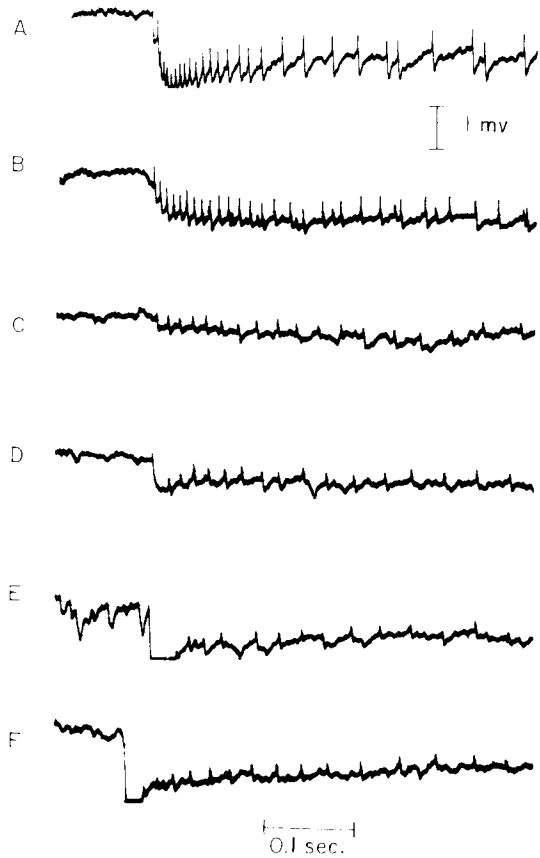


FIG. 2. Response of a labellar chemosensory hair to a variety of stimuli. The stimuli are as follows: *A*, 1 M NaCl. *B*, 0·1 M sucrose. *C*, H_2O. *D*, 0·1 M DL-methionine. *E*, 0·01 M DL-phenylalanine. *F*, 0·01 M DL-leucine. The recordingel ectrode is at the side of the hair; recording is DC. Positive at the recording electrode is up; the voltage and time calibrations as shown below *A* and *F* respectively apply to all records. In all records the stimulus was applied a short time before the initial negative shift in the baseline.

receptor potential as shown in the same figure. This difference occurred throughout our experiments and is thus probably not merely an artifact of sampling. The presence of this receptor potential could conceivably result in a subtle effect: since the amino acids produce greater negative potentials than water, they may be partially depolarizing one of the other chemore-

ceptive neurons and thus could act synergistically if the amino acid and another compound were presented in the same solution (but see below for a discussion of the possible lack of significance of the size or sign of the receptor potential).

The dicarboxylic amino acids, glutamic and aspartic, also have no effect, except when presented in nearly saturated concentrations. In this case, complete inhibition of the normally present water impulses occurs, as well as an exaggerated post-inhibitory rebound of the salt fiber (Fig. 3). A similar but less pronounced inhibition is produced by high concentrations of glutathione (0·3 M, pH 3·0). The mechanism of inhibition by these

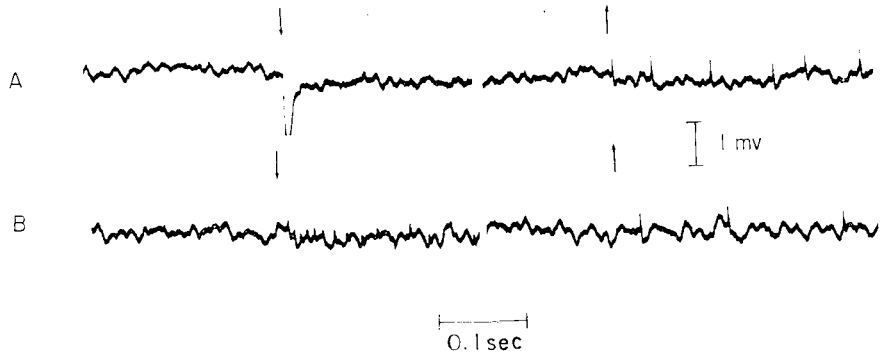


FIG. 3. Response of a labellar chemosensory hair to *A*, 0·05 M glutamic acid; *B*, H_2O. The recording electrode is at the side of the hair, DC recording. Positive at the recording electrode is up. The stimulus was applied where arrows point down and removed where the arrows point up. Approximately 2 sec have been removed from the center of each record. Voltage and time calibrations apply to both records.

compounds has not been determined, although since they are all acidic, the most obvious possible cause is the low pH. High hydrogen ion concentration has been shown to be a factor in producing inhibition of the water fiber (O. Blaumanis, personal communication). However, it is doubtful that the pH alone can be entirely responsible for the observed inhibition. For example, 5×10^{-2} M glutamic acid (pH 3·4) causes complete inhibition, but a ten-fold decrease in concentration (to 5×10^{-3} M) gives a solution which has about the same pH (3·6) but causes little or no inhibition.

C. Receptor Potential

The various types of slow potential changes in response to amino acid stimulation indicated that a more complete investigation of this phenomenon was required for interpretation of our results. The receptor poten-

tial seems to be dependent on the stimulus and the previous history of stimulation of the hair. The origin of this potential change is not clearly understood. Morita (1959) assumed it to be a "generator potential" produced at the chemoreceptor region of the neuron. He reported that those chemicals which stimulate nerve impulses produce a negative DC shift, whereas those which inhibit action potentials are accompanied by a positive "generator potential". However, in actuality the situation is more complex than that. The relation between the structure of the hair and the origin of the receptor potential has been discussed in some detail by Wolbarsht (1965). He has suggested that since several neurons are present, it is more likely that the observed receptor potential is a combination of the differential effects of the chemical and its solvent on the individual chemoreceptor neurons.

A schematic diagram of the anatomy of the chemosensory hair together with the electrode placement is shown in Fig. 1. With reference to this figure, we feel that the origin of the receptor potential is explained by the following considerations: the external fluid medium of the dendrites is effectively isolated from the external fluid medium of the cell body by the tight constriction at the base of the hair. Upon the application of a stimulating chemical, an increased current will flow into a receptor site because of the decreased resistance there and an increased negativity will be produced in the surrounding external media, relative to the body fluid bathing the cell body of the neuron. Conversely, any hyperpolarization of the cell caused by decreased leakage current flow through the receptor site will result in an increased local positivity in the external medium surrounding the receptor sites.

In order to test this hypothesis, we measured the relation between the change in resistance and the absolute potential (receptor potential) recorded at the same time. (Similar changes in resistance have been measured by Morita and Takeda (1957) in the chemosensory hair of the butterfly leg.) When the receptor potential was plotted versus resistance, the points fell on a straight line: negative receptor potentials were associated with a decrease in resistance; positive receptor potentials with an increase in resistance. Calcium salts produced a very large increase in the resistance of the membrane, which was accompanied by a positive potential. This is in good agreement with the effect postulated by Goldman (1964) and others for nerve membranes in general.

As has been mentioned above, the dendrites of several chemosensory neurons are present. Each of these various chemoreceptive membranes is affected by the stimulus differently with respect to the ionic flux leaking

across it. The total change is thus the algebraic sum of the changes in these various leakage currents. An example of an interaction between depolarization and hyperpolarization can be seen in Fig. 4, where water is the stimulus. A very slow spontaneous rate of the salt fiber can be observed before the onset of stimulation by water. During the stimulation period, no salt impulses appear, but upon removal of the water the salt fiber

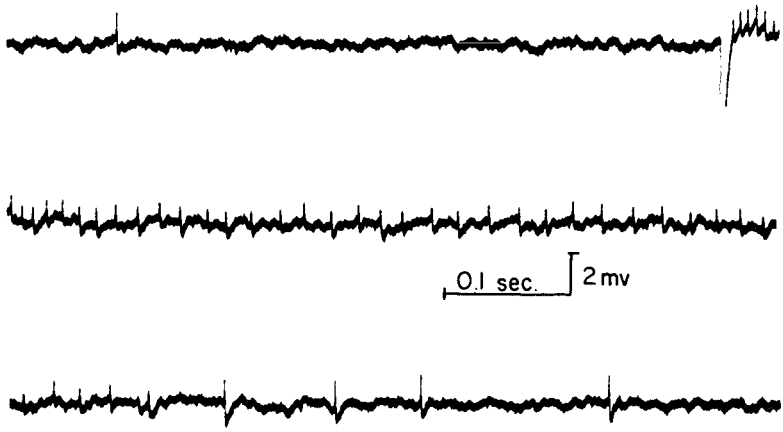


FIG. 4. Response of the labellar chemosensory hair to H_2O. The stimulating electrode is applied near the end of the top trace and removed near the beginning of the bottom trace. The impulses before and after stimulation are from the salt fiber. The three records are continuous. Side wall recording, direct coupled.

shows a greatly increased rate of firing, a post-inhibitory rebound effect. This indicates that water has caused a hyperpolarization of the salt fiber. Since no DC shift in the positive direction occurred in this instance, one must assume that it was counterbalanced by a negative potential from the water fiber.

An additional indication that the salt fiber is hyperpolarized by a water stimulus is shown by the increase in the size of the negative phase of the impulse following stimulation, which can be seen more clearly in Fig. 5. The negative phase of the impulse results from the impulse invading the dendritic portion of the cell (Wolbarsht and Hanson, 1965). In the long hairs which were used in these experiments, the normal impulse appears to stop before it reaches the end of the dendrite. However, hyperpolarization at the end of the dendrite (where the receptor sites are located) causes a post-inhibitory rebound in the receptor region following the hyperpolarization. This rebound allows the impulse to invade the tip of the dendrite.

In general, the receptor potential following the application of a stimulating chemical is negative, indicating that depolarization of the responsive

cell is greater than the sum of the effects due to hyperpolarization of the other cells. However, occasionally upon application of the stimulating chemical we have seen positive DC shifts which are accompanied by impulses (bottom record, Fig. 6). The presence of impulses rules out the possibility that the positive DC shift observed here is due to a hyperpolarizing

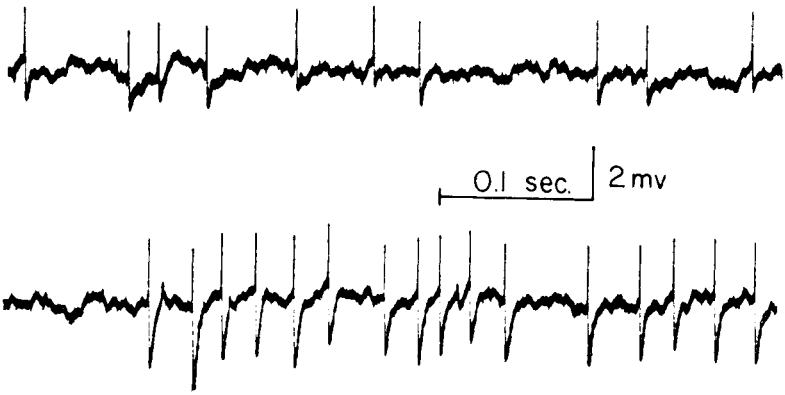


FIG. 5. Side wall recording from a labellar chemosensory hair. The top trace is prior to stimulation with a 0·01 M alcohol solution in water. The bottom record shows the response immediately following the removal of this mixture. This response is the same as has been observed following stimulation by distilled water. Note the enhancement of the negative phase of the impulses, in addition to the post-inhibitory rebound in the frequency of the salt fiber. Capacity coupled recording.

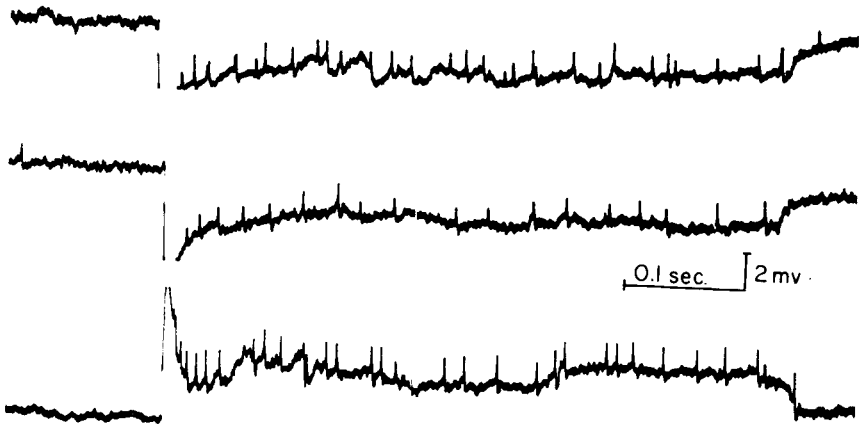


FIG. 6. Response of a labellar chemosensory hair to: top record, 0·1 M sucrose; middle record, H_2O; bottom record, 1 M NaCl. This series of records shows that impulse stimulation is independent of the direction of the receptor potential. DC side wall recording. Positive at the recording electrode is up. Voltage and time calibrations apply to all records. See text for further discussion.

generator potential of the stimulated neuron as would be suggested by Morita's (1959) discussion of the problem.

Thus, we may sum up our views on the receptor potential in the following manner. The receptor potential reflects the changes in polarization across the receptor membranes. It is a summed potential from several cells and thus does not give an accurate indication of the response of any particular cell to any particular stimulus. However, with certain precautions, the receptor potential can be analyzed in such a way as to further our understanding of the interaction of the stimulus and the receptor site.

The receptor potentials that were recorded in response to amino acid stimulation were of two types: a negative going one accompanied by water fiber impulses, and a positive going potential (or in some cases no change) with inhibition of the salt fiber during stimulation and a post-inhibitory rebound afterwards. The negative receptor potential from amino acid stimulation was larger than from pure water, although the impulse resptonse of the water fiber during stimulation (Fig. 2) was the same or less. This may indicate that some of the other fibers—salt, sugar, etc.—are stimulated but below the threshold for an impulse response. Stimulation by a combination of amino acids may give an impulse response; this remains to be tested. It is also possible that the amino acids would synergistically stimulate with any carbohydrates in the stimulating solution.

The positive going potential (or no change) from the acidic amino acids (glutamic and aspartic) may be partially a pH effect. However, it is possible that impulse stimulation could take place with a positive receptor potential, as is shown in Fig. 6.

Our experiments do not show any response from an amino acid stimulus that would indicate acceptance of the test solution by the fly, but they do not rule out the possibility of a subtle facilitatory effect.

CONCLUSIONS

The foregoing results do not support the data of other workers that would indicate the presence of specific amino acid or protein receptors in the blowfly. However, some data were obtained which suggest amino acids may act synergistically with other stimuli. Our testing of the amino acids, peptides, and proteins has been far from exhaustive; and we feel that more rigorous studies using chemically defined stimuli and several different species will be required to further elucidate the problem of protein detection in insects. Studies on the receptor potential indicated that it is a summed potential from several cells and not the generator potential of a single responsive neuron.

SUMMARY

1. A series of amino acids and proteins has been tested for electrophysiological and behavioral responses. No consistent positive responses were found. The proteins and amino acids were, with the exception of the very acidic amino acids, the equivalent of water.

2. When the dicarboxylic amino acids and glutathione (pH about 3) were used as stimulus, the water fiber was not stimulated, but the salt fiber was inhibited during stimulation, and showed a post-inhibitory rebound following stimulus.

3. Measurements of the receptor potential indicated that it is a summed potential from several neurons. It could not be regarded as a true generator potential, especially in the relation between the size and polarity of the receptor potential and the presence or absence of a certain type of impulse in any particular neuron.

ACKNOWLEDGEMENTS

The authors are grateful to Mr. Joseph Anderson for assisting with many of the more difficult experiments and to Mr. Otis Blaumanis for making available information on his unpublished research.

This article was prepared at Bethesda, Maryland, with the sponsorship of the U. S. Navy, Bureau of Medicine, Navy Department, Research Task MR-005.09-1401.04. Reproduction for official use by the United States Government is permitted. Portions of the work were done while Dr. Hanson was supported by a U.S.P.H.S. Training Grant, No. S-T1-GM-517, to the University of Pennsylvania.

REFERENCES

CASE, J. and GUILLIAM, G. F. 1963. Amino acid detection by marine invertebrates. *Proc. 16th Int. Zool. Cong.*, Washington, D.C. **3**, 75.

DETHIER, V. G. 1961. Behavioral aspects of protein ingestion by the blowfly, *Phormia regina* Meigen. *Biol. Bull.* **121**, 456–470.

DETHIER, V. G. and CHADWICK, L. E. 1947. Rejection thresholds of the blowfly for a series of aliphatic alcohols. *J. Gen. Physiol.* **30**, 247–253.

EVANS, D. R. and MELLON, DE F. JR. 1962. Electrophysiological studies of a water receptor associated with the taste sensilla of the blowfly. *J. Gen. Physiol.* **45**, 487–500.

GOLDMAN, D. E. 1964. A molecular structural basis for the excitation properties of neurons. *Biophys. J.* **4**, 167–188.

LAVERACK, N. S. 1963. Aspects of chemoreception in crustacea. *Proc. 16th Int. Zool. Cong.*, Washington, D.C. **3**, 72–74.

MORITA, H. 1959. Initiation of spike potentials in contact chemosensory hairs of in-

sects. III. D.C. stimulation and generator potential of labellar chemoreceptor of *Calliphora*. *J. Cell. Comp. Physiol.* **54,** 189–204.

MORITA, H. and TAKEDA, K. 1957. The electrical resistance of the tarsal chemosensory hair of the butterfly, *Vanessa indica*. *J. Fac. Sci. Hokkaido Univ. Ser. 6 Zool.* **13,** 465–469.

ROBBINS, W. E., THOMPSON, M. S., YAMAMOTO, R. T. and SHORTINO, T. J. 1965. Feeding stimulants for the female house fly, *Musca domestica* Linneaus. *Science,* **147,** 628–630.

WOLBARSHT, M. L. 1960. Electrical characteristics of insect mechanoreceptors. *J. Gen. Physiol.* **44,** 105–122.

WOLBARSHT, M. L. 1965. Receptor sites in insect chemoreceptors. *Cold Spring Harbor Symp. Quant. Biol.* **30,** 281–288.

WOLBARSHT, M. L. and HANSON, F. E. 1965. Electric activity in the chemoreceptors of the blowfly. III. Dendritic action potentials. *J. Gen. Physiol.* **48,** 673–683.

MAXILLARY CHEMORECEPTORS IN THE SILKWORM

SHIGEO ISHIKAWA

Sericultural Experiment Station,
Suginami-ku, Tokyo

INTRODUCTION

Feeding in phytophagous insects involves a sequence of stereotyped behavioral components (cf. Thorsteinson, 1960; Beck, 1965). The feeding in *Bombyx mori* L. has been shown to depend on three types of chemical stimuli: attractants, incitants (biting factors), and feeding stimulants (swallowing factors) (Hamamura, 1959; Hamamura *et al.*, 1962). With the recent progress of studies on the artificial diets of this insect, many chemical stimuli evoking positive feeding response have been reported (Hamamura, 1959; Hamamura *et al.*, 1962; Ito, 1960, 1961a, b, c; Horie, 1962; Nayar and Fraenkel, 1962; Niimura and Ito, 1964; and others).

On the other hand, it has so far been demonstrated that the maxillae of lepidopterous larvae play a significant role in host plant selection (Dethier 1937; Torii and Morii, 1948; Tazima, 1954; Ito *et al.*, 1959; Waldbauer and Fraenkel, 1961; Waldbauer, 1963). Torii and Morii (1948) and Ito *et al.* (1959) have reported that the maxillectomized larvae of the silkworm would feed for some time on a number of non-hostplant leaves, indicating that maxillectomy apparently destroyed this insect's sensitivity to some feeding deterrents. The water-soluble fractions from some non-hostplants have been shown to invariably have inhibitory effects on the feeding (Nayar and Fraenkel, 1962).

However, for a fuller understanding of the food preference or even the host plant selection we must know more about the functions of the chemoreceptors concerned by application of electrophysiological methods.

This presentation will be limited to our work on the gustatory sense with particular emphasis on the specific sensitivities of various contact chemoreceptors associated with the maxillary hairs. All the data have been derived from examination of newly moulted, fifth instar larvae of the silkworm, *Bombyx mori* L.

SENSE ORGANS

Saigo (1933) figured the maxilla of *Bombyx mori* L., which consists of maxillary lobe (or headpiece), maxillary palpus, and other parts (palpifer and stipes). The maxillary lobe bears two large and one small sensilla styloconica, two sensilla trichodea, and one sensillum chaeticum. The third segment of the palpus bears at its apex a group of six to eight small sensilla basiconica (Fig. 1).

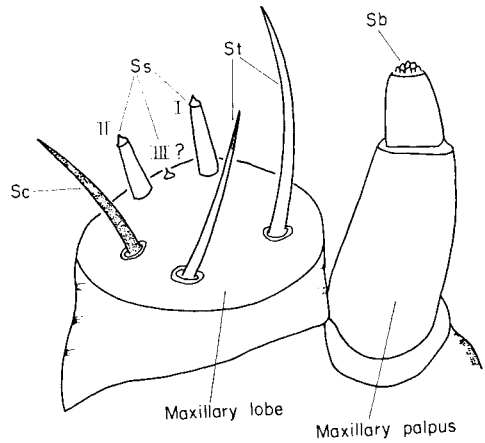


FIG. 1. Schematic representation of the maxilla of silkworm larva. Ss: Sensilla styloconica, Sb: Sensilla basiconica, St: Sensilla trichodea, Sc: Sensilla chaetica.

The sensilla trichodea on the head of the silkworm have been identified as mechanoreceptors electrophysiologically (Ishikawa and Hirao, 1961). A chemoreceptive function of the maxillae of lepidopterous larvae has been assigned to the styloconic and basiconic sensilla (Dethier, 1937; Tazima, 1954; Waldbauer and Fraenkel, 1961).

Accordingly, in the present study the functions of the sensilla styloconica were examined by electrophysiological methods. Afferent impulses which were generated in the chemoreceptor neurons associated with the hairs were recorded by techniques similar to those of Hodgson *et al.* (1955). It consisted of placing a fluid-filled micropipette over the tip of a hair and employing it both as a recording electrode and as a source of chemical stimulation. The signal was amplified, displayed on a cathode ray oscilloscope, and photographed. By the technique these two large sensilla styloconica (Ss-I and II) were identified as taste organs containing certain chemoreceptors, whereas the function of the Ss-III was unable to be deter-

mined because of inapplicability of the technique to recording from such an extremely small hair. The function of the sensilla basiconica on the maxillary palpus was not yet examined.

SENSITIVITIES OF RECEPTORS

Electrophysiological studies have revealed that several chemoreceptors are associated with both Ss-I and II hairs, and that each receptor is specifically different with regard to the chemicals to which it is sensitive. At the present time, nine chemoreceptors areable to be discriminated in both hairs: five in Ss-I hair (a sugar receptor, an inositol receptor, a salt receptor, and other two receptors) and four in Ss-II hair (a bitter substance receptor, a water receptor, and two salt receptors). Tactile stimulation demonstrated the absence of the mechanoreceptor in these hairs.

Sugar Receptor (designated Ls)

In the Ss-I hair there is one receptor neuron specifically sensitive to sugars. The detail story of the effect of sugars on the feeding of this insect has been constructed from behavioral studies (Ito, 1960; Hamamura *et al.*, 1962). The task now presented to electrophysiology is that of supporting the story, filling in the details, and revising where necessary.

This neuron is highly specific and unequally sensitive to various carbohydrates. Comparison of threshold concentrations and that of impulse frequencies in a constant concentration were employed to study relative effectiveness of different carbohydrates on this receptor activity. The results are summarized in Table 1. This receptor revealed a spectrum of activity from complete unresponsiveness to extreme sensitivity. Sucrose showed the extremely low threshold value, indicating the most effective stimulant. Furthermore, the results demonstrated that there are many similarities in the sensitivities of the sugar receptors between the silkworm and the fly. Namely, it is clear that the structural configuration of a sugar is its most important determinant as an effective stimulus for both receptors: (1) effective compounds possess an α-D-glucopyranoside link in the molecule; (2) the α-form of a sugar is more stimulating than the β-form; (3) D-arabinose is more stimulating than L-arabinose (cf. Dethier, 1962; Hodgson, 1964).

The responses of this receptor by stimulation with different concentrations of sucrose are shown in Fig. 2. The response began at a high

TABLE 1. ELECTROPHYSIOLOGICAL THRESHOLDS IN THE SUGAR RECEPTOR (L_s) AND THE INOSITOL RECEPTOR (L_I) FOR CARBOHYDRATES

Receptor	Carbohydrate	Threshold in molar concentration
L_s	Sucrose	$1{\cdot}11\times10^{-4}\sim10^{-4}$
	Maltose	$3{\cdot}33\times10^{-4}\sim2{\cdot}5\times10^{-4}$
	Methyl-α-D-glucoside	$1{\cdot}25\times10^{-3}\sim1{\cdot}11\times10^{-3}$
	D-glucose, D-arabinose, L-rhamnose	$2\times10^{-3}\sim1{\cdot}67\times10^{-3}$
	L-sorbose	$5\times10^{-3}\sim3{\cdot}33\times10^{-3}$
	L-arabinose, D-fructose, D-galactose, trehalose, raffinose, melezitose	$10^{-2}\sim5\times10^{-3}$
	D-xylose, D-ribose, D-mannose, lactose, cellobiose, melibiose, methyl-α-D-mannoside, phenyl-β-D-glucoside, sorbitol mannitol, dulcitol	± or inert in 5×10^{-2}
L_I	Inositol	$1{\cdot}43\times10^{-4}\sim1{\cdot}25\times10^{-4}$

Each stimulating solution contains 10^{-2} M NaCl. This condition is the same in the other tables and figures concerning the sugar receptor and the inositol receptor unless otherwise described.

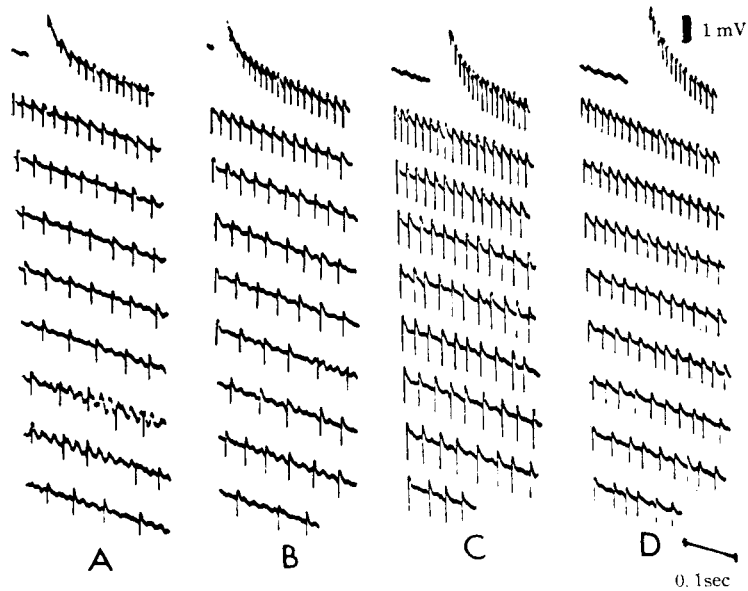


FIG. 2. Responses to different concentrations of sucrose in the L_s receptor. *A*: 2×10^{-4} M, *B*: 10^{-3} M, *C*: 10^{-2} M, *D*: 10^{-1} M. A downward deflection represents an increase in positivity at recording electrode. (From Ishikawa and Hirao, 1963.)

frequency and rapidly declined during first short period. Thereafter, it gradually decreased and finally reached nearly a steady level. This response was observed to maintain even thirty minutes later with a prolonged stimulus. The activity of impulses also showed a consistent correlation with the concentration of the stimulant (Fig. 2 and Table 2).

TABLE 2. THE NUMBER OF IMPULSES DURING FIRST 6 SEC WITH DIFFERENT CONCENTRATIONS OF A STIMULANT FOR L_s OR L_I RECEPTOR

Stimulant	Receptor	Number of impulses		
		Exp. 1	Exp. 2	Exp. 3
2×10^{-4} M sucrose	L_s	104	114	104
10^{-3} M sucrose		116	154	164
10^{-2} M sucrose		178	240	293
10^{-1} M sucrose		196	281	313
10^{-3} M inositol	L_I	108	181	184
10^{-2} M inositol		165	216	188
10^{-1} M inositol		179	226	225

(From Ishikawa and Hirao, 1963.)

TABLE 3. EFFECTS OF MIXTURES OF TWO STIMULANTS ON THE L_s RECEPTOR ACTIVITY

Stimulating solution	Number of impulses*
10^{-3} M sucrose	39
10^{-3} M sucrose + 10^{-2} M D-glucose	27
10^{-3} M sucrose + 2×10^{-2} M D-glucose	22
10^{-3} M sucrose + 10^{-1} M D-glucose	30
10^{-3} M sucrose + 5×10^{-1} M D-glucose	32
2×10^{-2} M D-glucose	8
10^{-1} M D-glucose	15
5×10^{-1} M D-glucose	32
10^{-3} M sucrose	34
10^{-3} M sucrose + $3{\cdot}3\times10^{-3}$ M α-D-methylglucoside	31
10^{-3} M sucrose + 10^{-2} M α-D-methylglucoside	18
10^{-3} M sucrose + $2{\cdot}5\times10^{-2}$ M α-D-methylglucoside	28
$2{\cdot}5\times10^{-2}$ M α-D-methylglucoside	25

* Derived from counts of impulses during 3·5 sec, beginning 3 sec after administration of the stimulus.

(From Ishikawa and Hirao, 1963.)

The effects of various mixtures of two stimulants on this receptor activity were compared quantitatively (Table 3). The impulse frequencies in response to any mixtures were not the simple sums of those obtained when the individual constituents were applied separately. Conversely, a slight, competitive inhibition was observed. This effect might be explained by interaction between the chemicals in the stimulating solution or at the receptor site.

Inositol Receptor (designated L_I)

Hamamura *et al.* (1962) have reported that inositol is one of the important feeding stimulants in the silkworm. The present electrophysiological studies supported this importance from the side of the sensory mechanism of this insect.

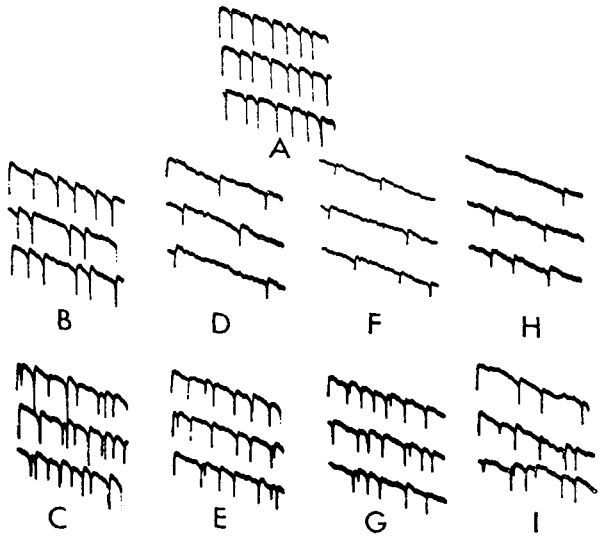


FIG. 3. The L_I and L_s receptor activities. *A*: Inositol, *B*: Sucrose, *C*: Inositol+sucrose, *D*: Maltose, *E*: Inositol+maltose, *F*: Methyl-α-D-glucoside, *G*: Inositol+methyl-α-D-glucoside, *H*: D-arabinose, *I*: Inositol+D-arabinose. Final concentration of each compound: $2{\cdot}5\times10^{-2}$ M. (From Ishikawa and Hirao, 1963.)

As shown in Fig. 3, the mixtures of a certain stimulant for the L_s receptor and inositol evoked two kinds of impulses, which were discriminated on the basis of spike height and electrical summations. This result indicates that the Ss-I hair contains one neuron specifically sensitive to inositol other than the L_s, and that the L_s receptor is not sensitive

to inositol. The amplitude of the impulses from the inositol-sensitive neuron is slightly larger than that from the L_s.

Such a specific type of receptor neuron has not yet been reported in other insects. The differentiation of the inositol receptor in the silkworm seems to be important in the light of the fact that mulberry leaves contain a certain amount of inositol (Ishikawa and Hirao, 1963).

Extremely low threshold value was obtained for inositol in this receptor (Table 1). No other stimulant to the receptor has thus far been found. The time course of the response of this receptor was similar to that of the L_s. A consistent correlation was also shown between the response and the concentration of the stimulant (Table 2).

The phenomena of adaptation and disadaptation occurring in this receptor neuron were examined (Table 4). The sensitivity of the receptor was hardly lowered by 5 seconds' duration of stimulation with 10^{-1} M inositol. At the prolonged stimuli, however, a long period after the end of the stimulus was needed for the disadaptation, viz., the recovery of the sensitivity, to appear.

TABLE 4. TIME TAKEN FOR THE DISADAPTATION IN THE L_I NEURON TO APPEAR AFTER VARYING FIRST STIMULATION WITH 10^{-1} M INOSITOL

Time elapsed after first stimulation (sec)	Duration of first Stimulation		
	5 sec	1 min	3 min
0*	86	77	75
10	87	54	12
180	85	61	16
300	86	75	23
600	—	—	54
900	—	—	50
1200	—	—	75

Each data shows the number of impulses during 3 sec after administration of the stimulus, which was applied for about 3 sec at the time indicated.

* This means the first stimulation in an unadapted state.

The symbol, "—", indicates that no test was made.

(From Ishikawa and Hirao, 1963.)

The mutual inhibitory effect on the activities of both the L_I receptor and the salt receptor N_1, which will be described later, was observed when the stimulating mixture solutions of inositol and a certain salt were applied to the hair. As shown in Fig. 4, the effect depended on the mixing ratio between inositol and NaCl. The similar effect was observed

between activities of the L_s and N_1 (Ishikawa, 1963). This effect might be explained by interaction of the components in the stimulating mixture solution, in which the movement of one component is slowed by the other component present (cf. Wolbarsht, 1958; Barton-Browne and Hodgson, 1962).

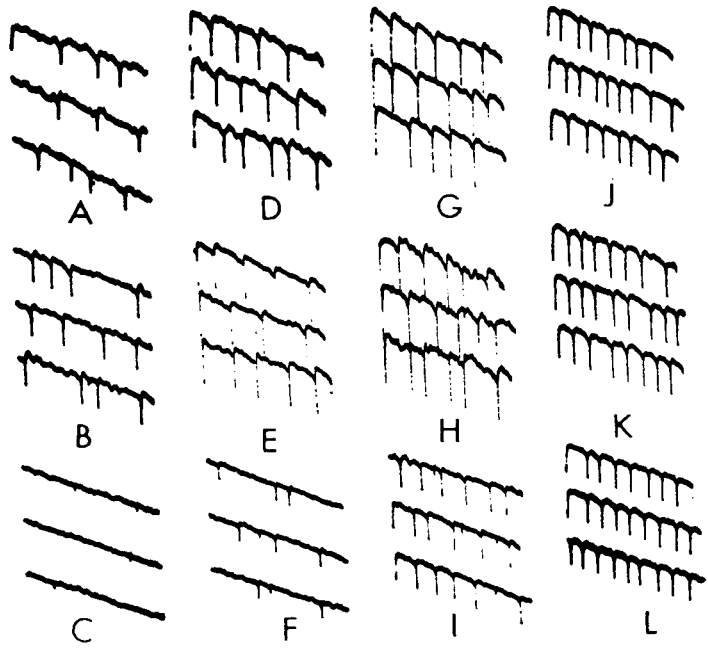


FIG. 4. Responses to mixtures of inositol and NaCl. All recordings: Inositol+ NaCl. Concentration of inositol: *A*–*C*, 5×10^{-4} M; *D*–*F*, 2×10^{-3} M; *G*–*I*, 10^{-2} M; *J*–*L*, 10^{-1} M. Concentration of NaCl: *A*, *D*, *G* and *J*, 10^{-2} M; *B*, *E*, *H* and *K*, 2×10^{-2} M; *C*, *F*, *I* and *L*, 10^{-1} M. (From Ishikawa and Hirao, 1963.)

Bitter Substance Receptor (designated R)

When aqueous extract solutions from many unacceptable plant leaves were applied to the tip of the Ss-II hair, the activity of one kind of large impulses was detected. The impulse frequency increased with increasing concentration of the extract. This receptor neuron was also shown to be specifically sensitive to many bitter substances: alkaloids, glycosides, and others. Electrophysiological threshold values for these substances were shown in Table 5. There are some differences in sensitivities for bitter substances between the *R* receptor and the human bitter receptor,

TABLE 5. ELECTROPHYSIOLOGICAL THRESHOLD IN THE BITTER SUBSTANCE RECEPTOR (*R*) FOR VARIOUS BITTER SUBSTANCES

Bitter substance	Threshold in molar concentration
Strychnine nitrate	10^{-7}
Salicin, Brucine, Pilocarpine hydrochloride, Berberine hydrochloride	2×10^{-7}
Nicotine	2×10^{-5}
Choline chloride	2×10^{-3}
Quinine hydrochloride, Quinine sulphate, Rutin, Quercitrin	Positive response in 5×10^{-4}*
Hyoscine hydrochloride	Positive response in $2{\cdot}3\times10^{-3}$*
Morin, Isoquercitrin, Amygdaline, Carmic acid, Phlorizin, Betaine, Phynylthiourea, $MgSO_4$	Inert in considerably high concentration

Each stimulating solution contains 2×10^{-2} M NaCl.
* Threshold concentration was not examined.
(From Ishikawa, 1966.)

although there exist many similarities (cf. Pfaffmann, 1959). For instance, phenylthiourea, betaine, and $MgSO_4$, which stimulate the human receptor, were inert for the *R* receptor. Isoquercitrin and morin have been shown to stimulate the feeding of the silkworm, whereas rutin and quercitrin to inhibit slightly (Hamamura *et al.*, 1962; Horie, 1962). The present electrophysiological results showed that the *R* receptor is sensitive only to rutin and quercitrin of these flavonoids.

The response of this receptor exhibited a relatively slow adaptation process (Fig. 5A). When sodium chloride was not mixed in the stimulating solutions of relatively high concentrations of a bitter substance, both the *R* and the water receptor *W*, which will be mentioned later, were activated, indicating that bitter substances do not affect the activity of the water receptor (Fig. 5B).

As shown in Table 6, the frequency of the impulses of this bitter substance receptor increased according to the increase in the concentration of mixed NaCl ranging from 10^{-2} M to 4×10^{-2} M. On the contrary, when the concentration of mixed NaCl was above 10^{-1} M, the *R* receptor activity was suddenly and completely abolished and salt receptor impul-

ses (N_2 and N_2'), which will be described later, appeared instead of R impulses. These results indicate that the effect of NaCl on the R receptor activity is synergistic at relatively low concentrations, but antagonistic at relatively high concentrations.

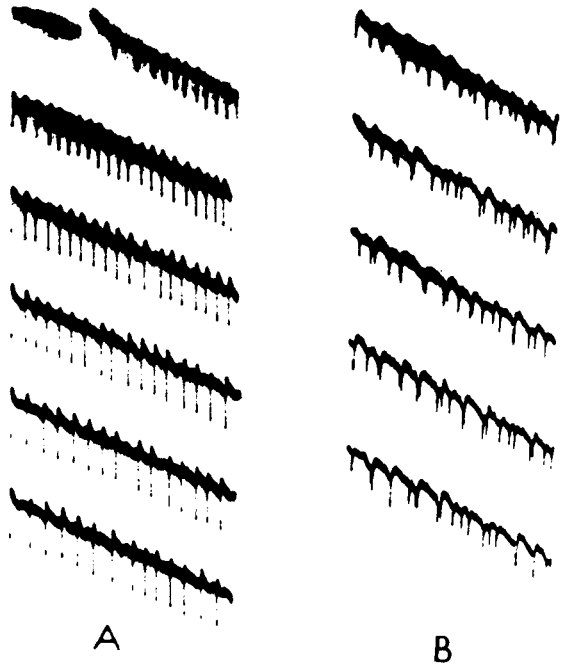


FIG. 5. The R and W receptor activities. A: 2×10^{-6} M Strychnine nitrate $+2\times10^{-2}$ M NaCl (R impulses), B: 2×10^{-6} M Strychnine nitrate (large impulses: W, small ones: R). (From Ishikawa, 1966.)

TABLE 6. EFFECTS OF MIXED NaCl ON THE ACTIVITY OF THE BITTER SUBSTANCE RECEPTOR (BITTER SUBSTANCE: $3{\cdot}3\times10^{-7}$ M STRYCHNINE NITRATE)

Concentration of mixed NaCl	Impulses/sec 3 sec after beginning of the stimulus	
	R impulses	N_2 impulses
10^{-2} M	11	0
2×10^{-2} M	26	0
4×10^{-2} M	48	0
10^{-1} M	0	14
2×10^{-1} M	0	16

(From Ishikawa, 1966.)

The specific stimuli for this receptor were found to significantly depress the normal feeding response of the silkworm (Ishikawa, 1966). Accordingly, it is clear that this neuron functions as a feeding deterrent receptor on the feeding of the silkworm.

Water Receptor (designated W)

Mellon and Evans (1961) and Evans and Mellon (1962a) have shown electrophysiologically the presence of a water receptor in the taste sensilla of the blowfly.

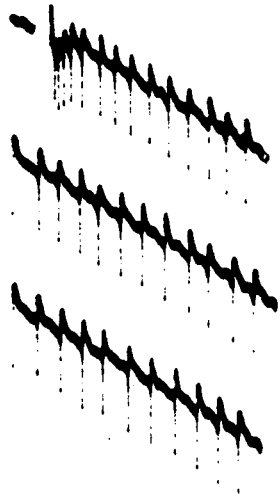


FIG. 6. The *W* receptor activity evoked by pure water. (From Ishikawa and Hirao, 1963.)

When the stimulating-recording electrode filled with distilled water was applied to the tip of the Ss-II hair of the silkworm, a rapid train of impulses of large amplitude was recorded (Fig. 6). In a long continued recording the impulses were maintained at an almost constant rate except a slightly high rate during the initial short period, indicating that this receptor adapts very slowly for the stimulus. This adaptation process seems to be quite different from those of the L_s, L_I and R neurons.

When ascending concentration series of aqueous sugar solutions were examined, the water response was found to decrease with increasing sugar concentrations (Fig. 7). Furthermore, a series of NaCl or $CaCl_2$ concentrations and pure water were tested for their effect on the water response (Fig. 8). In contrast to inhibition by sugars, the inhibition by NaCl had a sudden onset and rapidly became complete at about 10^{-2} M. At higher

concentrations than 10^{-2} M, the activities of the salt receptors (N_2 and N_2') appeared. Whereas, only the inhibition of water response was observed by $CaCl_2$. These phenomena are quite similar to those obtained from the water receptor of the blowfly (Evans and Mellon, 1962b).

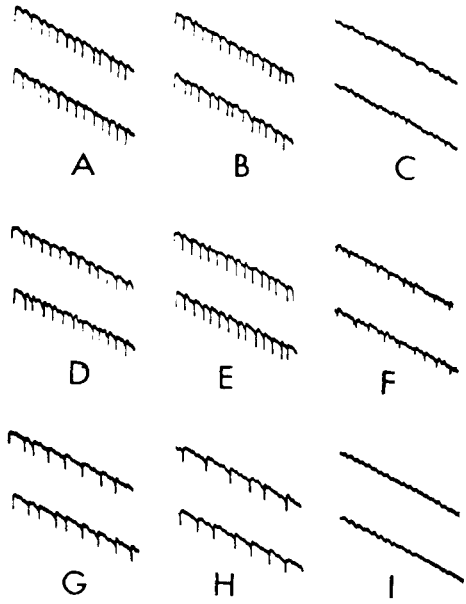


FIG. 7. Effect of mixed sugars on the *W* receptor activity. *A–C*: Melibiose, *D–F*: D-glucose, *G–I*: Sucrose. Concentration: *A*, *D* and *G*, 10^{-3} M; *B*, *E* and *H*, 10^{-2} M; *C*, *F* and *I*, 10^{-1} M. (From Ishikawa and Hirao, 1963.)

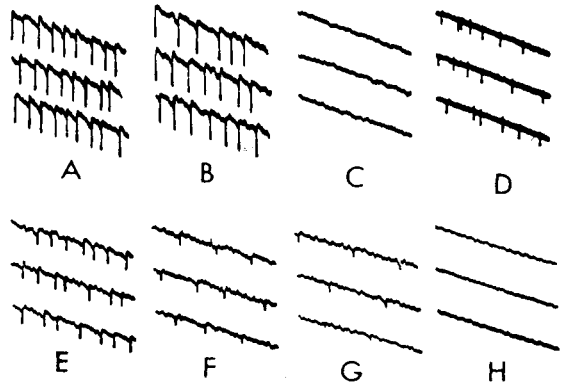


FIG. 8. Effect of mixed salts on the *W* receptor activity. *A* and *E*: Water, *B–D*: NaCl, *F–H*: $CaCl_2$. Concentration: *B* and *F*, 2×10^{-3} M; *C* and *G*, 10^{-2} M; *D* and *H*, 10^{-1} M. (From Ishikawa and Hirao, 1963.)

Salt Receptors (designated N_1 in Ss-I hair and N_2 and N_2' in Ss-II hair)

The silkworm was found to possess three salt receptors in the maxillary hairs: one (N_1) in the Ss-I hair and two (N_2 and N_2') in the Ss-II hair. The amplitudes of the impulses generated in these salt receptors were rather small. Of these three, the N_2 impulse was slightly larger than others. In some cases, hence, the N_1 or N_2' impulses were unable to be discriminated from the noise level.

None of these units showed an initially high discharge frequency or marked adaptation; on the contrary, there appeared to be continuous activity which waxed and waned in apparently random fashion (Fig. 9A).

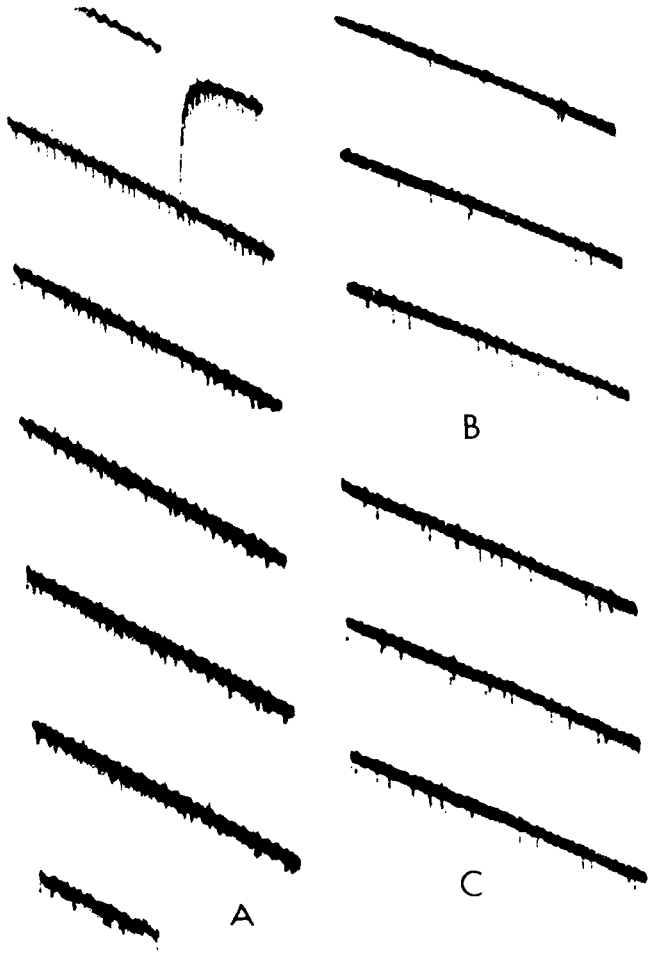


FIG. 9. The N_2 and N_2' receptor activities. *A*: 10^{-1} M NH_4Cl, *B*: 2×10^{-1} M NaCl, *C*: 5×10^{-1} M NaCl.

There was, however, a correlation between the frequency of the salt receptor impulse and concentration of salt in the electrode (Fig. 9B and C). These features are quite similar to the salt receptor in the papillae of the blowfly (Dethier and Hanson, 1965).

All these receptor neurons were sensitive to various electrolytes, such as inorganic salts, organic salts, and organic acids, with relatively high threshold concentrations. The stimulation by these electrolytes was chiefly dominated by the cations involved. Generally, monovalent cations (NH_4^+, Na^+, K^+, etc.) were effective stimuli, whereas the effects of divalent cations were weak (Mg^{++}) or inert (Ca^{++}). Choline chloride was found not to stimulate any salt receptors. This situation seems to be similar to the salt receptors in other insects (Frings, 1946; Hodgson, 1951; Evans and Mellon, 1962b).

The pH relationship of stimulation indicated that non-physiological, abnormal excitation of the receptor neurons included in the hair applied was elicited by electrolytes which have extremely low or high pH values, typically by high concentrations of oxalic acid, Na_3PO_4, K_3PO_4, and Na_2CO_3. This phenomenon has been reported also in the salt receptor of the blowfly (Evans and Mellon, 1962b).

Other Receptors

Besides the above-mentioned receptors two receptor neurons were found to be associated with the Ss-I hair.

When this hair was stimulated with D-glucose, one kind of small impulses was recorded in company with L_s impulses. Both frequencies of the L_s and these small impulses increased with increasing concentration of D-glucose. This receptor was designated *G* receptor. Although this receptor was shown to be activated typically by D-glucose, it could not be confirmed that the receptor is sensitive to D-glucose alone. The responsiveness of the *G* receptor seems to be similar to that of the non-sugar receptor obtained when L-arabinose or D-arabinose was applied to the tip of the papilla of the blowfly (Dethier and Hanson, 1965).

The presence of another receptor was found by the stimulation of the Ss-I hair with the aqueous leaf-extract solutions of cabbage *(Brassica oleracea* var. *capitata)*, turnip *(Brassica rapa)*, a variation of rape *(Brassica napus* var. *komatsuna)*, and radish *(Raphanus sativum)*. The extract solution elicited an initial rapid train of impulses whose amplitude was between those of the L_I and L_s impulses. The initial rapid response steeply declined and, thereafter, gradually decreased. This response pattern

seems to be similar to that of the L_s or the L_I (Fig. 10). Although these stimulating plant leaves are known to belong to Cruciferae, active substances to which this receptor is sensitive have not yet been identified.

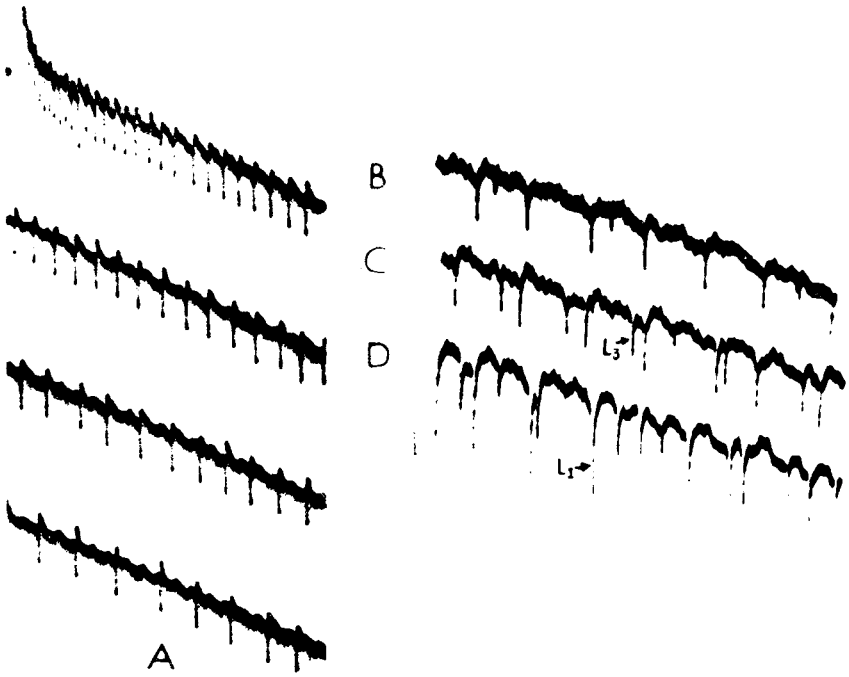


FIG. 10. The activity of the receptor which is sensitive to the aqueous leaf-extracts of some plant species in Cruciferae. *A*: Turnip extract, *B*: Cabbage extract, *C*: Cabbage extract $+ 2 \times 10^{-3}$ M sucrose, *D*: Cabbage extract $+ 2 \times 10^{-3}$ M inositol. (From Ishikawa, unpublished.)

CONCLUSION

With electrophysiological methods it was possible to work out some details of taste receptor function in the silkworm. Single unit taste receptors studied so far are of the sensilla styloconica type on the maxillae. At the present time, six types of receptors were able to distinctly be discriminated: those specifically sensitive to general sugars (L_s), to inositol (L_I), to bitter substances (R), to water (W), to electrolytes (N_1, N_2, and N_2'), and to unknown compounds found in some plant species of Cruciferae. The function of the G receptor which typically responded to D-glucose was not clearly illustrated yet.

The correlations between these receptor functions and the feeding behavior of the silkworm have partially been demonstrated. Sucrose

and inositol which are the effective stimuli for the L_s and L_I receptors respectively have been shown to be the most effective feeding stimulants (Hamamura *et al.*, 1962; Ito, 1960; Niimura and Ito, 1964). In addition, the specific stimuli for the R receptor have been found to have the function as the feeding deterrents (Ishikawa, 1966).

However, many events concerning these correlations have remained unsolved. It is perhaps worthwhile to define some areas in which intensive investigation is necessary:

1. Further illustration of the peripheral discrimination mechanism. The following studies will be involved: (1) identification of new receptors or taste organs, (2) determination of the range and threshold concentrations of the specific stimuli for each receptor, (3) analysis of inhibitors or synergizers affecting on each receptor activity, and (4) elucidation of the mutual interactions between activities of receptors associated with a sensory hair (organ).

2. Investigation of the integration mechanism in the central nervous system. Actually, a feeding response might be triggered in consequence of the integration of afferent impulses from various taste receptors which are respectively sensitive to feeding stimulants or deterrents involved in a given diet.

ACKNOWLEDGEMENT

Mr. Tuneo Hirao collaborated with the author in many phases of the research reported here.

REFERENCES

BARTON-BROWNE, L. and E. S. HODGSON. 1962. Electrophysiological studies of arthropod chemoreception. IV. Latency, independence, and specificity of labellar chemoreceptors of the blowfly, *Lucilia*. *J. Cell. Comp. Physiol.*, **59**, 187–202.

BECK, S. D. 1965. Resistance of plants to insects. *Ann. Rev. Ent.*, **10**, 207–232.

DETHIER, V. G. 1937. Gustation and olfaction in lepidopterous larvae. *Biol. Bull.*, **72**, 7–23.

DETHIER, V. G. 1962. Chemoreceptor mechanisms in insects. *Biological Receptor Mechanisms*. Cambridge University Press.

DETHIER, V. G. and F. E. HANSON 1965. Taste papillae of the blowfly. *J. Cell. Comp. Physiol.*, **65**, 93–100.

EVANS, D. R. and DeF. MELLON 1962a. Electrophysiological studies of a water receptor associated with the taste sensilla of the blowfly. *J. Gen. Physiol.*, **45**, 487–500.

EVANS, D. R. and DeF. MELLON 1962b. Stimulation of a primary taste receptor by salts. *J. Gen. Physiol.*, **45**, 651–661.

FRINGS, H. 1946. Gustatory thresholds for sucrose and electrolyte for the cockroach, *Periplaneta americana* (Linn.). *J. Exp. Zool.*, **102**, 23–50.

HAMAMURA, Y. 1959. Food selection by silkworm larvae. *Nature*, **183**, 1746–1747.

HAMAMURA, Y., K. HAYASHIYA, K. NAITO, K. MATSUURA and J. NISHIDA 1962. Food selection by silkworm larvae. *Nature*, **194**, 754–755.

HODGSON, E. S. 1951. Reaction thresholds of an aquatic beetle, *Laccophilus maculosus* Germ., to salts and alcohols. *Physiol. Zool.*, **24**, 131–140.

HODGSON, E. S. 1964. Chemoreception. *The Physiology of Insecta*. Vol. I. Academic Press, New York–London.

HODGSON, E. S., J. Y. LETTVIN and K. D. ROEDER. 1955. The physiology of a primary chemoreceptor unit. *Science*, **122**, 417–418.

HORIE, Y. 1962. Effects of various fractions of mulberry leaves on the feeding of the silkworm, *Bombyx mori* L. *J. Seric. Sci. Japan*, **31**, 258–264.

ISHIKAWA, S. 1963. Responses of maxillary chemoreceptors in the larva of the silkworm, *Bombyx mori*, to stimulation by carbohydrates. *J. Cell. Comp. Physiol.*, **61**, 99–107.

ISHIKAWA, S. 1966. Electrical response and function of a bitter substance receptor associated with the maxillary sensilla of the larva of the silkworm, *Bombyx mori* L. *J. Cell. Physiol.*, **67**, 1-11.

ISHIKAWA, S and T. HIRAO. 1961. Tactile sense organ on the head of silkworm larva *(Bombyx mori)*. *J. Seric. Sci., Japan*, **30**, 17–22.

ISHIKAWA, S. and T. HIRAO. 1963. Electrophysiological studies of taste sensation in the larvae of the silkworm, *Bombyx mori*. Responsiveness of sensilla styloconica on the maxilla. *Bill. Sericul. Exp. Sta., Tokyo*, **18**, 297–357.

ITO, T. 1960. Effect of sugars on feeding of larvae of the silkworm, *Bombyx mori*. *J. Ins. Physiol.*, **5**, 95–107.

ITO, T. 1961a. Nutrition of the silkworm, *Bombyx mori*. I. Nutritive effects of soybean oil for the larva. *Bull. Sericul. Exp. Sta., Tokyo*, **16**, 311–348.

ITO, T. 1961b. Nutrition of the silkworm, *Bombyx mori*. III. Requirements for sterols and their effects on feeding. *Bull. Sericul. Exp. Sta., Tokyo*, **17**, 91–117.

ITO, T. 1961c. Nutrition of the silkworm, *Bombyx mori*. IV. Effects of ascorbic acid. *Bull. Sericul. Exp. Sta., Tokyo*, **17**, 119–136.

ITO, T., Y. HORIE and G. FRAENKEL 1959. Feeding on cabbage and cherry leaves by maxillectomized silkworm larvae. *J. Seric. Sci. Japan*, **28**, 107–113.

MELLON, DeF. and D. R. EVANS 1961. Electrophysiological evidence that water stimulates a fourth sensory cell in the blowfly taste receptor. *Am. Zool.*, **1**, 372.

NAYAR, J. K. and G. FRAENKEL 1962. The chemical basis of hostplant selection in the silkworm, *Bombyx mori* (L.). *J. Ins. Physiol.*, **8**, 505–525.

NIIMURA, M. and T. ITO 1964. Feeding on filter paper by larvae of the silkworm, *Bombyx mori* L. *J. Ins. Physiol.*, **10**, 425–430.

PFAFFMANN, C. 1959. The sense of taste. *Handbook of Physiology*. Sec. 1, Vol. I. American Physiological Society, Washington, D.C.

SAIGO, T. 1933. Distribution of the sensory neurons in the sensory organs of the head in silkworm larvae. *Japanese J. Appl. Zool.*, **5**, 225–231.

TAZIMA, Y. 1954. Alteration of food selecting character by artificial mutation in the silkworm. *Bull. Internatl. Silk. Assoc.*, **20**, 27–29.

THORSTEINSON, A. J. 1960. Host selection in phytophagous insects. *Ann. Rev. Ent.*, **5**, 193–218.

TORII, K. and K. MORII 1948. Studies on the feeding habits of silkworms. *Bull. Res. Inst. Sericult. Sci.*, **2**, 6–12.

WALDBAUER, G. P. 1963. Maxillectomized insects in the study of host plant specificity. *Proc. N. Central. Br. Entomol. Soc. Am.*, **18**, 28.

WALDBAUER, G. P. and G. FRAENKEL 1961. Feeding on normally rejected plants by maxillectomized larvae of the tobacco hornworm, *Protoparce sexta* (Lepidoptera, Sphingidae). *Ann. Ent. Soc. Am.*, **54**, 477–485.

WOLBARSHT, M. L. 1958. Electrical activity in the chemoreceptors of the blowfly. II. Responses to electrical stimulation. *J. Gen. Physiol.*, **42**, 413–428.

STUDY ON THE PARTICIPATION OF METALS IN THE OLFACTORY RESPONSE OF HOUSEFLY

KEIICHIRO HOSHISHIMA and KATSUO IZUMI

Department of Hygiene, Fukushima Medical College,
Fukushima, Japan

IN OUR laboratory, the genetical (Izumi *et al*, 1963 a, b,; 1964) and biochemical studies on the olfactory response are being made, in order to clarify the mechanism of this sense using houseflies as an experimental animal. The present study is the first step in biochemical investigation, and is concerned with the participation of metals in this sense.

Baradi and Bourne (1951 a) noted cytochemically that vanillin produced a marked inhibition of gustatory phosphatase reactions in rabbits, and these enzyme reactions in nasal mucosa, kidney, gut, and bone were also inhibited in a certain degree by this reagent. On the other hand, vanillin is generally accepted as a complex forming agent with various cations (*Beilsteins Handbuch der Organischen Chemie*, 1925, 1931, 1948).

The information obtained from above-mentioned facts forms the basis of the present study.

METHODS

Measurement of the Olfactory Response

A newly-designed olfactometer shown in Fig. 1 was employed for this experiment. A thin box (2 cm in depth) was divided into three chambers, *A*, *B* and *C*, by two partition plates with domeshaped port. A door was fitted to each partition plate. The box was made of transparent plastic, while the partitions were of non-transparent white plastic. In chamber *A* there were two inlet holes for gas, and at each end of chamber *B* there was an outlet hole for gas. When the gas was turned on, the gas filled chamber *A*, and flowed into chamber *B*. No gas entered chamber *C*, as the gas was drawn off through the outlets in chamber B by suction. This was confirmed by using smoke. When a housefly was attracted by a certain gas, it crawled

into chamber *A*, and in the case of rejection it moved to chamber *C*. When neither was attracted or repelled by the gas, the fly remained in chamber *B*. The measurement of the response was made at 25 °C with illumination of about 100 lux. The tests were run as follows:

(1) The single fly to be tested was taken from the stock of flies, transferred to chamber *B*. Five minutes was allowed for the fly to adapt itself to the chamber.

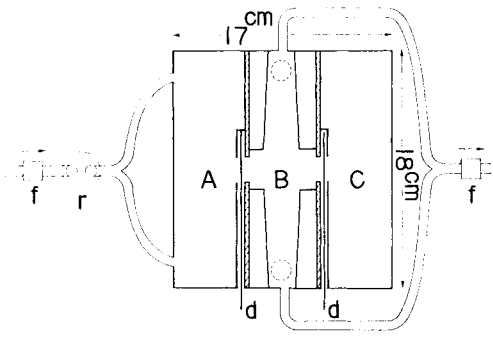


FIG. 1. Olfactometer. Explanation in text. *f*, gas-flowmeter; *r*, bottle in which reagent solution contained; *d*, door; The arrow shows the direction of gas flow.

(2) When the fly was at rest in the position shown by the dotted circle in chamber *B* (Fig. 1), the odorous gas was pumped in. Air which passed through the column of activated carbon was turned into odorous gas by bubbling a solution of odorous compound in the bottle. The air flow was set at the rate of 15 ml per second. The concentration of the gas was altered by changing the concentration of the compound which was dissolved in ethylenglycol.

(3) After the initial one-minute period of pumping, two doors were opened simultaneously. The time which was required for the fly to crawl from chamber *B* to either chamber *A* or *C* was recorded from this point. The time obtained was transformed into a response scale on the basis shown in Table 1. In each experiment, 20 flies were tested and the response was calculated.

Activity Measurement

The general activity of the fly was checked by recording the time which was required for the fly to crawl towards the light source (6 V, 1·4 W) through a glass tube (75 cm in length, and 0·6 cm in inner diameter).

TABLE 1. RESPONSE SCALE

Response time (second)	Response scale	
	Attraction	Rejection
0– 29	+4	−4
30– 59	+3	−3
60– 89	+2	−2
90–119	+1	−1
120–	0	

The tube was coated with black paint to prevent the dispersion of light. In this case, 20 flies were also tested in each experiment.

Anesthesia and Injection Procedures

For anesthesia, the fly was exposed to the cold (−20 °C) for about 4 min. This method was also used for the purpose of separating male and female flies after emergence.

The intrathoracic injection of 1·3 μl of reagent was given to each anesthetized fly with the aid of Micrometer Burette (Roger Gilmont Instruments, Inc., New York, N.Y., U. S. A., Cat. No S-1100-A) equipped with glass capillary (70–80 μ in diameter). The dose was regulated by changing the concentration of reagent solution.

RESULTS

Certain strains of houseflies were tested with the gases from several lower alcohols (Izumi *et al.*, 1963a, b). The authors found that the olfactory response of male fly of NIDAM strain towards *n*-butanol was most distinctive, and the variance of response among each fly was the smallest. The reaction of this fly was measured, and a concentration of 2·2 M of *n*-butanol was used as the stimulant throughout this experiment.

Effect of Vanillin on the Olfactory Response

When increasing doses of vanillin (up to 6 μg per one fly; 1·3 μl of 3×10^{-2} M solution of vanillin) were injected to these flies, the inhibition of response became more marked, while the general activity of flies was unaffected (Table 2). This shows that the effect of vanillin was specific to the olfactory response. It will be noted that the injection procedure caused

TABLE 2. EFFECT OF VANILLIN ON THE OLFACTORY RESPONSE:

Injection of vanillin was given within 24 hours after emergence. The measurements of general activity and olfactory response were made 24 hours after injection. Ephrüssi-Beadle-Ringer solution was given as a control solution

Concentration of vanillin (M)	General activity (second)	Response
Untreated	34·3±1·12	−3·30±0·29
Ringer solution	33·1±1·48	−3·00±0·37
$3{\cdot}7\times10^{-3}$	34·2±1·45	−1·82±0·66
$7{\cdot}4\times10^{-3}$	35·5±1·25	−0·50±0·42
$1{\cdot}5\times10^{-2}$	32·7±1·47	−0·45±0·53
$3{\cdot}0\times10^{-2}$	32·1±1·56	+0·85±0·54

no harm to the fly, as no difference was observed between the olfactory response and the general activity of the flies injected with Ringer solution and those of untreated flies.

Inhibitory Effects of Metal Chelating Agents on the Response

These results are summarized in Table 3. In order to make the survey of the possibility of effective metals, two of the metal chelating agents, o-phenanthroline and α,α′-dipyridyl, were tested as the first step. The inhibitory effect on the olfactory response was demonstrated by the injection of o-phenanthroline, while the injection of α,α′-dipyridyl exhibited no inhibition on the response. The concentrations of the reagents used in the experiment described in this section was the maximum concentration which did not

TABLE 3. EFFECT OF METAL CHELATING AGENTS ON THE OLFACTORY RESPONSE:

Experimental conditions were the same as those in Table 2.

Metal chelating agent (M)		General activity (second)	Response
Untreated		34·3±1·12	−3·30±0·29
o-Phenanthroline	$(1{\cdot}0\times10^{-2})$	37·3±1·19	−0·75±0·41
α, α′-Dipyridyl	$(1{\cdot}5\times10^{-2})$	35·6±2·56	−2·45±0·35
Nitroso-R-salt	$(1{\cdot}5\times10^{-2})$	35·8±2·03	−0·55±0·53
Ferron	$(5{\cdot}0\times10^{-3})$	35·5±2·10	−1·45±0·63
Dimethylglyoxime	$(3{\cdot}0\times10^{-3})$	35·5±1·63	−0·80±0·53

affect the general activity of the fly. These results indicate that cobalt, iron (III) and/or nickel had some bearing on the olfactory response. Subsequently the possibility of the participation of these three metals in the response was checked. The olfactory response of the fly injected with nitroso-R-salt, a potent cobalt chelating agent, showed strong inhibition. When injected with dimethylglyoxime, nickel chelator, moderate inhibition was observed. Whereas only a slight effect was demonstrated in the case of ferron, iron (III) chelating agent.

As shown in Table 4, the effect of nitroso-R-salt on the response remained unchanged up to 48 hours after injection.

TABLE 4. RECOVERY OF RESPONSE BY THE ADMINISTRATION OF METALS FROM THE INHIBITORY EFFECT OF METAL CHELATING AGENTS:

Metals (as chloride) were injected 24 hours following the injection of metal chelating agents. The tests were made 24 hours after the injection of metals. The concentration of metal chelating agents used was the same as that in Table 3.

Metal chelating agent	Response (48-hours after injection)	24 hours after injection of metals		
		Metals (M)	General activity (second)	Response
Nitroso-R-salt	$-0{\cdot}75\pm0{\cdot}85$	$CoCl_2$ ($1{\cdot}5\times10^{-2}$)	$36{\cdot}5\pm2{\cdot}15$	$-2{\cdot}80\pm0{\cdot}68$
Ferron	$-1{\cdot}15\pm0{\cdot}62$	$FeCl_3$ ($2{\cdot}0\times10^{-3}$)	$34{\cdot}0\pm1{\cdot}74$	$-1{\cdot}50\pm0{\cdot}49$
Dimethylglyoxime	$-0{\cdot}75\pm0{\cdot}68$	$NiCl_3$ ($5{\cdot}0\times10^{-3}$)	$36{\cdot}2\pm2{\cdot}01$	$-1{\cdot}25\pm0{\cdot}74$

Recovery of the Olfactory Response by the Administration of Metals from the Effect of Metal Chelating Agents

Whereas the injection of cobalt nullified the inhibitory effect of nitroso-R-salt, no restoration of the response was obtained by the administration of nickel and iron (III) to the flies which had been injected with dimethylglyoxime and ferron (Table 4). These results shown in Table 3 and 4 strongly suggest that cobalt ion is responsible for the olfactory response of housefly towards *n*-butanol. The inhibitory effect of dimethylglyoxime seen in Table 3 seems to be more or less due to the chelating activity of this reagent with cobalt. Dimethylglyoxime is known to form a complex with cobalt, besides with nickel, under certain conditions (Feigl, 1954).

DISCUSSION

It has been demonstrated by the present experiment that there exists a certain relationship between cobalt ion and the olfactory response of housefly.

Cobalt is known as an activator for alkaline phosphatase (Freiman, 1956; Clark and Porteous 1963) besides for certain other enzymes.

Baradi and Bourne (1951b) had postulated that the excitation of olfactory and gustatory receptors occurs as the result of the interference in one or more enzymes, including phosphatases, by the substances possessing the properties of odor or taste. This enzymatic theory of chemoreceptor stimulation was criticism encountered about certain points from Beidler (1961).

Recently, Rakhawy (1963) made the discussion about the possible role of phosphatases in the gustatory phenomenon.

The olfactory response and the sense of taste may be placed under the same category of chemoreception. Considering these discussions mentioned above, it does not seem likely that enzymes are directly responsible for the activation of the olfactory receptors. This does not, however, imply that phosphatases or cobalt as an enzyme activator do not participate in the olfactory process.

It has been reported that vitamin B_{12} was detected in an insect—silkworm (Takahashi, 1955). This compound contains cobalt ion in its molecule, and has been shown to play an important role in the metabolism of nucleic acids (Bernhauer *et al.*, 1964). Baradi and Bourne (1951b) showed that 5-nucleotidase and 3-nucleotidase were distributed in the basal cells of the olfactory mucosa in rabbits. Considering these facts, it is possible to suppose that the metabolism of nucleic acids may be involved in the olfactory response.

Recently, it has been reported that certain divalent metal ions showed certain effects upon the surface-area curve of cardiolipin monolayer (Shah and Schulman 1965). As far as the authors are aware, there is no report concerning the role of cobalt in this field. The well known facts that certain phospholipids play an important role in biological membranes lead the authors to make a speculation about the possibility that the cobalt may have certain effects upon the function of membranes.

ACKNOWLEDGEMENTS

Aided in part by the grant from Tohoku Yakult Company Ltd., Fukushima, Japan.

Special appreciation is due to Mr. Y. Saito for his excellent technical assistance.

REFERENCES

BARADI, A. F. and BOURNE, G. H. 1951 a. Theory of taste and odors. *Science*, **113**, 660–661.

BARADI, A. F. and BOURNE, G. H. 1951 b. Localization of gustatory and olfactory enzymes in rabbit, and the problem of taste and smell. *Nature*, **168**, 977–979.

BEIDLER, L. H. 1961. Taste receptor stimulation. *Progress in Biophysics and Biophysical Chemistry*. Pergamon Press, London, **12**, 107–151.

Beilsteins Handbuch der Organischen Chemie. 1925. Springer Verlag, Berlin, Bd. VIII, 247–254.

Beilsteins Handbuch der Organischen Chemie. 1931. Springer Verlag, Berlin, Ergänzungswerke, Bd. VIII, 604–606.

Beilsteins Handbuch der Organischen Chemie. 1948. Springer Verlag, Berlin, Ergänzungswerke II. Bd. VIII, 278–281.

BERNHAUER, K., MÜLLER, O. and WAGNER, F. 1964. Fortschritte auf dem Vitamin B_{12} Gebiet. *Adv. Enzymol.* **26**, 233–281.

CLARK, B. and PORTEOUS, J. W. 1963. Metal ion activation of the alkaline β-glycerophosphatase of rabbit small intestine. *Biochem. J.* **89**, 100–101.

FEIGL, F. 1954. *Spot Tests*, Vol. 1, Inorganic Applications. Translated by OOESPER, R. E. Elsevier Publishing Company, Amsterdam, The Netherlands.

FREIMAN, D. G. 1956. Metal activation of alkaline phosphatase and 5-nucleotidase; Histochemical studies. *Lab. Invest.* **5**, 338–347.

IZUMI, K. TAKASE, N. and HOSHISHIMA, K. 1963 a. The olfactory response of inbred strains of houseflies: I. A new type insect olfactometer and its application. *Med. & Biol.* **67**, 52–55.

IZUMI, K., TAKASE, N. and HOSHISHIMA, K. 1963 b. The olfactory response of inbred strains of houseflies: II. Response of housefly towards lower alcohols. *Ibid.* **67**, 72–74.

IZUMI, K., TAKASE, N. and HOSHISHIMA, K. 1964. The olfactory response of inbred strains of houseflies: III. Assimilation of the olfactory response with the method of artificial selection. *Ibid.* **68**, 265–267.

RAKHAWY, M. T. E. 1963. Alkaline phosphatases in the epithelium of the human tongue and a possible mechanism of taste. *Acta anat.* **55**, 323–342.

SHAH, D. O. and SCHULMAN, J. H. 1965. Binding of metal ions to monolayers of lecithins, plasmalogen, cardiolipin, and dicetyl phosphate. *J. Lipid Res.* **6**, 341–349.

TAKAHASHI, J. 1955. Biochemical studies on vitamin B_{12} in silk-worm: II. *Nippon Nogeikagaku Kaishi.* **29**, 711–715.

EFFECTS OF SALTS ON THE SUGAR RECEPTOR OF THE FLESHFLY

HIROMICHI MORITA

Department of Biology, Faculty of Science, Kyushu University
Fukuoka, Japan

THE receptor potential recorded from the chemosensory hair of insects indicates that the electrical current (the receptor membrane current) flows across the receptor membrane of the sugar receptor when a plain sucrose solution is applied at the hair tip (Morita, 1959; Morita and Yamashita, 1959). As to the structure of the labellar chemosensory hair of the fly, it has been generally accepted that at least three functionally differentiated chemoreceptors (sugar, salt and water receptors) send their distal processes up to the opening located at the hair tip. The receptors are accessible to stimuli through this opening only. When there is no stimulation, the surface of the receptor ending might be exposed to air, though a very thin extracellular film (of mucus or lipid) would prevent drying of the receptor surface. The salt receptor normally responds to monovalent inorganic cations. According to Evans and Mellon (1962), spontaneous activity of this receptor is low, and therefore, it is likely that there are few cations around the receptor membranes when the tip of the hair is exposed to air. Meanwhile, all investigators concerned have agreed that salts dissolved in sucrose solutions depress the response of the sugar receptor to sucrose (Hodgson, 1957; Wolbarsht, 1958; Morita and Takeda, 1959; Takeda, 1961). This action of salts has been shown to occur before or at the stage of generation of the receptor potential (Brown and Hodgson, 1962; Morita and Yamashita, 1966). Considering all these facts, it was a challenging problem for us to find what is the carrier of the receptor membrane current when the sugar receptor is stimulated by a plain sucrose solution.

METHODS

The fleshfly, *Boettcherisca peregrina* (kindly furnished by the Department of Medical Zoology, Tokyo Medical and Dental University), was used throughout this work. In this species of fly, the spikes from the sugar receptor were the largest among those from all the receptors in the same hair, and thus, were easily discriminated from the others (cf. Fig. 1).

The use of the side-wall recording was essential for the present work. A detailed description of this technique has been made previously (Morita, 1959; Evans and Mellon, 1962). In brief, impulses of the chemosensory neurons were recorded from a cracked part of the side wall of the hair by means of a glass capillary electrode, and stimulus solutions were applied at the tip of the hair, being contained in another glass capillary. As an electrolyte solution in the recording electrode, the Waterhouse's saline (in Buck, 1953) was used.

The different concentrations of salts were dissolved in 0·2 molal sucrose solutions. Molality was used to express the final salt concentration in a mixture, and when necessary, the mean ionic activity or the activity of the cation was calculated from the mean activity coefficient in the tables listed by Harned and Owen (1958). The activity of sucrose was obtained from the activity coefficient, γ_B, in terms of the mole fraction,

$$\gamma_B = e^{7.3(1-X^2_A)},$$

where X_A is the mole fraction of water (Morita, Steinhardt and Hodgson, 1966). The activity in the mixture will be different from the value obtained above. This does not, however, introduce any serious difficulties into the conclusions based on the present results, since the concentration of one of the solutes in the mixture was usually kept constant.

The stimulus duration was limited to one-half second, and the response magnitude was defined as impulse number during a period of 0·2–0·3 sec starting at 0·15 sec after stimulus beginning. It has been ascertained that the impulse frequency is in a steady state during this period (Morita, Steinhardt and Hodgson, 1966). In study on the concentration effects of salts, the magnitude of response was normalized so that the value to pure 0·2 molal sucrose was unity. The effects of any given salt was compared with those of NaCl in the same hair, since the latter varied with different hairs. It should be better to explain the actual procedure, referring to the particular example shown by Fig. 2, where the number attached to each circle represents the order of application. Lacking numbers (1, 2, 3, 6, 9, etc.) show applications of plain 0·2 molal sucrose. As can be seen in this figure, the order of application was ascending as to the concentration of

the salt, and application of the mixture of any given concentration of the salt was followed by the mixture of the same concentration of NaCl. (In the case of $CaCl_2$ only, the mixture of $CaCl_2$ was followed by that of NaCl ten times concentrated than $CaCl_2$. See Fig. 6.) Pure 0·2 molal sucrose was given before and after applications of the mixtures of the same concentrations of the salts. (Note the lacking numbers 6, 9, 12, 15, etc.) The relative magnitude of response was obtained by dividing the magnitude of response to the mixture by the average of those to 0·2 molal sucrose which was given before and after the mixture. The values for application Nos. 13 and 14, for example, were obtained by comparison with the average of responses to 0·2 molal sucrose given at application Nos. 12 and 15. Thus, changes in the resting conditions (due to possible deterioration of the preparation) were eliminated, and the interval between stimuli could be made short as 1·5–2·0 min. However, when making a Beidler type plot (Beidler, 1954) test solutions were applied 3 min after stimulation by sucrose lower than 0·2 molal, but 5 min after higher concentrations of sucrose. Experiments were done at room temperature (about 20°C), change in which was less than 0·5°C during one experiment.

RESULTS AND DISCUSSIONS

Response to Water, Sugar and Salt

Figure 1 shows records of typical responses to three different qualities of stimuli. It should be noticed that only a single train of impulses (of the salt receptor) occurred in the stimulation by 0·5 molal NaCl. Two trains of impulses result from the stimulation by water (distilled and deionized) and three in the case of 0·2 molal sucrose. It seems likely that impulses of the highest frequency in record *A* are those from the water receptor, and that those of the highest frequency in record *B* are from the sugar receptor. The small spikes in record *B* are those of the water receptor. We can see that the spikes not from the water receptor (low frequency) in record *A* have the same height as the spikes in record *C*. This suggests that impulses are initiated in the salt receptor as well as in the water receptor when water is applied at the hair tip. Without any exception in more than three hundred hairs tested, the spikes other than those of the water receptor, which were initiated by water, coincided in the spike height with those of the salt receptor in the same hair. Therefore, there is scarcely any doubt that water applied at the hair tip initiates impulses in the salt receptor as well as in the water receptor.

We have many records in which two different sizes of spikes are initiated by water applied at the tip of the labellar hair of *Lucilia* and *Calliphora*. In these cases, however, because of poor differentiation in spike height, it was impossible to identify one of the two as those of the salt receptor.

The conclusion that water initiates impulses in the salt receptor is important in the present work. As mentioned in the introduction we cited evidence for absence of any cations around the receptor region when no

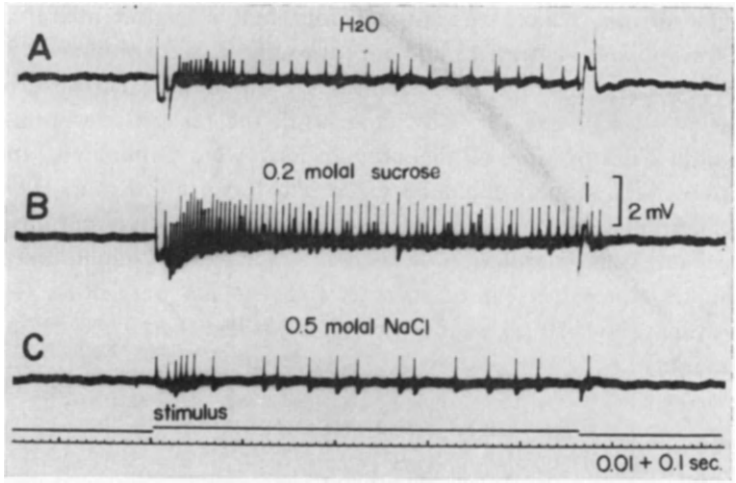


FIG. 1. Responses to: *A*, distilled and deionized water; *B*, 0·2 molal sucrose; *C*, 0·5 molal NaCl. (Morita, Hidaka and Shiraishi, unpublished.)

stimulus is given. Here, we have had an observation suggesting that some monovalent cations are released around the receptor region when water is applied to the hair tip.

Role of Cl^- in the Receptor Membrane Current

It is easy to show from the cable theory that in a stationary state the amplitude of the receptor potential recorded from a cracked part of the side wall of the hair is proportional to the receptor membrane current and to the potential change in the receptor membrane when the only receptor is chemically excited. It has been shown also that the frequency of the impulse is proportional to the amplitude of the receptor potential (Morita and Yamashita, 1966). Therefore, the frequency of impulses, the magnitude of the receptor potential, the receptor membrane current and the

magnitude of the displacement of the receptor membrane potential are all equivalent, since we are concerned only with the stationary state of the response and with its relative values in the following discussions.

The membrane current must be inward at the receptor membrane to initiate impulses at a proximal region of the receptor cell (for impulse initiation site, see Tateda and Morita, 1959; Morita, 1959). This means

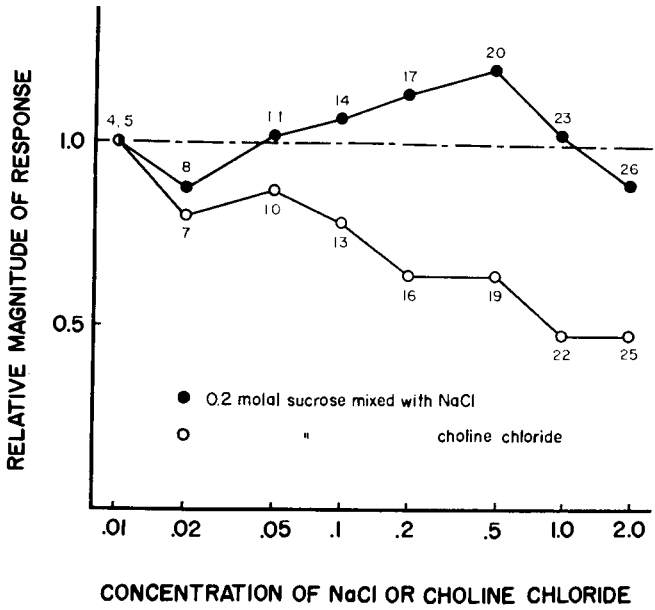


FIG. 2. Concentration effects of choline chloride and NaCl on the steady response of the sugar receptor to 0·2 molal sucrose. The response is expressed as relative to that to 0·2 molal sucrose. The number attached to each circle represents the order of application. Lacking numbers (1, 2, 3, 6, 9, etc.) show applications of pure 0·2 molal sucrose. The similar order was used in Figs. 3–6. (Morita, Hidaka and Shiraishi, unpublished.)

hat any ions other than anions inside, cations outside the receptor membrane cannot be the carriers of the receptor membrane current. (Here, we adopt the ordinary opinion that the membrane current is carried by ionic fluxes across the membrane.)

Before the result shown in Fig. 1 was obtained, we had no evidence indicating that cations exist around the receptor membrane when a plain sucrose solution is applied at the hair tip. Furthermore, there were only the papers reporting that the sugar receptor response to sucrose is depress-

ed by existence of salts such as NaCl, etc. Therefore, it would be reasonable to have assumed that some anion such as Cl^- exists on the inside of the receptor to carry the receptor membrane current. The effects of choline chloride (shown in Fig. 2) would support this assumption, since an increase in concentration of Cl^- on the outside of the membrane decreased the receptor membrane current. However, the effects of NaCl (shown in the same figure) indicates that the explanation is not so simple. Contrary to our expectations and to the results reported previously, NaCl increased

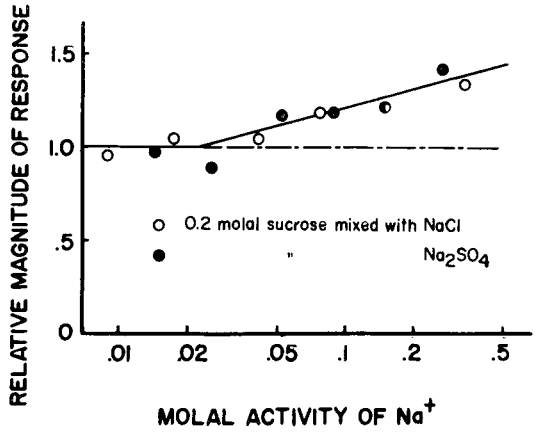


FIG. 3. Effects of NaCl and Na_2SO_4. (Morita, Hidaka and Shiraishi, unpublished.)

the response to sucrose at moderate concentrations. In this range of concentrations the increment of the receptor membrane current was roughly proportional to that of the chemical potential of NaCl in the sucrose solution. However, above 1·0 molal concentration NaCl always depressed the response of the sugar receptor.

The results in Fig. 2 show that salts of different cations and the same anion (Cl^-) have quite different concentration effects. However, salts of the same cation (Na^+) and different anions are equivalent (Fig. 3), if the activity of Na^+ is used as the concentration factor. These results suggest that Cl^- does not take a major part in the receptor membrane current. If we assume that the excited receptor membrane is impermeable to SO_4^{--} then the membrane is impermeable to Cl^- as is shown in Fig. 3. But if we assume, as another possibility, that Cl^- carries the receptor membrane current, then the membrane should be permeable to Cl^- just as twice as to SO_4^{--}. Thus, SO_4^{--} would carry the same amount of current as Cl^- at the same activity of Na^+. In any way, Cl^- has no special importance in the receptor membrane current.

Effects of Cations

As mentioned above, different cations of salts with the same anion have quite different effects on the sugar receptor response. In the following figures, the effects of a few cations (all with Cl^-) are shown in comparison with those of NaCl.

Figure 4 shows the effect of KCl. It can be seen that there is no difference in the excitatory effect between KCl and NaCl below 0.1 molal con-

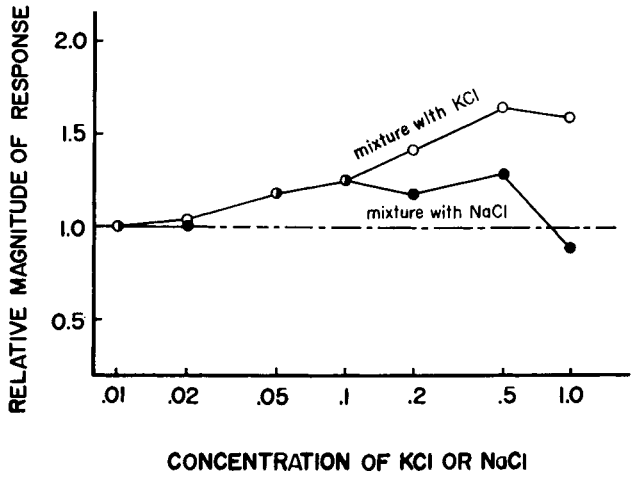


FIG. 4. Effects of KCl in comparison with NaCl. (Morita, Hidaka and Shiraishi, unpublished.)

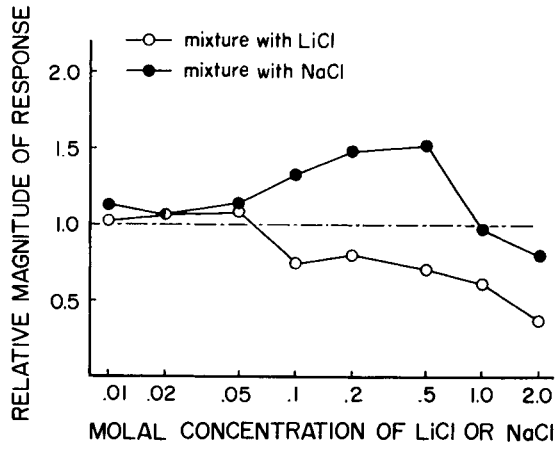


FIG. 5. Effects of LiCl and NaCl. (Morita, Hidaka and Shiraishi, unpublished.)

centration, while KCl is more effective at high concentrations. The excitatory effect of Cs^+ and of NH_4^+ was studied in the same way and was found to be significantly stronger than that of Na^+.

Among the monovalent inorganic cations tested, Li^+ was the only cation which showed no notable accelerating effect on the sugar receptor response (Fig. 5). Calcium ion also showed the inhibitory effect only, but this effect was shown at concentrations more than ten times as low as Li^+ (Fig. 6).

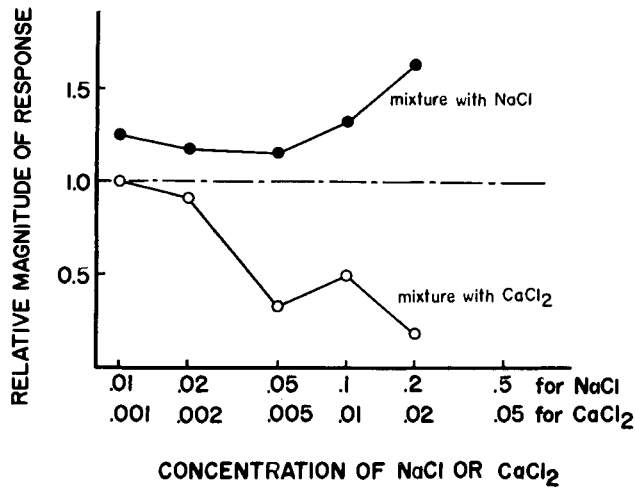


FIG. 6. Effects of $CaCl_2$ and NaCl. (Morita, Hidaka and Shiraishi, unpublished.)

Summarizing the results of the effects of cations, the order of strength in the excitatory effect is given as

$$Cs^+,\ NH_4^+ > K^+ \geqq Na^+ > Li^+ \gg Ca^{++} \tag{1}$$

Inhibition by Salts

As seen in the figures presented above, some inhibition is appearent at sufficiently high concentrations of salts. This inhibition is explained if we assume the following scheme:

$$A + S \overset{K}{\rightleftharpoons} AS, \tag{2.1}$$

$$I + S \overset{K_i}{\rightleftharpoons} IS, \tag{2.2}$$

$$IS + A \overset{K'}{\rightleftharpoons} AIS, \tag{2.3}$$

$$AS + I \overset{K_i'}{\rightleftharpoons} AIS. \tag{2.4}$$

In these equations A, S and I represent the molecule of sucrose, the receptor site and the inhibiting ion, respectively. K, K_i, K', K_i' are the equilibrium constants for reactions (2.1), (2.2), (2.3) and (2.4), respectively. Letting a, n and i activities or numbers of A, AS and I, respectively, and assuming that the complex AS only is responsible for response, the amplitude of the response (r) is given after Beidler's theory (Beidler, 1954) as

$$r = kn = \frac{ks/(1+ik_iK'/K)}{1+(1+ik_i)/a(K+ik_iK')}, \tag{3}$$

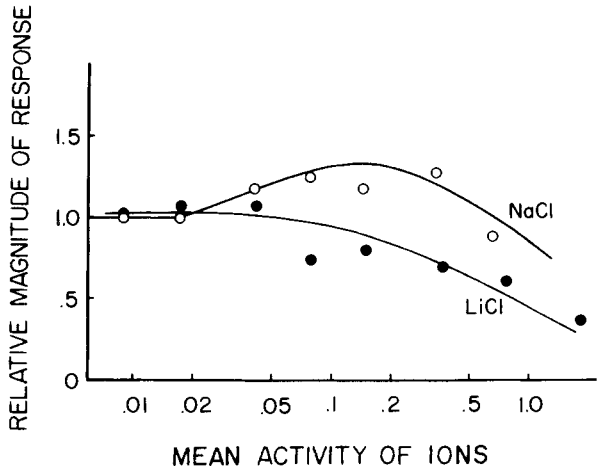


FIG. 7. The continuous curves were obtained from eqns. (3) and (4). The values of the constants are shown in Table 1. (Morita, Hidaka and Shiraishi, unpublished.)

where k represents a proportionality constant. We can induce an empirical equation for the excitatory effect of cations as

$$k = k_0 + k' \log \frac{i}{i_0}, \tag{4}$$

where k_0, k' and i_0 are constants varying with cations. Combining eqns. (3) and (4), we get a theoretical curve with appropriate values of the constants. In Fig. 7 calculated curves are compared with the experimental results. The values of the constants used are shown in Table 1. As seen in Fig. 7, the calculated curves cover the experimental values fairly well. Equation (3) corresponds to that for the mixed type of inhibition in the enzymatic kinetics, and is reduced to the fully competitive type in the case of LiCl.

TABLE 1.

	NaCl	LiCl
k_0	1·20	1·26
k'	0·78	0·08
$1/aK$	0·2	0·2
K_i	6·0	10·0
K'/K	0·1	$\ll$0·1

Figure 8 gives another way in which the inhibition was explained in terms of enzymatic kinetics. Beidler's plot (Beidler, 1954) in this figure is represented by the straight lines for plain sucrose solutions and for mixtures with 0.1 molal NaCl. In this particular preparation, the presence of 0.1 molal NaCl is shown to reduce the value of K_b (the value of $-1/A_0$, with A_0 being the X-axis intersect) to less than half that for plain sucrose solutions. In terms of enzyme kinetics, it can be said that the affinity of the receptor site for sucrose was reduced by 0.1 molal NaCl.

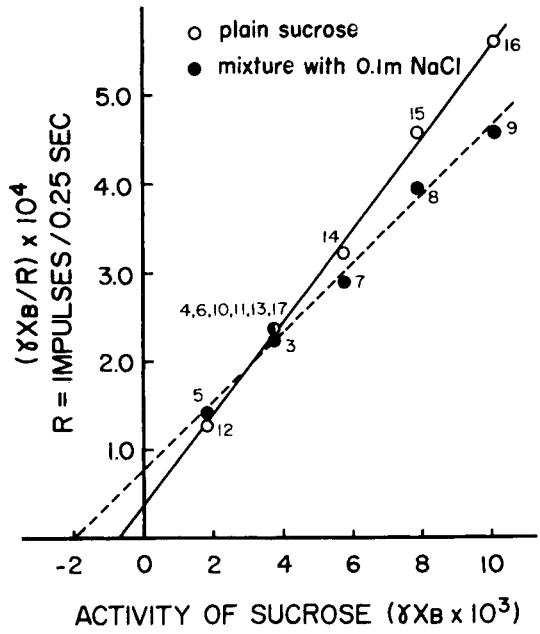


FIG. 8. Plots after Beidler's theory. The numbers attached to the circles show the application order. (Morita, Hidaka and Shiraishi, unpublished.)

It should be noticed here that K_b is related to the equilibrium constants in eqn. (2), if the assumption is correct, as

$$K_b = (1 + iK_i)/(K + iK_iK'). \quad (5)$$

Equation (5) shows that the graphically obtained values of Beidler's constant (K_b) are a function of the concentration of ions outside the receptor membrane. To avoid complications, the existence of ions other than Li^+ or Cl^- was neglected in calculating the curve for LiCl in Fig. 7. However, as shown before, we cannot imagine that there are no cations outside the membrane even when non-electrolyte solutions are applied at the hair tip. At present we do not know the exact concentrations of the ions, and therefore, it is impossible to obtain the value of K in eqn. (2.1).

We may ascribe the inhibition by salts either to cations or to anions or to both. However, here also, the cations are more important, since chlorides of different metals showed different effectiveness. If such is the case, the anionic groups in the receptor membrane, whose existence is suggested by the pH-effect as discussed elsewhere (Morita, Steinhardt and Hodgson, 1966), would be the inhibition centers.

CONCLUSION

The following picture for the receptor membrane current is not conclusively proved by any of the present results, but is the most probable judging from all the results reported here and elsewhere on the labellar chemoreceptors and from the character of other excitable membranes. The application of a plain sucrose solution at the hair tip enables ions to diffuse out from the extracellular fluid inside the hair to the outside of the receptor membrane. Among the diffused ions, cations flow into the receptor activated by sucrose molecules. This influx of cations is the receptor membrane current.

The diffusion of cations is strongly suggested by record *A* in Fig. 1. Wolbarsht and Hanson (1965) observed that anesthetics applied at the hair tip diffused into the inside of the hair and blocked the backward conduction of impulses. Since we cannot doubt existence of the receptor potential, it is quite certain that the inside of the hair is filled with an electrolytic solution. If any solute diffuses into the inside, electrolytes should diffuse from it when a suitable solvent for ions fills the cavity containing the receptor regions (Adams, 1963).

The importance of cations in the receptor membrane current is indicated by the results shown in Figs. 2–6. As illustrated as an example in Fig. 3, all the excitatory cations seem to have increased the response in a linear

manner against logarithm of their concentrations (i. e. against their chemical potentials), though this was limited within a somewhat narrow range. This suggested that the excited receptor membrane is non-selectively permeable to cations. According to this view, eqn. (1) shows the order of the velocity at which cations pass the membrane. In fact, this order is the same as that of the ionic mobility.

An alternative explanation for the excitatory effect of cations would be as an activator for the reaction between sucrose and the receptor site. However, this is excluded by the result in Fig. 8, which shows that 0·1 molal NaCl decreased the affinity. Thus, the excitatory effect of cations should be considered to represent the augmentation of displacement in the receptor membrane potential with increase in their chemical potentials.

REFERENCES

ADAMS, J. R. 1963. The fine structure of insect contact chemoreceptors. *Proc. XVI Intern. Congr. Zool.*, **3**, 93–95.

BEIDLER, L. N. 1954. A theory of taste stimulation. *J. Gen. Physiol.*, **38**, 133–139.

BROWN, L. B. and HODGSON, E. S. 1962. Electrophysiological studies of arthropod chemoreception. IV. Latency, independence, and specificity of labellar chemoreceptors of the blowfly, *Lucilia.*

BUCK, J. B. 1953. Physical properties and chemical composition of insect blood. In *Insect Physiology*. Ed. by ROEDER, K. D. JOHN WILEY and Sons, Inc. New York.

EVANS, D. R. and MELLON, DeF. 1962. Electrophysiological studies of a watcr receptor associated with the taste sensilla of the blowfly. *J. Gen. Physiol.*, **45**, 487–500.

HARNED, H. S. and OWEN, B. B. 1958. *The Physical Chemistry of Electrolytic Solutions.* Reinhold Publishing Corp. New York.

HODGSON, E. S. 1957. Electrophysiological studies of arthropod chemoreception. II. Responses of labellar chemoreceptors of the blowfly to stimulation by carbohydrates. *J. Ins. Physiol.*, **1**, 240–247.

MORITA, H. 1959. Initiation of spike potentials in contact chemosensory hairs of insects. III. D. C. stimulation and generator potential of labellar chemoreceptor of *Calliphora. J. Cell. and Comp. Physiol.*, **54**, 189–204.

MORITA, H. and TAKEDA, K. 1959. Initiation of spike potentials in contact chemosensory hairs of insects. II. The effect of electric current on tarsal chemosensory hair of *Vanessa. J. Cell. and Comp. Physiol.*, **54**, 177–188.

MORITA, H., STEINHARDT, R. A. and HODGSON, E. S. 1966. In preparation.

MORITA, H. and YAMASHITA, S. 1959. Generator potential of insect chemoreceptor. *Science*, **130**, 922.

MORITA, H. and YAMASHITA. S. 1966. In preparation.

TAKEDA, K. 1961. The nature of impulses of single tarsal chemoreceptors in the butterfly, *Vanessa indica. J. Cell. and Comp. Physiol.*, **58**, 233–245.

TATEDA, H. and MORITA, H. 1959. Initiation of spike potentials in contact chemosensory hairs of insects. I. The generation site of the recorded spike potentials. *J. Cell. and Comp. Physiol.*, **54**, 171–176.

WOLBARSHT, M. L. 1958. Electrical activity in the chemoreceptors of the blowfly. II. Responses to electrical stimulation. *J. Gen. Physiol.*, **42**, 413–428.

WOLBARSHT, M. L. and HANSON, F. E. 1965. Electrical activity in the chemoreceptors of the blowfly. III. Dendritic action potentials. *J. Gen. Physiol.*, **48**, 673–683.

SOME FACTORS GOVERNING OLFACTORY AND GUSTATORY RESPONSES OF INSECTS

K. N. SAXENA

Department of Zoology, University of Delhi,
Delhi 7, India

ODOURS and tastes of various food-sources are known to play a varying degree of role in the perception, discrimination, selection and ingestion of food by various animals. In some animals viz., man, the role of olfactory and gustatory receptors is subordinate to that of some other receptors particularly visual organs. On the other hand, in some other animals the olfactory and gustatory receptors play a much more important role than other receptors in the selection and ingestion of food. To this category belong many insects which are known to feed on food-sources having agreeable odours/tastes and reject those with disagreeable odours/tastes.

The chemical constituents of a diet serving as sources of odour, i. e. olfactory, and taste, i. e. gustatory, stimuli may influence three main types of responses of an insect which, according to Dethier (1953) and Feir and Beck (1963), follow one after another to culminate in the ingestion of food. Of these three types of responses, the first involves the orientation of the insect in relation to the source of stimulus. This response can be influenced by the olfactory and not by the gustatory stimulants. The orientational response of the insect may involve its attraction to or repulsion from the source of stimulus. The olfactory stimulants resulting in the attraction of the insects have been termed as "attractants" and those repelling the insects as "repellents" by Beck (1965). The orientational response of the insect, if resulting in its attraction to the stimulant, is followed by its "initial" and then by its "continued" feeding response. The last two types of responses can be determined by both the olfactory and gustatory stimulants. Those stimulants which favour or "incite" an insect to show the "initial feeding response" have been termed "incitants" and those which suppress this type of response have been termed

"suppressants" by Beck (1965). Similarly, the chemicals favouring the "continued feeding response" of an insect have been referred to as "stimulants" and those inhibiting this type of response as "deterrents" by Beck (1965).

The relationship between the presence or absence of the above-mentioned types of chemical stimulants in various food-sources and the feeding responses of insects has been examined by several workers. Most of this work deals with surveys of the chemical constituents of various food-sources which serve as olfactory/gustatory stimuli for different insects. Such surveys give information on the distribution of naturally occurring olfactory/gustatory stimulants, their extraction, purification, chemical characterization and synthesis, etc. The literature on these aspects has been reviewed by a number of authors (Dethier, 1947, 1956; Fraenkel, 1959; Green *et al.*, 1960; Thorsteinson, 1960; Hocking, 1963; Beck, 1965). However, it has often been observed that an insect may not show the same response to an olfactory or a gustatory stimulant at all times. The factors which might be responsible for such differences in the responses of an insect to the same chemical stimulant(s) at different times have not been considered sufficiently well. Some of these factors, particularly a few of the physiological conditions of insects, which may influence their responses to gustatory stimulants have been discussed by Dethier and Chadwick (1948). Nevertheless, there is need for further examination of these as well as of other factors which govern the responses of various insects to not only gustatory but also olfactory stimuli. The role of a number of these factors has been considered in this paper on the basis of the existing literature and the writer's work on the red cotton bug, *Dysdercus koenigii* (Fabr.) (Heteroptera: Pyrrhocoridae).

The procedure adopted to measure the olfactory/gustatory responses of *Dysdercus* is described in detail elsewhere (Saxena, 1962, 1963, 1966). The intensities of "attraction", "initial-" as well as "continued-feeding" responses of the insect towards the test olfactory/gustatory stimulants were measured and expressed as the percentages of the test individuals showing these types of responses in a period of 30 min, as described before (Saxena, 1962, 1966). The olfactory and gustatory stimulants towards which the insect's responses were measured were of two types: (i) a fraction of the ether-soluble constituents of cottonseed consisting of a mixture of the triglycerides and an unidentified Factor *X* (Saxena, 1964a, 1966); and, (ii) certain essential oils of cotton leaf (Saxena, 1964a 1966). The ether-soluble constituents of cottonseed serve as both olfactory and gustatory stimulants. The olfactory stimulation by these stimulants

elicits "attraction", "initial-" as well as "continued-feeding" responses of the insect. The gustatory stimulation by the same stimulants elicits only "initial-" and "continued-feeding" responses but not "attraction" of the insect. In other words, the "attraction", "initial-" and "continued-feeding" responses of the insect to the above-mentioned seed stimulants represent both olfactory and gustatory responses (Saxena, 1964a, 1966). The essential oils of cotton leaf serve as mainly olfactory stimulants eliciting "attraction" and "initial-feeding" response but not the "continued-feeding" response of the insect (Saxena, 1964a, 1966).

It was important to carefully control the experimental conditions, particularly the concentration of the chemical stimulants, the physiological state of the insect, the temperature and the relative humidity of the laboratory for the measurement of the olfactory/gustatory responses of *Dysdercus*. The concentration of the chemical stimulants, mentioned above, was maintained at the same level as that obtaining in their natural sources. Unless otherwise stated, the insects were maintained at *28–30° C* between *40–60%* r. h., and were allowed to ingest water freely so that they remained *water satiated*. Other conditions of the experiments, especially the insect's physiological state, were varied in different tests as indicated in the text and/or the figures below. The observations presented in this paper are reproducible within statistical limits only under the experimental conditions specified.

The factors whose role on the olfactory/gustatory responses of insects is considered in this paper may be grouped under two main categories: (i) Physiological condition of the insects, and, (ii) Interaction of the olfactory/gustatory stimuli with other types of stimuli.

INFLUENCE OF PHYSIOLOGICAL CONDITION OF INSECTS ON THEIR OLFACTORY AND GUSTATORY RESPONSES

Various physiological conditions of insects whose influence on their olfactory/gustatory responses is considered in this paper include (a) starvation, (b) stage of development, (c) moulting cycle, (d) desiccation, (e) reproductive maturity, and (f) rearing medium of the insects.

Influence of Starvation of Insects

It has been observed by many workers (vide review of Dethier and Chadwick, 1948; Evans and Dethier, 1957) that most insects, when fed to satiety on a gustatory stimulant, stop showing any feeding response to the latter due to an elevation in the threshold of their gustatory receptors.

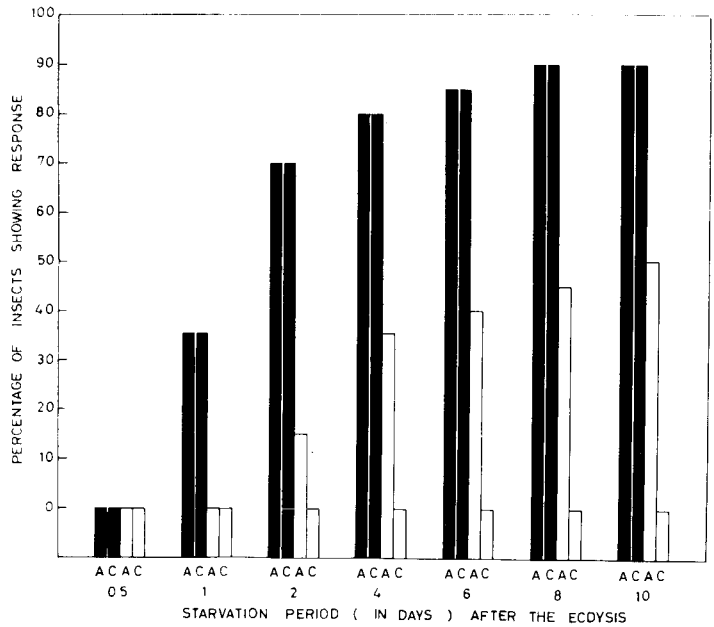


FIG. 1. Orientational and feeding responses of water-satiated fifth instar nymphs of *Dysdercus koenigii* to the ether-soluble olfactory/gustatory stimulants of cottonseed and cotton leaf after starving the insects for varying periods since the preceding ecdysis.
Solid rectangles, responses to the cottonseed stimulants; *Clear rectangles*, responses to the cotton leaf stimulants; *A*, attraction and "initial-feeding" responses (combined); *C*, "continued-feeding" responses.

The gustatory response of such individuals is restored after varying periods of starvation. According to Dethier and Bodenstein, (1958), *Phormia* adults fed to satiety on sugars cease to show any response to the stimulants because of the inhibitory impulses in the recurrent nerve caused by the presence of the ingested food in the foregut. When the latter is vacated due to the backward passage of the food, the inhibitory impulses cease and the insect begins to show the feeding response to the gustatory stimulants. Further information on the influence of food-intake and star-

vation of insects on not only their gustatory but also olfactory responses has been obtained in the writer's laboratory with reference to the red cotton bug, *Dysdercus*.

The "attraction", "initial-" as well as "continued-feeding" responses of *Dysdercus* to the ether-soluble stimulants of cottonseed increased with

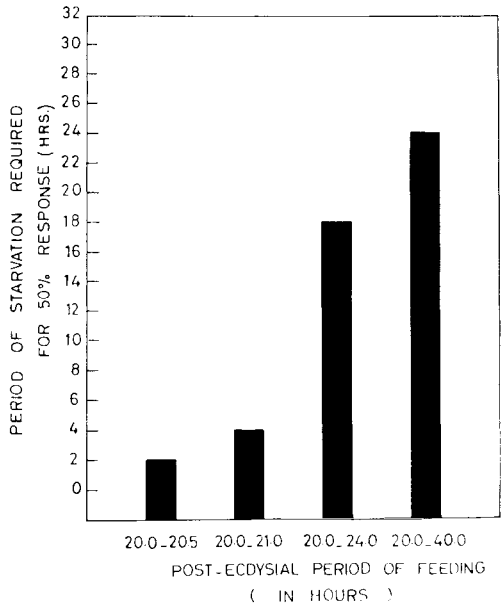


FIG. 2. Influence of starvation of water-satiated fifth instar nymphs of *Dysdercus koenigii* on their orientational and feeding responses to the ether-soluble cotton-seed-stimulants after feeding the insects on the seeds for varying durations since the preceding ecdysis.
The "attraction", "initial-" and "continued-feeding" responses in each test were identical and are therefore represented by single rectangles.

the starvation of the insect (Figs. 1 and 2). Similarly, the insect's "attraction" and "initial-feeding" responses, but not the "continued-feeding" response, to the essential oils of cotton leaf increased with the period of starvation (Fig. 1). However, the rate of increase in the intensity of each type of response with the period of starvation was much greater for the cottonseed stimulants than for the essential oils of the cotton leaf (Fig. 1).

The period of starvation required to elicit a certain level of olfactory/gustatory response of the insect varied with the degree of prior ingestion of food. Of the insects in fifth nymphal instar starved since the preceding

moult, 50% started showing all the three types of responses mentioned above by the 2nd postmoult day and 80% by the fourth postmoult day (Fig. 1). If the fifth instar nymphs starved since the preceding moult were allowed to feed on their natural diet, i. e. cottonseed, for varying durations after the 20th postmoult hour and then forced to stop feeding before satiety, they no more showed any response to the cottonseed stimulants. Such a response was lacking immediately after the interruption of feeding but the same individuals would still resume feeding on their natural diet. Such a resumption of feeding on the natural diet soon after the forced cessation of the previous feeding was not due to any chemical stimulation but mainly due to other types of stimuli emanating from the seeds as explained elsewhere (Saxena, 1966). In other words, the olfactory/gustatory responses of the insect would decline not only after a "complete" but even after a "partial" feed. Nevertheless, the partially fed insects started showing the responses again after starvation for periods which varied according to the degree of "partial feeding" (Fig. 2). The individuals starved up to the 20th postmoult hour and then fed for half an hour started showing 50% olfactory/gustatory responses to the cottonseed stimulants in about 2 hours (Fig. 2). When the duration of partial feeding was extended to 1 hour, the period of starvation required to restore the same intensity (50%) of responses was 4 hours (Fig. 2). The insects of the same age, when fed for 4 hours, required starvation for about 18 hours to show the same degree of the responses (Fig. 2). On further prolonging the period of feeding to about 20 hours, i. e. from the 20th to the 40th postmoult hour, the olfactory/gustatory responses of the insects were restored in about 24 hours (Fig. 2).

Influence of Developmental Stage of Insects

Various insects are known to show olfactory/gustatory responses to chemical stimulants present in their food-sources in the adult or immature or in both these stages (Dethier, 1947; Fraenkel, 1959; Thorsteinson, 1960; Beck, 1965). The extent to which even different instars of the immature stages of an insect may differ in their olfactory/gustatory responses is evident from the writer's observations on *Dysdercus*.

In its first two nymphal instars, *Dysdercus* did not show any olfactory/gustatory response to the ether-soluble cottonseed stimulants even if it was starved throughout each of these instars (Table 1). Both the first and the second instar nymphs moulted to the succeeding instars without any food-intake. In view of this, the nymphs could be starved from the

TABLE 1. RESPONSES OF DYSDERCUS KOENIGII IN ITS DIFFERENT DEVELOPMENTAL STAGES TO THE ETHER-SOLUBLE CHEMICAL STIMULANTS EXTRACTED FROM COTTONSEEDS

Developmental instar	Response in relation to post-ecdysial period of starvation	Remarks
First	No response even after being starved throughout the instar	Moults without any food-intake.
Second	No response even after being starved throughout the instar	Moults without any food-intake.
Third	Shows attraction, initial and continued feeding responses after 1 day's post-ecdysial starvation period	Moults only after being allowed to feed on cotton or other suitable plant seeds.
Fourth	Shows the above responses after 1 day's post-ecdysial starvation period	Moults as above.
Fifth	Shows the above responses after 1 day's post-ecdysial starvation period	Moults as above.
Adult	Shows the above responses after 3 day's starvation after the previous ecdysis	Ovaries developed and eggs produced only after feeding on cotton or other suitable seeds from 3rd/4th to 7th/8th day.

time they emerged from the eggs in the first instar up to the end of the second instar but they failed to show any olfactory/gustatory response to the above mentioned stimulants. The third, fourth and fifth instar nymphs, however, started showing the olfactory/gustatory responses to the same stimulants after 1 day's postmoult starvation (Table 1). The adult insect required a longer postmoult starvation period, i. e. about 3 days, before it started showing similar responses (Table 1).

Influence of Moulting Cycle of Insects

Another little explored physiological condition of an insect which may influence its responses to olfactory/gustatory stimulants is its moulting cycle. During each instar the physiological changes associated with the

moulting of *Dysdercus*, like those in other insects (Wigglesworth, 1953), are initiated as a result of ingestion of food. These physiological changes include deposition of the new cuticle, discharge of the moulting fluid in between the new and the old cuticle and, finally, the shedding of the old cuticle. If the insect is allowed continuous access to its diet, i. e. cottonseed, throughout the moulting cycle covering the above-mentioned physiological changes, it begins to ingest food only after an initial "quiescence phase" after the preceding ecdysis (Saxena, 1962). For the fifth instar nymph, this quiescence phase lasts for about 16 hours after which the insect begins to feed (Saxena, 1962) (Fig. 3; upper half). As the postecdysial period advances, the feeding activity of the insect increases and reaches the maximum during the 28th/32nd postecdysial hours (Fig.

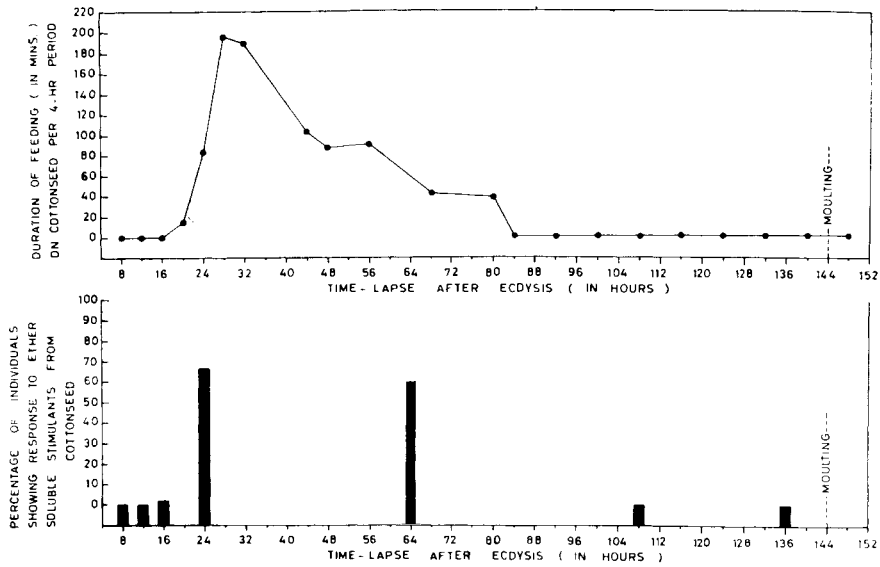


FIG. 3. Orientational and feeding responses of water-satiated fifth instar nymphs of *Dysdercus koenigii*, allowed continuous access to cottonseeds since the preceding ecdysis, to the same seeds (upper half) and to the ether-soluble olfactory/gustatory stimulants of cottonseeds (lower half) at different stages in their moulting cycle.

The individuals tested in the 8th, 12th and 16th hour after the ecdysis had been starved since the last day of the previous instar; those tested in the 24th postecdysial hour were fed after the 16th to the 20th hour and then starved for the subsequent four hours; the individuals tested during the 64th hour were fed from the 20th to the 40th postecdysial hour and then starved for 24 hours; those tested in the 108th and 136th hour were fed up to the 80th postecdysial hour and then starved for 28 and 56 hours respectively. The "attraction", "initial-" and "continued-feeding" responses in each test were identical and are represented by single rectangles.

3; upper half). Thereafter, the feeding activity of the fifth instar nymph declines and the insect completely stops feeding about the 80th postecdysial hour until after the next moult which occurs at about 144–150th hour after the preceding ecdysis (Fig. 3; upper half). These differences in the feeding activity of the insect, though allowed constant access to its natural diet, are evidently related to its moulting cycle as discussed elsewhere (Saxena, 1962).

Somewhat similar changes were observed in the olfactory/gustatory responses of the insect to the ether-soluble cottonseed stimulants in different stages of its moulting cycle (Fig. 3; lower half). These observations were made on the individuals which had been removed from the food and starved for suitable periods prior to tests in each stage of the moulting cycle of the insect. Such starvation would insure that the preceding food intake was not responsible for any decline in the insects' olfactory/gustatory responses. The period of starvation prior to tests in each stage of the moulting cycle of the insect varied according to the length of time for which the individuals had been allowed to feed since the previous ecdysis, as explained above (Fig. 2). The fifth instar nymphs of *Dysdercus*, even though starved since the last day of the preceding instar, did not show any olfactory/gustatory response to the cottonseed stimulants up to at least 16th postmoult hour (Fig. 3; lower half). Subsequent to this time interval, the nymphs started showing the olfactory/gustatory responses whose intensity increased with the advance in the postmoult period (Fig. 3, lower half) more or less in the same manner as the feeding activity of the insects on the natural diet (Fig. 3; upper half). In the 24th postmoult hour the insects, which had been allowed to feed up to the 20th hour and then starved (Fig. 2; Fig. 3; upper half), showed a fairly high intensity of olfactory/gustatory responses (Fig. 3; lower half). In the later stages of the moulting cycle, the olfactory/gustatory responses of the nymphs were determined after starving them for 24 hours to avoid any decline in their responses due to food intake, as explained before (Fig. 2). For instance, the fifth instar nymphs, allowed to feed up to the 40th postmoult hour (Fig. 3; upper half) and subsequently starved for 24 hours (Fig. 2) to restore their responses, would be in the 64th hour of the moulting cycle when they showed somewhat lower intensity of olfactory/gustatory responses (Fig. 3; lower half). When the nymphs were allowed to feed as and when they liked up to the 80th postmoult hour and tested after starvation for 24–28 hours, i.e. in the 104–108th hour of the moulting cycle, they did not show any olfactory/gustatory response at all (Fig. 3; lower half). Similarly, the individuals in the 136th hour of the moulting cycle failed to show any such response (Fig. 3; lower half) in spite

of their having been starved for 28–56 hours prior to tests. These observations indicate that the above mentioned differences in the olfactory/gustatory responses of the insect are not due to those in the periods of their starvation but due to certain physiological changes associated with the process of moulting in the insect.

Influence of Desiccation of Insects

The state of desiccation of insects has been found to influence their responses to water and humidity stimuli by a number of workers (vide review of Bursell, 1964). But little is yet known about the effect of desiccation of insects on their responses to olfactory/gustatory stimuli. The author's work on this aspect indicates that the olfactory/gustatory responses of *Dysdercus* are markedly influenced by its state of desiccation. If the fifth instar nymphs were deprived of water for varying periods, maintaining the period of their starvation since the previous ecdysis and the stage of the moulting

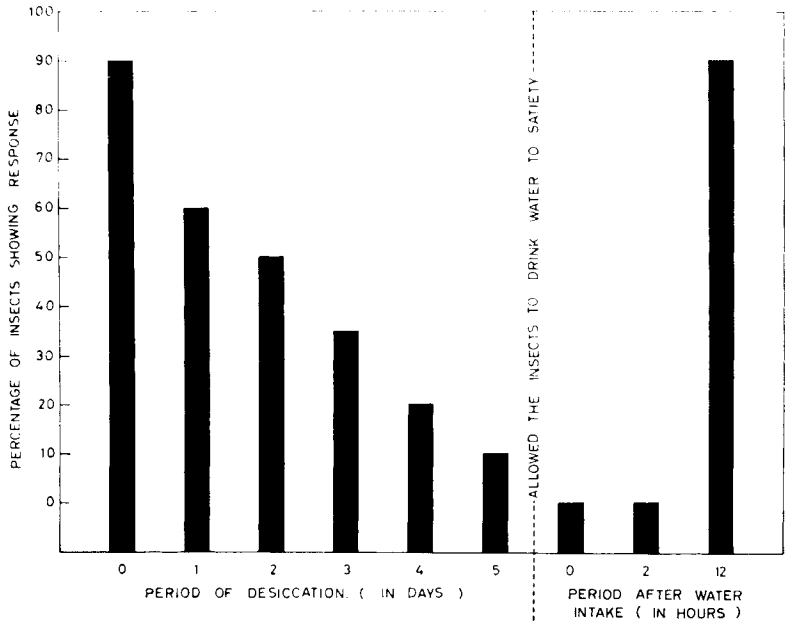


FIG. 4. Orientational and feeding responses of 8-day old and starved fifth instar nymphs of *Dysdercus koenigii* to the ether-soluble olfactory/gustatory stimulants of cottonseed after desiccating the insects for varying periods at 28–30°C between 40–60% r.h.
The "attraction", "initial-" and "continued-feeding" responses in each test were identical and are represented by single rectangles.

cycle constant, their responses to the olfactory/gustatory stimulants of cottonseed declined with the advance in the desiccation period (Fig. 4). Finally, the insects stopped showing any response after a desiccation of about five days (Fig. 4). If these desiccated individuals were allowed to ingest water to satiety, they did not immediately start showing the olfactory/gustatory responses again (Fig. 4). These responses were restored about 12 hours after the water intake by the desiccated insects (Fig. 4).

Influence of the Reproductive State of Insects

The olfactory/gustatory responses of adult insects towards a food-source which they select for their own use and/or for their younger stages seem to be influenced by the stage of maturity of their gonads, especially ovaries, in two ways: In some insects viz., houseflies (Pospišil, 1958), the intensity of response to a chemical stimulant increases with the maturity of the ovaries and reaches its maximum in the gravid females. On the other hand, in some other insects the responses of adult females to olfactory/gustatory stimuli may decline with the maturity of the ovaries. To this category belongs *Dysdercus*.

As stated before, the adults of *Dysdercus* starved since the preceding moult began to show olfactory/gustatory responses to the ether-soluble cottonseed stimulants on about the 3rd postmoult day (Fig. 5 a, b). By the fifth postmoult day about 80% of the adults, starved since the preceding ecdysis, showed the olfactory/gustatory responses (Fig. 5 c). Up to this stage the ovaries of the insects remained very small in size, weighing between 2 and 6 mg (Fig. 5 a, b, c), and their development had not started by then. On allowing the adult females to feed on their natural diet (cottonseed, from the 3rd–4th day onwards, their ovaries began to grow in size and developed eggs which were laid by the 8th–9th postmoult day. Such individuals having their ovaries in different stages of development were removed from the food and starved for 24 hours after which their olfactory/gustatory responses to the cottonseed stimulants were tested. The results showed that the intensity of responses of the females declined as the size of their ovaries increased due to the development of oocytes (Fig. 5 d, e, f). Finally the gravid females stopped showing any response at all to the olfactory/gustatory stimulants (Fig. 5 f). After the eggs had been laid by these gravid females, their ovaries again got reduced to the original size but their olfactory/gustatory responses were not restored up to 1–2 days after the oviposition (Fig. 5 g). About the third day after the oviposition the females started showing the responses again (Fig. 5 h).

It is at present difficult to state whether the above-mentioned decline in the olfactory/gustatory responses of the maturing females was due directly to the developing ovaries or due to changes in other physiological processes associated with the development of the ovaries.

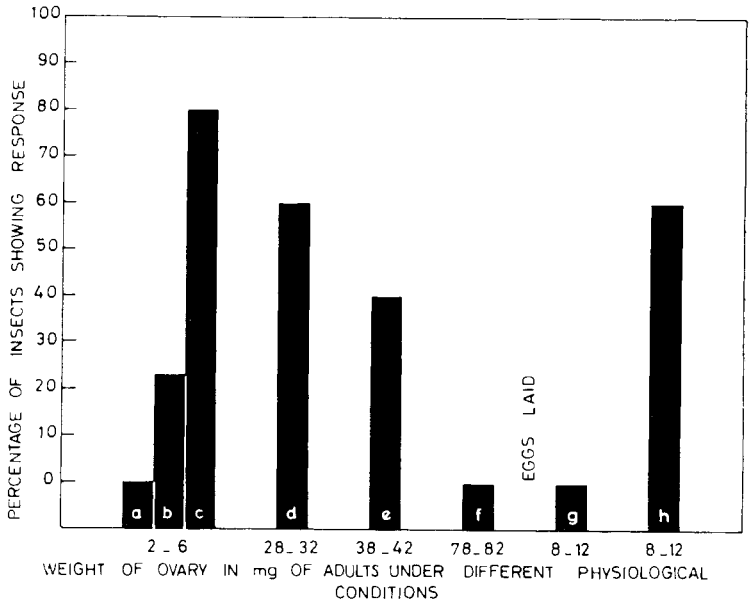


FIG. 5. Orientational and feeding responses of water-satiated female adults of *Dysdercus koenigii*, having their ovaries in different stages of development, to the ether-soluble olfactory/gustatory stimulants of cottonseed.

a, response of the insects having immature ovaries on the first postecdysial day after starving them since the preceding ecdysis; b, response of 3-day old and starved insects having immature ovaries; c, response of 5-day old and starved insects having immature ovaries; d, response of the insects having maturing ovaries after feeding them on cottonseeds from the third postecdysial day onwards and starving for 24 hours prior to the test; e, response of the same insects but with the ovaries in a more advanced stage of development; f, response of the same insects but with the ovaries in fully gravid stage; g, response of the same insects one day after they laid the eggs; h, same three days after the egg-laying; the "attraction", the "initial"- and "continued- feeding" responses in each test were the same and are represented by single rectangles.

Influence of the Rearing Medium of Insects

It has been shown by a number of workers (Frings, 1941; Thorpe, 1938, 1963; Thorpe and Jones, 1937) that some insects can be trained in their own life-time to show attraction and/or feeding responses to chemical stimulants to which they were previously indifferent or showed repulsion. Such

types of "habituation" or "associative learning" (Thorpe, 1963; Carthy, 1965) are generally short lived and are not transmitted to the progeny. On the other hand, there are a few reports to indicate that rearing an insect on a culture medium which incorporates a new type of olfactory/gustatory stimulant is likely to make the insect show preference to that chemical. For instance, Pospišil (1961) observed that rearing *Musca domestica* on a medium containing menthol resulted in a decline in the insect's repulsion to this chemical and even in developing slight attraction of the adult flies to the stimulant. The extent to which presence or absence of the ether-soluble cottonseed stimulants or of some other chemicals in a diet would influence the olfactory/gustatory responses of *Dysdercus* has been examined by the writer.

For this study, freshly emerged first instar nymphs were made to feed and develop on an unusual diet, i.e. peanut kernels, which are not infested by the insect in fields (Saxena, 1964b). Although the insect's growth and reproductive activity on peanut kernels were significantly lower than those on its natural diet, i.e. cottonseeds, it was possible to rear and breed *Dysdercus* on the former diet for successive generations. The nymphs of the third generation on peanut kernels were used for comparison of their olfactory/gustatory responses with those of cottonseed-reared insects.

The ether-soluble stimulants of cottonseed, particularly the triglycerides, are not present in peanut kernels though the latter has other types of triglycerides and lipoid constituents (Bailey, 1948; Hilditch, 1956). The olfactory/gustatory responses of both cottonseed-reared and peanut-reared *Dysdercus* to the ether-soluble constituents of cottonseed as well as of peanut kernel were compared. The first and second instar nymphs of the third generation of the peanut-reared insects, like those of the cottonseed-reared insects (Table 1), did not show any olfactory/gustatory response to cottonseed- or peanut-extracts even though the nymphs were starved throughout the first two instars. However, the third instar nymphs, starved during the first two instars, from the cottonseed-bred culture showed equal response to cottonseed-extract or to peanut-extract offered as a single choice (Fig. 6A a, b). When the same nymphs were offered both the extracts at the same time to choose from, the olfactory/gustatory responses to cottonseed-extract were of the same intensity as those to peanut-extract (Fig. 6A c). Similar results were also obtained with the third instar nymphs in the third generation of the peanut-reared insects (Fig. 6B a, b, c). If the same nymphs were allowed to feed and develop up to the fifth instar on their respective diets, the olfactory/gustatory responses of the cottonseed-reared, starved, fifth instar nymphs for cottonseed-extract were much greater than

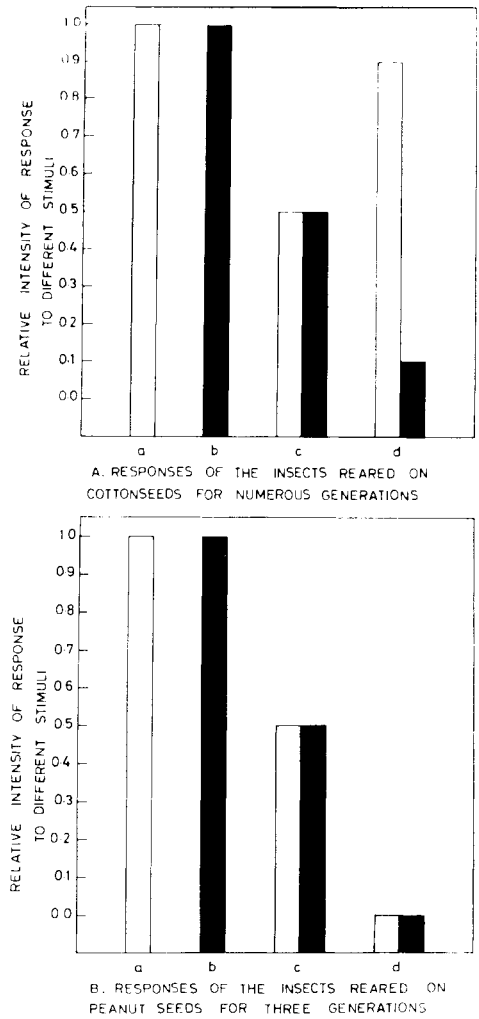


FIG. 6. Orientational and feeding responses of *Dysdercus koenigii*, reared on different media, to the extracts of cottonseed and peanut.

A, responses of the insects reared on cottonseed for numerous generations; B, same for the insects reared on peanut kernels for three generations; a, response of the third instar nymphs to the cottonseed extract offered singly after starving the insects from the first nymphal instar; b, response of the same insects to the peanut extract offered singly; c, responses of the same insects to both the cottonseed and peanut extracts offered together; d, responses of 4-day old and starved fifth instar nymphs to cottonseed and peanut extracts offered together after rearing the insects up to the end of the fourth nymphal instar on their respective media. Each rectangle represents the attraction, initial as well as the continued feeding response of the insects.

for peanut-extract (Fig. 6A d). In other words, these nymphs showed a decided preference for the cottonseed stimulants after, but not before, having been allowed to feed on cottonseed from the third to the fifth instar. On the other hand, the starved fifth instar nymphs reared on peanut showed no response to either peanut-extract or to cottonseed-extract even after starvation of 7–8 days (Figs. 6B d; 7) when the cottonseed-reared insects showed a high intensity of response to cottonseed extract (Fig. 7). It was

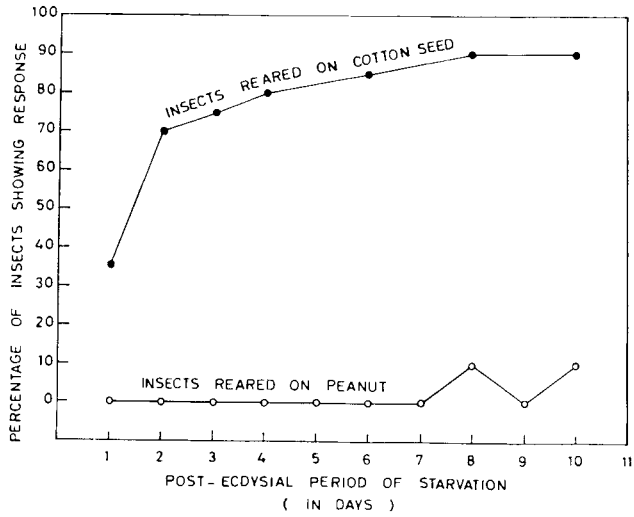


FIG. 7. Orientational and feeding responses of water-satiated fifth instar nymphs of *Dysdercus koenigii*, reared on cottonseed or on peanut, to the ether-soluble cottonseed stimulants after varying periods of starvation since the preceding ecdysis.
Each circle represents the "attraction", "initial-" as well as "continued feeding" response of the insect in each test.

only after starvation for about 8 days that peanut-reared fifth instar nymphs showed a slight olfactory/gustatory response to cottonseed-extract (Fig. 7).

The above observations suggest that *Dysdercus* is capable of developing a preference for the olfactory/gustatory stimulants of the cottonseed as a result of food-intake from this diet during its life time in earlier instars. But, it cannot develop similar preference for the corresponding constituents of peanut kernel even though the insect can feed and grow and breed on this diet. Nevertheless, deprivation of the cottonseed stimulants in the rearing medium viz., peanut kernel, reduces the intensity of olfactory/gustatory responses of the insect to these chemicals.

INTERACTION OF OLFACTORY AND GUSTATORY STIMULI WITH OTHER TYPES OF STIMULI

Responses of insects to olfactory/gustatory stimuli may also be modified by a number of non-olfactory/non-gustatory stimuli including humidity, temperature, light, etc. The pattern of such an influence is determined by the triangular relationship between the insect, the olfactory/gustatory stimulus and the non-olfactory/non-gustatory stimulus under test. This triangular relationship may be of three types as described below.

In one, the sources of the olfactory/gustatory as well as the non-olfactory/non-gustatory stimuli are situated close together or overlap each other and both the types of stimuli show a gradient in their intensity from their sources towards the insect. But, the non-olfactory/non-gustatory stimulus is *within* and the olfactory/gustatory stimulus *beyond* the range of perception of the insect. Under such conditions, the non-olfactory/non-gustatory stimulus generally determines the primary orientation of the insect resulting in its arrival within or its movement away from the range of perception of the olfactory/gustatory stimulus. For instance, photo- and thermostimuli emanating from the human body are primarily responsible for drawing the mosquitoes within the range of perception of the chemical stimuli emanating from the same source (Brown, 1956; Brown *et al.*, 1951; Burgess and Brown, 1957; Schaerffenberg and Kupka, 1959; Brown and Carmichael, 1961). Similarly, photo- and certain other non-olfactory/non-gustatory stimuli emanating from plants appear to be important in bringing the aphids within the range of perception of the olfactory/gustatory stimuli emanating from the same sources (Kennedy and Stroyan, 1959; Kennedy *et al.*, 1961).

In the second type of relationship between the insects and various stimuli, both the olfactory/gustatory and the non-olfactory/non-gustatory types of stimuli are within the range of perception of the insect. But, only the olfactory/gustatory stimulus shows a gradient in its intensity from its source towards the insect whereas the intensity of the non-olfactory/non-gustatory stimulus remains almost uniformly distributed around the insect as well as the olfactory/gustatory stimulant. Such a condition would obtain when an insect is allowed access to the olfactory/gustatory stimulant in a chamber maintaining a constant condition of the non-olfactory/non-gustatory stimulus viz., light, humidity, temperature, etc., whose effect is to be tested. Under this condition, the non-olfactory/non-gustatory stimulus does not compete with the olfactory/gustatory stimulus for eliciting the insect's orientational/feeding response. Consequently, the insect would show its orientational/feeding response to the olfactory/gustatory and not

to the non-olfactory/non-gustatory stimulus. In view of this, a change in the latter would, if at all, alter the insect's orientational/feeding response to the olfactory/gustatory stimulus without a counterchange in its similar response to the non-olfactory/non-gustatory stimulus itself. Information on this type of relationship has been given by some workers who studied the effects of light intensity, temperature and humidity on the gustatory responses of various insects (vide review of Dethier and Chadwick, 1948; Frings and Cox, 1954; Dethier and Arab, 1958). None of these stimuli, under the above-mentioned conditions, appear to modify the gustatory response of the insects examined.

In the third type of relationship between the insects and various stimuli, the sources of the olfactory/gustatory as well as the non-olfactory/non-gustatory stimuli lie close together or overlap each other and both are within the range of perception of the insect. Each of the two types of stimuli shows a gradient in its intensity from its source towards the insect. Under this condition, the non-olfactory/non-gustatory stimulus competes with the olfactory/gustatory stimulus for eliciting the orientational/feeding response of the insect. Consequently, a change in the non-olfactory/non-gustatory stimulus would, if at all, alter the insect's orientational/feeding response towards itself and thereby effect a counterchange in its response towards the olfactory/gustatory stimulus. Information on this type of relationship has been obtained by the writer in the case of *Dysdercus* and the results may be illustrated with reference to water/humidity stimulus emanating from the vicinity of the olfactory/gustatory stimulants of cottonseed mentioned above.

When 8-day starved and water-satiated fifth instar nymphs of *Dysdercus* were allowed a choice between water (on soaked cotton swab), and the olfactory/gustatory stimulants (on impregnated dry cotton swab) placed side by side, all the insects showed attraction, initial and continued-feeding response to the latter and none to water (Fig. 8 c). Their response to the olfactory/gustatory stimulant was of the same order (about 90%) as that when the stimulants were offered singly without any water stimulus (Fig. 1). If, however, the water-satiated insects were exposed to the chemical stimulants on the dry impregnated swab placed in the centre of a water-soaked swab, the intensity of their response to the former was lowered to about one-fourth (Fig. 8 d) of the original value (Fig. 8 c). It would be so because the water satiated insects are known to show a negative orientational response to water or high humidity zone (Saxena, 1960, 1964 and 1966). Such water satiated insects would therefore be prevented from arriving at and responding to the olfactory/gustatory stimulant since

their avoidance reaction to the water or high humidity zone is much more predominant than their attraction to the former.

On the other hand, if the insects starved for the same period, i.e. 8 days, but desiccated for 2 days were given a choice between water soaked swab and dry swab impregnated with the olfactory/gustatory stimulants placed side by side, all of them responded to the former and none to the latter (Fig. 8 a). The same insects showed 50% response to the olfactory/gustatory stimulants when the latter were offered alone without any water stimulus,

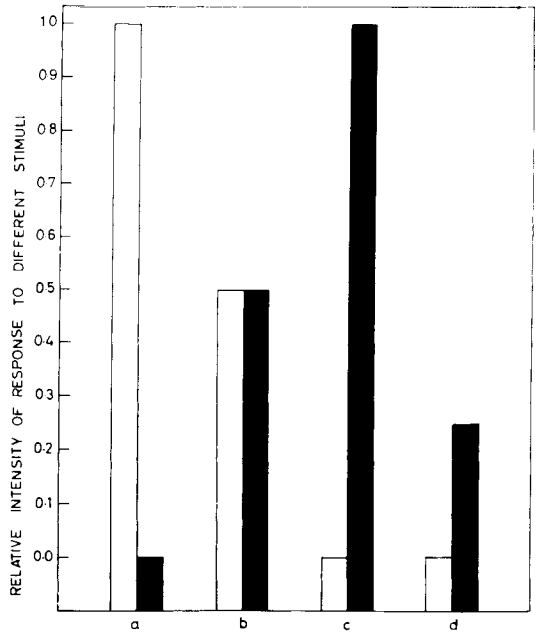


FIG. 8. Relative intensity of orientational and feeding response of 8-day old and starved fifth instar nymphs of *Dysdercus koenigii* to the olfactory/gustatory and water/humidity stimuli offered together.

Clear rectangles, relative intensity of response to the water/humidity stimulus offered as water-soaked, disc-shaped, swab of cotton wool; *Solid rectangles*, relative intensity of response to the ether-soluble cottonseed stimulants impregnated on to disc-shaped dry or water-soaked swab of cotton wool; a, response of 2-day desiccated insects offered a choice between a water-soaked swab and a dry swab impregnated with the cottonseed stimulants placed side by side; b, response of the same insects offered a plain water-soaked swab by the side of a cottonseed-stimulant-impregnated water-soaked swab; c, response of water-satiated insects offered a water-soaked swab by the side of a dry swab impregnated with the cottonseed stimulants; d, response of water-satiated insects offered cottonseed-stimulant-impregnated dry swab in the centre of a larger water-soaked swab. Each rectangle represents the "attraction", "initial-", and "continued-feeding" response of the insects in each test.

as stated before (Fig. 4). In other words, the presence of the water stimulus alongside the olfactory/gustatory stimulus served to shift the insect's response to the former thereby eliminating its response to the latter. Even if the desiccated insects were allowed a choice between the olfactory/gustatory stimulants placed on water-soaked swab and plain water-soaked swab, they showed equal response to both (Fig. 8 b). This again shows that the insect's response to water or humidity stimulus masked the effect of the olfactory/gustatory stimulus so that the insect failed to show any preference towards the latter.

CONCLUSIONS

The role of various chemical stimulants including attractants, repellents, feeding stimulants and inhibitors in the control of insect pests is being increasingly examined by a number of workers (Dethier, 1947, 1956; Green *et al.*, 1960; Hocking, 1963). The first step in adopting such a method of insect control is to survey and prepare various chemicals which serve as the stimulants for different insects. But, the facts presented in this paper indicate that a chemical stimulant may not be always effective in eliciting the desired response of an insect. In view of this, the success of controlling an insect by a chemical stimulant would depend on the knowledge of various factors which influence the insect's behavioural responses to the olfactory/gustatory stimulant. It is therefore desirable to extend the investigation of the factors considered in this paper as well as of others to other insects and chemical stimulants.

ACKNOWLEDGEMENTS

The writer takes pleasure in thanking Prof. B.R. Seshachar for suggestions in the preparation of this paper and for providing facilities for this work.

REFERENCES

BAILEY, A. E. (1948). Cotton Seed Oil. In *Cottonseed and Cottonseed Products*. Ed. A. E. BAILEY. Interscience Publishers Inc., N. Y. 364–408.

BECK, S. D. (1965). Resistance of plants to insects. *Ann. Rev. Ent.* **10**, 207–232.

BROWN, A. W. A. (1956). Factors which attract *Aedes* mosquito to humans. *Proc. X Intern. Congr. Entomol.* **3**, 757–763 (1958).

BROWN, A. W. A. and CARMICHAEL, A. G. (1961). Lysine and alanine as mosquito attractants. *J. econ. Ent.* **54**, 317–324.

BROWN, A. W. A., SARKARIA, D. S. and THOMPSON, R. P. (1951). Studies of the responses of the female *Aëdes* mosquito. Part I. The search for attractant vapours. *Bull. ent. Res.* **42**, 105–114.

BURGESS, L. and BROWN, A. W. A. (1957). Studies of the responses of the female *Aëdes* mosquito. Part VIII. The attractiveness of beef blood to *Aëdes aegypti* (L.). *Bull. ent. Res.* **48,** 783–793.

BURSELL, E. (1964). *Environmental Aspects: Humidity.* In the *Physiology of Insecta*, Vol. I. Ed. MORRIS ROCKSTEIN. Academic Press, New York. 324–358.

CARTHY, J. D. (1965). *The Behaviour of Arthropods.* Univ. Revs. in Biol. Ser. Oliver & Boyd, Edin. & Lond.

DETHIER, V. G. (1947). *Chemical Insect Attractants and Repellents.* Blackiston Co. Philadelphia.

DETHIER, V. G. (1953). Host plant perception in phytophagous insects. *Trans. IX Intern. Congr. Entomol.* **2,** 81–88.

DETHIER, V. G. (1956). Repellents. *Ann. Rev. Ent.* **1,** 181–202.

DETHIER, V. G. and ARAB, Y. M. (1958). Effect of temperature on the contact chemoreceptors of the Blowfly. *J. Ins. Physiol.* **2,** 153–161.

DETHIER, V. G. and BODENSTEIN, D. (1958). Hunger in Blowfly. *Zeitsch. Tierpsychol.* **15,** 129–140.

DETHIER, V. G. and CHADWICK, L. E. (1948). Chemoreception in insects. *Physiol. Rev.* **28,** 220–254.

EVANS, D. R. and DETHIER, V. G. (1957). The regulation of taste thresholds for sugars in the Blowfly. *J. Ins. Physiol.* **1,** 3–17.

FEIR, D. and BECK, S. D. (1963). Feeding behaviour of the large milkweed bug, *Oncopeltus fasciatus. Ann. ent. Soc. Amer.* **56,** 224–229.

FRAENKEL, G. (1959). The chemistry of host specificity of phytophagous insects. In *Biochemistry of Insects*, Ed. L. LEVENBOOK. Pergamon Press. 1–14.

FRINGS, H. (1941). The loci of olfactory end organs in the blowfly *Cynomia cadaverina* Desvoidy. *J. Exp. Zool.* **88,** 65–93.

FRINGS, H. and COX, B. L. (1954). The effects of temperature on the sucrose thresholds of the tarsal chemoreceptors of the flesh fly *Sarcophaga bullata. Biol. Bull.* **107,** 360–363.

GREEN, N., BEROZA, M. and HALL, A. S. (1960). Recent Developments in Chemical Attractants for Insects. In *Advances in Pest Control Research*, Vol. III. Ed. R. L. METCALF. Interscience Publishers, N.Y. 129–179.

HILDITCH, T. P. (1956). *The Chemical Constitution of Natural Fats.* Chapman & Holt Ltd.

HOCKING, B. (1963). The use of attractants and repellents in vector control. *Bull. Wld. Hlth. Org.* **29,** 121–126.

KENNEDY, J. S. and STROYAN, H. L. G. 1959. Biology of aphids. *Ann. Rev. Ent.* **4,** 139–160.

KENNEDY, J. S., BOOTH, C. O. and KERSHAW, W. J. S. (1961). Host finding by aphids in the field. III. Visual attraction. *Ann. appl. Biol.* **49,** 1–21.

POSPÍŠIL, J. (1958). Některé problémy čichu u saprofilních much. *Acta Soc. Ent. Čechosloveniae* **55,** 316–334.

POSPÍŠIL, J. (1961). Vliv chovného prostredi larev na chemotaxi dospělých much. *Folia Zoologica* **10,** 219–222.

SAXENA, K. N. (1960). Physiological factors governing establishment of *Dysdercus koenigii* (Fabr.) (Pyrrhocoridae) on its host plants—I. Role of hygroreception in orientation and feeding behaviour. *Proc. Ind. Sci. Congr. Abst.* **47,** 461.

SAXENA, K. N. (1962). Food intake in relation to the growth of *Dysdercus koenigii. Ann. ent. Soc. Amer.* **55,** 218–225.

SAXENA, K. N. (1963). Mode of ingestion in a heteropterous insect, *Dysdercus koenigii. J. Ins. Physiol.* **9,** 47–71.

SAXENA, K. N. (1964a). Control of orientation and feeding behaviour of red cotton bug. *Dysdercus koenigii* by chemical constituents of plants. *Proc. XII Intern. Congr. Entomol. London*, 294 (1965).

SAXENA, K. N. (1964b). Physiological factors governing susceptibility and resistance of plants to red cotton bug, *Dysdercus koenigii*. *Proc. XII Intern. Congr. Entomol. London*, 541 (1955).

SAXENA, K. N. (1966). Control of orientation and feeding behaviour of red cotton bug, *Dysdercus koenigii* (Fabr.) (under publication).

SCHAERFFENBERG, B. and KUPKA, H. (1959). Der attraktiv Faktor des Blutes für blutsaugende Insekten. *Naturwiss*, **46**, 457–458.

THORPE, W. H. (1938). Olfactory conditioning. *Proc. roy. Soc. B* **126**, 370–397.

THORPE, W. H. (1963). *Learning and Instinct in Animals*. Methuen. London.

THORPE, W. H. and JONES, F. G. W. (1937). Olfactory conditioning and its relation to the problem of host selection. *Proc. roy. Soc. B* **124**, 56–81.

THORSTEINSON, A. J. (1960). Host selection in phytophagous insects. *Ann, Rev. Ent.* **5**, 193–218.

WIGGLESWORTH, V. B. (1953). *The Principles of Insect Physiology*. Methuen, London.

DIFFERENTIAL IMPEDANCE CHANGES OF THE OLFACTORY MUCOSA WITH ODOROUS STIMULATION

ROBERT C. GESTELAND

Northwestern University, Evanston, Illinois

IF A small alternating current is applied to the olfactory mucosa through an electrode touching the surface and the mucosa is then stimulated with a puff of odor, changes can be seen in the a.c. signal recorded by a second electrode located nearby. This is not surprising as nervous activity is inherently a process of membrane conductance change. However, we may ask whether such a measure can contribute to our understanding of either receptor mechanisms or coding of odor information. Measurements of the slow voltages (EOG voltages) across the mucosa or spike activity of the receptor cells caused by odors have not yielded enough information to allow unique answers to our questions about how the nose works. I present here some preliminary results which suggest that new information can be gained using alternating current as test probe.

We can think of the change in the recorded a.c. signal as a measure of change in the transfer impedance of the patch of receptor tissue which connects the injection electrode to the recording electrode, since the output is a sinusoidal voltage and the input a current. This impedance change can be characterized by a change in impedance magnitude and a change in phase angle and is measured by the recorded voltage amplitude change and phase shift caused by odorous stimulation. Lettvin and I have made use of this measure to separate excitatory and inhibitory mechanisms in the olfactory receptors. This is reported elsewhere (Gesteland, *et al.*, 1965).

PROCEDURE

The experimental method is as follows. Frogs (*R. pipiens*) were pithed and then the roof of the nasal cavity was removed, exposing the *eminentia olfactoria* on which the receptor cell epithelium is found. Particular care

must be taken to maintain a vigorous circulation in the animal and to prevent seepage of blood onto the mucosa. A stream of moist air plays continously on the exposed epithelium to prevent dehydration. Recording and stimulating electrodes are glass pipettes filled with a stiff gelatin solution made up in 0·9% NaCl solution. Tip diameters are between 4 and 50 micra and contact with the electrolyte gel is made with Ag/AgCl electrodes. The common electrode is a gauze-wrapped Ag/AgCl plate placed in the frog's mouth. The gauze is wet with 0·9% NaCl solution. For stimulation, puffs of odorous vapors are injected into the moist air stream playing on the mucosa. These puffs are delivered at a flow rate which is small compared to the moist air flow to minimize aerodynamic artifacts. When puffs of clean air are substituted for odorous puffs, no response is evoked.

The recording method is shown schematically in Fig. 1. The signal injection electrode is positioned so that the tip just makes contact with the mucus surface of the eminentia. A sinusoidal current, small enough so that

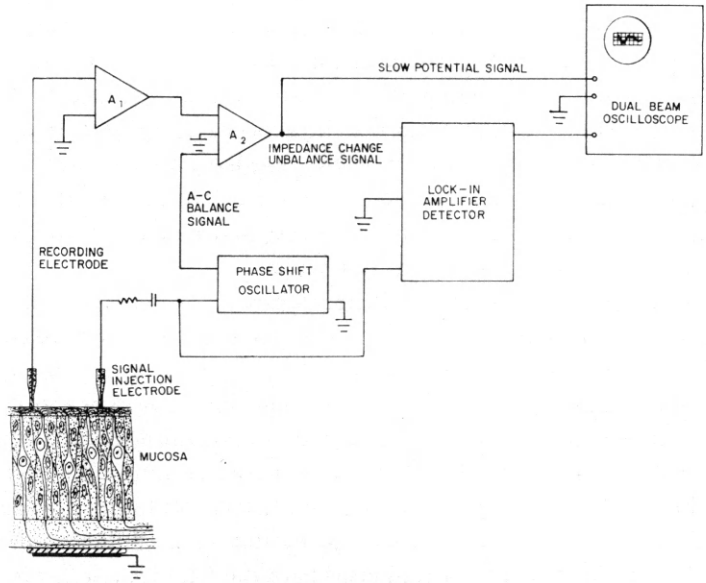


FIG. 1. This is a schematic diagram of the apparatus used for measuring impedance change. The EOG voltage is displayed on one beam and the impedance change on the other of a dual-beam oscilloscope. The a.c. balance signal used to null out the resting level a.c. signal recorded from the mucosa comes from a Hewlett-Packard Type 203A Variable Phase Function Generator, which has two output voltages, one of which may be varied in phase with respect to the other. A section of the mucosa is drawn under the electrodes. The preparation consisted of a pithed frog with vigorous circulation, not an isolated piece of olfactory epithelium.

it does not electrically excite the receptor cells, is continuously passed through the injection electrode. The recording electrode is positioned so that its tip touches the mucus a few tip diameters away from the injection electrode. It is directly coupled to an electrometer preamplifier and thus records both the voltage waves due to olfactory stimulation (the EOG) and the a.c. signal voltage due to the current passed through the injection electrode. Since we are interested in detecting amplitude changes of a few per cent and phase shifts of a few degrees in the a.c. signal and since the a.c. voltage at the electrode has a peak amplitude of the order of a millivolt, a coherent detection system is used. It has the double advantage of greatly improving the signal-to-noise ratio compared to an amplifier and filter combination and of allowing amplitude changes and phase changes to be separated.

In the figure, A_1 is the electrometer-preamplifier and A_2 serves as a virtual bridge. That is, the amplified signal from the recording electrode is subtracted from a balancing signal in the A_2 differential amplifier. The balancing signal is the variable amplitude and phase output from the oscillator and is adjusted so that in the resting state (no odorous stimulation) it is in phase and slightly larger than the signal from the recording electrode. The output of A_2 is fed to one channel of an oscilloscope to display the EOG. It is also fed to the detector, a Princeton Applied Research Type JB-5 or JB-6 Lock-In Amplifier. The reference signal to the Lock-In Amplifier is the same as the signal feeding the current injection electrode. Under small signal conditions (small amplitude and phase changes) the detector output will be proportional primarily to amplitude changes if the reference signal is in phase with the output of A_2 and primarily to phase changes if the reference signal is in quadrature with the output of A_2. A phase-shifter for the reference signal is included in the detector. An integrating time of 0·1 sec at the detector output is the best compromise at the measuring frequencies of interest, yielding a reasonable signal-to-noise ratio without seriously degrading the rise time of the response.

Changes in the recorded a.c. signal with odor stimulation occur using any frequency from 1 cps to 10 kc. However, over most of this band these are not very informative. Either the magnitude and angle changes follow similar time courses, or one changes with little change in the other. In the band between 20 and 50 cps, on the other hand, the variety of phenomena is richer. Both orthogonal components of the transfer impedance may change with independent time courses. The changes are of both polarities, i.e. the voltage magnitude at the recording electrode may increase or decrease and the voltage phase angle may either lead or lag. The particular

frequency band where this occurs is no doubt dependent upon the size of the electrodes and the mucus layer thickness and conductivity, but certainly there ought to be a band of frequencies where both components of the transfer impedance change with the same order of magnitude so that a change of one does not swamp changes in the other. An immediate conclusion which can be drawn from the finding of independent magnitude and angle changes is that there must be at least two odor-dependent processes occuring in the receptor cells.

In a healthy frog the patterns of the odor-evoked changes in transfer impedance are the same each time a similar strength and duration puff of the same odor is delivered. The first sign of degeneration of the cells of the mucosa is failure of an odor to produce a repeatable response. Action potentials recorded from axons of single receptor cells show the degeneration less clearly. It is usually seen as a tendency of a cell to be excited by most all stimuli or to be unresponsive to most stimuli, whereas a healthy cell is more selective in its responses. Changes in the EOG waveform are the least sensitive indicator of degeneration. A poor mucosa has lower amplitude EOG voltages and less complex ones. In particular the initial positive voltage swing evoked by some odors is quite labile.

RESULTS

Responses to eighteen different stimulating odors are shown in Fig. 2. The top tracing in each group is the EOG voltage. The polarity is such that an upward deflection occurs when the mucosa surface is positive with respect to the ground electrode in the frog's mouth. The duration of all traces is 10 sec. Odor puffs were 2 sec long unless otherwise noted and of an intensity such that the evoked EOG had a peak amplitude of 1–3 mV. The center trace in each group shows changes in the amplitude of the recorded a.c. signal, hence is proportional to the transfer impedance magnitude changes. Points above zero represent an increase in voltage magnitude (hence a decrease in the impedance coupling the injection and recording electrodes or an increase in the shunt impedance from the two electrodes to the ground electrode or some combination of the two effects). The lower trace in each group shows changes in the phase angle of the recorded signal with values above the axis representing a leading phase angle. The responses are tracings of oscillograms recorded on Polaroid film. In these examples the magnitude and phase angle curves were obtained from successive puffs of odor and only those pairs where an identical EOG voltage was evoked were used. I took particular care to align the tracings so that the

onset of the odor puff for the magnitude tracing is directly above the onset of the puff for the phase angle tracing. Each odor was used at least twice during each experiment with at least a half-hour between trials. If identical responses were not obtained from the two trials, the experiment was rejected. Further, it must be emphasized that these are preliminary findings and the examples shown are not intended to be an exhaustive collection of possible patterns nor as a substitute for a set of averaged responses with some measure of frog-to-frog variance. These are being pursued but it is a slow process to find responses of many frogs to each of many odors with variations of stimulus parameters.

If these impedance parameters are to have any significance for study of receptor function, it is necessary to show that the patterns of change seen are primarily dependent upon odor rather than upon a particular location of the recording electrode, particular stimulus intensity and flow rate, or the particular frog used. Figure 2a shows the responses of two different frogs (separate experiments on different days) to approximately identical puffs of benzonitrile. In the curve shown as a dotted line the odor puff had a somewhat higher flow rate, hence sharper onset, as can be seen from the faster rise of the EOG voltage. The shapes of all three measures of response are quite alike for both animals. Figure 2b shows responses to nitrobenzene, a substance with an odor somewhat similar to benzonitrile. Here the responses at two different recording electrode locations on the mucosa are shown, one by the solid lines, the other by the dotted lines. The stimulus was again about the same for the two experiments. Again the shapes of all three measures of response are preserved in spite of the lower amplitudes of the solid-line curve. The amplitude of the EOG was shown by Ottoson (1956) to depend on the receptor cell density in the vicinity of the recording electrode and this varies over the mucosa surface. This experiment shows the same effect for impedance measures. For the latter, the separation of the current injection electrode and the recording electrode will also affect the amplitude of the responses but not the shapes. In Fig. 2c the solid lines are responses to a two-second puff of spearmint extract. The dotted lines are the responses to a five-second puff of the same odor. The intensity (flow rate of odorized air) was reduced for the longer puff resulting in changes in peak amplitudes and slower rise and fall times. Again the patterns of the two responses are similar but the longer, slower onset puff causes a stretch in the time axis. In addition the positive peak of the impedance magnitude is higher for the longer, weaker puff, as if the early response is an inhibition which is less for weaker onsets. Figure 2d shows responses to a low flow rate two-second puff of menthol as solid lines and

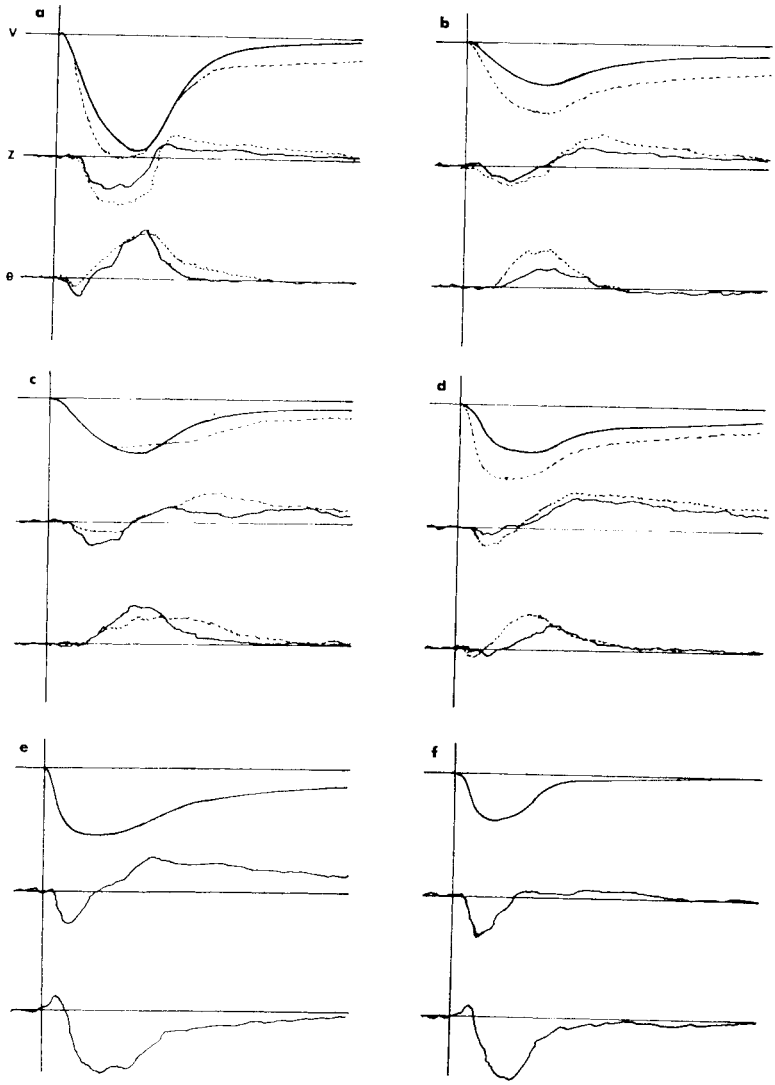
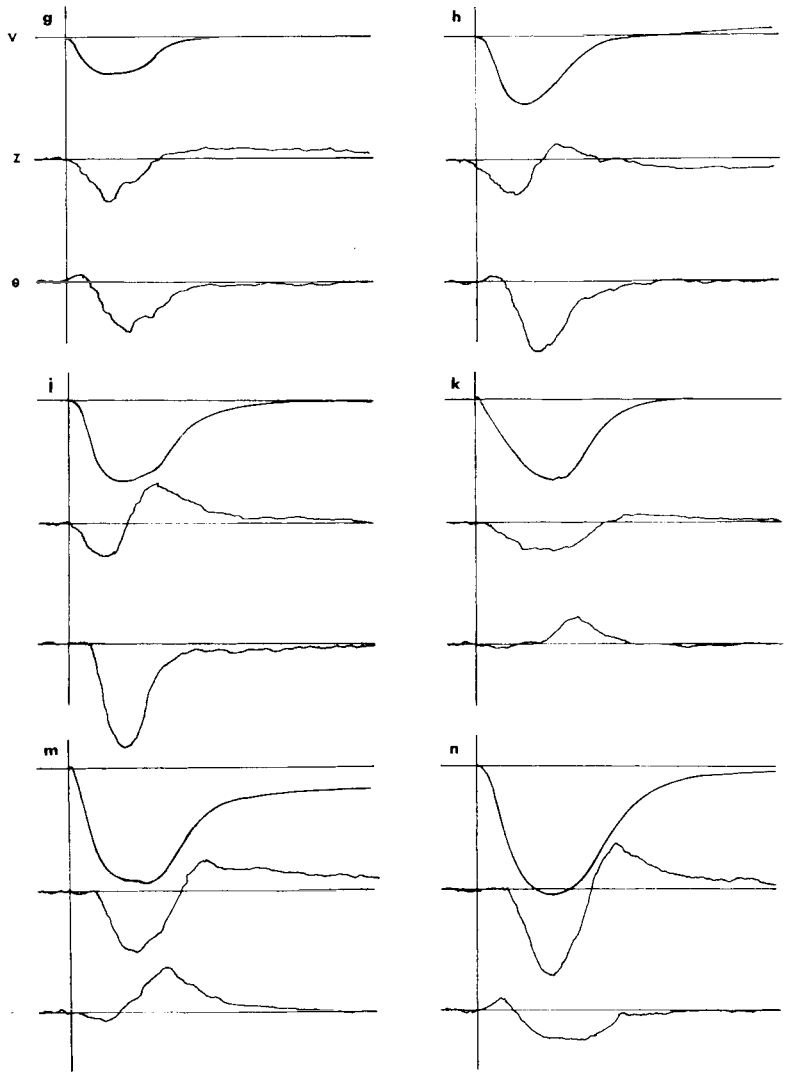
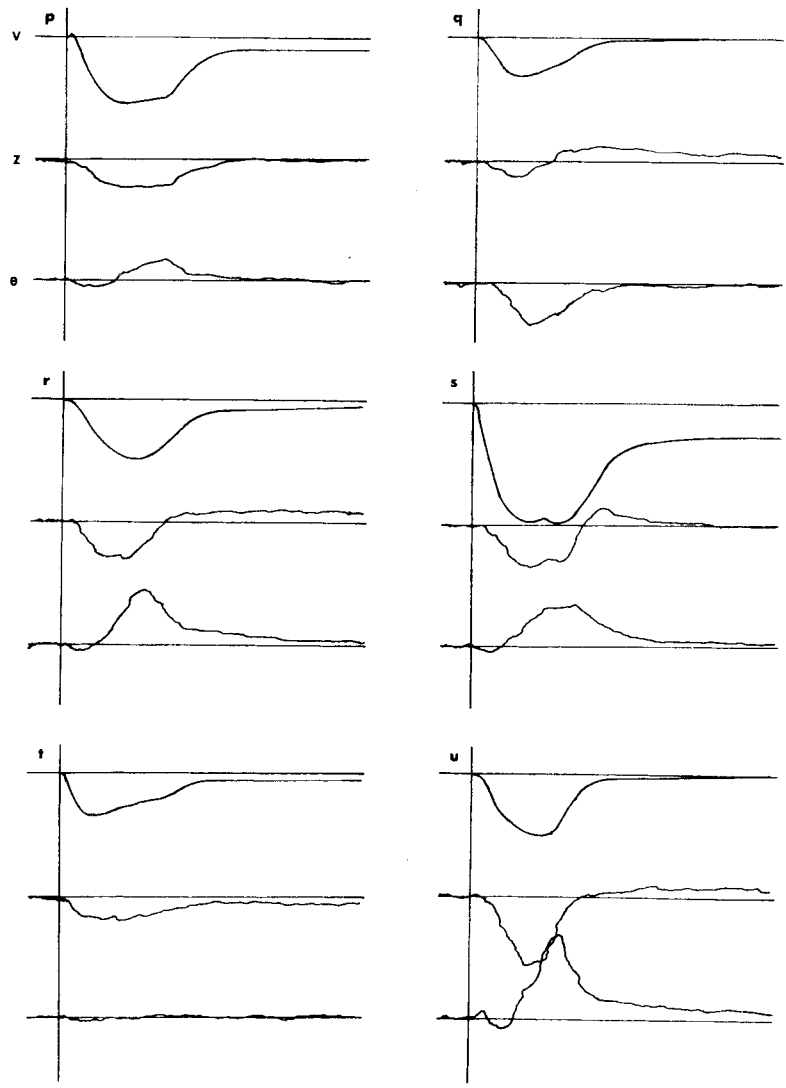


FIG. 2. Responses evoked by different odors. The top trace in each group is the EOG voltage (V). The center trace is the transfer impedance magnitude change (Z). The lower trace is the transfer impedance phase angle change (θ). Odor puffs began shortly before the vertical axis line and lasted 2 sec except in c. where the dotted lines represent responses to a 5 sec odor puff. Time base is 10 sec. For the voltage trace positive polarity is upward. For the impedance magnitude trace,



points above the axis represent increase in the a.c. voltage at the recording electrode. For the phase angle tracing, points above the axis represent a leading shift in the phase angle of the signal at the recording electrode. a. Responses of two frogs (one shown as a solid line, the other as a dotted line) to benzonitrile. b. Responses to nitrobenzene with the recording electrode at two different locations on the mucosa surface. The dotted curves are for a position closer to the injection



electrode than the curves shown as solid lines. c. Responses to a short puff of spearmint (solid lines) and a longer puff (dotted lines). d. Responses to a flow-rate puff of menthol (solid lines) and a higher flow-rate puff (dotted lines). Other responses shown are evoked by e, camphor; f, civet; g, coumarin; h, cyclopentanone; j, diallyl disulfide; k, 2-ethylaminoethanol; m, geraniol; n, limonene; p, phenylethyl alcohol; q, menthone; r, peppermint; s, salicylaldehyde; t, musk xylene; and u, ylang-ylang.

to a higher flow rate two-second puff of the same substance as dotted lines. The stronger puff causes faster rise times, still preserving the general shapes of the responses, as if the time axis had been compressed. As in 2b and 2c, the relative amplitudes of peaks does change with stimulus parameters, but no new peaks due to the odor presented one way drop out when the same odor is presented in another way. To a great extent, the changes observed in the responses are what we would predict if we knew the way in which the stimulus parameters or electrode location were changed.

Figures 2e through 2t show responses to two-second puffs of camphor, civet, coumarin, cyclopentanone, diallyl disulfide, 2-ethylaminoethanol, geraniol, limonene, phenylethyl alcohol, menthone, peppermint, salicylaldehyde, 2,4,6-trinitro-3, 5-dimethyl-*tert* butylbenzene (musk xylene) and ylang-ylang. Some are relatively pure laboratory reagents, others are very complex mixtures, essences extracted from natural sources. These are merely examples selected from a much larger group of substances I have used. Some have similar chemical properties, some have similar odors. As a group they represent a wide variety of odors and a wide range of chemical properties. Menthone and menthol smell similarly and benzaldehyde and nitrobenzene smell similarly. Few people, however, would have trouble distinguishing between the members of the pairs. All of the curves shown came from one of five experiments. All could be repeatably elicited during the course of the experiment and the mucosae in these experiments lasted for many hours before there were signs of degeneration. The degraded mucosa, caused by poor circulation, drying, over stimulation, or irritation and damage due to the electrode shows the following signs. The amplitudes of the impedance and phase angle curves are diminished, repetition of a stimulus results in responses which are not identical (usually losing a phase or two), and after a short while, responses to practically all odors become similar for both impedance and phase angle. The pattern is generally a downward swing followed by an upward swing. All responses begin to look alike, but sloppily so.

At first glance the collection of responses in Fig. 2 seems rather like a random collection of disturbances. However, what is true here and what is particularly striking after watching a large number of experiments, is that each pair of impedance magnitude and angle curves seem unique. Substances which smell different, even slightly so, evoke clearly distinctive patterns. After a series of experiments my intuitive feeling is that I can guess the odor used from the patterns on the oscilloscope. It is not certain yet and it will take considerably more experimentation to prove that there is not a smooth gradation of pattern types as the number of odors is greatly in-

creased. However, it is at least possible that impedance patterns can be as separate and distinctive for odors as are words separate and distinctive in a language, neither forming a continuum. In fact, since very similar odors produce startlingly different patterns (Figs. 2a–2b and 2d–2r) it will be surprising if the patterns from a great number of odors can be ordered to form a continuum.

The variety of patterns is large. Note the impedance curves in Figs. 2e, 2n, 2s, and 2t and the phase curves in 2a, 2f, 2k, 2s, and 2t. Onset times, time relations between magnitude and angle, duration of after effect, rise times, and notches are all functions of the odor.

DISCUSSION

Responses of the receptor cells of the frog's olfactory mucosa are most simply characterized by the statement that each cell orders odors and combinations of odors from most exciting to most inhibiting in a unique way. Thus every cell "looks" at odor space from a different point of view (Lettvin and Gesteland, 1965). However, the EOG waveforms are quite consistent for the mucosa and show similar shapes for homologous series of chemicals and some clearly different types of patterns for different odors (Ottoson, 1956; Takagi and Shibuya, 1959; Gesteland, 1964). The changes in transfer impedance magnitude and phase angle presented here are also of a variety of different patterns and consistently alike for the same odor anywhere on the mucosa and from any frog. Thus the ensemble of receptor cells has an average response which, measured grossly by the EOG and the impedance change, allows us to distinguish a large number of different odors. At the level of the receptor cells, odor space has as many dimensions as there are differently-responding receptor cells. However, since only three measures on the ensemble of cells are apparently enough to distinguish as many odors as I have been able to try, it must be possible to construct a space of as few dimensions to describe the physical chemistry of odorous substances with respect to their action on receptor membranes. (To identify odors from the grossly recorded electrical changes, it is necessary to know about the duration and the intensity of the sniff or puff, but this information is available from proprioceptor responses and receptors in the nose which signal aerodynamic information.) Since there are only three ways in which a cell can respond, exaltation of firing rate, depression of firing rate, or no change in firing rate, it is not unnatural to expect that this number of orthogonal parameters should be enough to characterize odors (as suggested by Chalazonitis and Chalazonitis earlier in this sym-

posium). These gross measures which I describe will not shed much information on receptor molecular processes since the mucosa is a sheet of cells with both shunt impedance and transverse impedance changing due to odor. The most that can be hoped is that with a more extensive series of experiments my suggestion that three dimensions are enough to separate all distinguishable odors can be more firmly established. The principal enigmas remain. One is a question requiring chemical answers, i.e. what are the molecular mechanisms by which sensitive biological tissue reacts with stimulus molecules? The other is how does the brain sort out the holistic representation of odor space which occurs at the receptor level to construct notions of identity, similarity, and odor quality? There must be some such transformation done on the odor signals coming from the primary cells since there are so few second ordor cells compared to the number of receptors.

ACKNOWLEDGMENTS

Part of this work was done at the Scientific Engineering Institute, Waltham, Massachusetts supported by a grant from the Dearborn Foundation. Some of the odorous materials were donated by Firmenich, Inc. and by Fritzsche Brothers, Inc. Mrs. Diane Kidder assisted with the experiments.

REFERENCES

CHALAZONITIS, A. and CHALAZONITIS, N. (1966). Molecular control of the excitability of giant neuron in relation to olfactory perception mechanisms. This volume.

GESTELAND, R. C. (1964). Initial events of the electro-olfactogram. *Ann. N. Y. Acad. Sci.* **116**, 440–447.

GESTELAND, R. C., LETTVIN, J. Y. and PITTS, W. H. (1965). Chemical transmission in the nose of the frog. *J. Physiol.*, **181**, 525–559.

LETTVIN, J. Y. and GESTELAND, R. C. (1965). Speculations on smell. *Cold Spring Harbor Symposium*, **30**, 217–225.

OTTOSON, D. (1956). Analysis of the electrical activity of the olfactory epithelium. *Acta Physiol. Scand.* **35**, suppl. 122, 1–83.

TAKAGI, S. F. and SHIBUYA, T. (1959). "On"- and "off"-responses of the olfactory epithelium. *Nature*, **184**, 60.

LIST OF PARTICIPANTS

AMOORE, J. S., U.S. Agricultural Research Service, Albany, USA.
ARVANITAKI-CHALAZONITIS, I.N.P., 2 Laboratoire de Neurophysiologique Cellulaire, Marseille 9e, France.
ASRATYAN, E. A., Institute of Higher Nervous Activity, Moscow B-17 USSR.
ADACHI, AKIRA., Dept. of Oral Physiology, Osaka Univ., Dental School., Japan.
AMATSU, MASAHIRO, Dept. of Physiology, Kumamoto Univ., Medical School Kumamoto, Japan.
BARDACH, JOHN E., Fisheries, Univ. of Michigan, Ann Arbor, USA.
BEIDLER, L. M., Biological Science, Florida State Univ., Tallahassee, USA.
BELL, F. R., Royal Veterinary College Field Station, Hatfield, Herts, UK.
BOECKH, J., Psychiatrie, Max Planck-Institute, 8 München 23, Germany.
BORG, G., Otorhinological Clinic, Umeå and the Department of Physiology, Sweden.
BOTT, E., Howard Florey Laboratories of Experimental Physiology, Univ. of Melbourne, Parkville, N. 2., Australia.
CHACKO, L. W., Anatomy, All India Institute of Medical Science, New Delhi-16, India
CHALAZONITIS, N., I.N.P. 2 Laboratoire de Neurophysiologique Cellulaire 31, Marseille 9e, France.
CHAUNCEY, H. H., Dental Medicine, Tufts Univ., Boston, USA.
CARRERAS, M., Clinica delle Malattie Nervese e Mentali, Università di Parma, Umana-Università, Italia.
COLLISTER, T., Univ of Detroit, School of Dentistry, Mich. USA.
DEMERDACHE, A., British Columbia Research Council, Vancouver, Canada.
DENTON, DREK A., Physiology, Univ. of Melbourne, Victoria, Australia.
DIAMANT, H., Otorhinological Clinic, Umeå and the Department of Physiology, Sweden.
DRAVNIEKS, A., IIT Research Institute, Chicago, USA.
FELDMAN, S.E., Poultry Husbandry, Univ. of California, Berkeley, USA.
FELLER, R.P., Dept. of Oral Pathology, Tufts Univ., School of Dental Medicine, Boston, Mass., USAF, Brooks Air Force Base, Texas., USA.
FISHER, G. L., Brown University, School of Dentistry, Mich., USA.
FRANK, M. K., Univ. of Maryland.
FREGLY, MELVIN J., Physiology, Univ. of Florida, Gainesville, USA.
FUJIYA, MASARU, School of Natural Resourses, Univ. of Michigan, Ann Arbor, USA.
FUNAKOSHI, MASAYA, Oral Physiology, Dental School, Osaka Univ., Japan.
GAFFEY, C. T., Univ. of California, Donner Laboratory, Berkeley, USA.
GANTT, W. H., Pavlovian Lab., The Johns Hopkins Univ., Baltimore, USA.
GESTELAND, R. C., Scientific Engineering Institute, Waltham, Mass. USA.
HALPERN, B. P., Physiology, State Univ. of N.T., Upstate Medical Center, Syracuse N.Y., USA.
HANAOKA, TOSHIMASA, School of Husbandry, Nara Women's Univ., Japan.
HANSON, F. E. Biophysics Division U. S. Naval Med. Res. Inst. Bethesda, USA.
HARTKE, G. T., Dept. of Anatomy, College of Veterinary Medicine, Kansas State Univ., Manhattan, USA.
HAYASHI, TAKASHI, Physiology, Keio Univ., Tokyo, Japan.
HELLEKANT, GÖRAN, Physiology, Kungle, Veterinärhögskolan, Stockholm, Sweden.

HENDRIX, D. E., Neurophysiology and EEG, Neurology and Psychiatry, Northwestern Univ., Medical School, Chicago, Illinois, USA.
HENKIN, R. I., National Heart Institute, NIH, Bethesda, USA.
HODGSON, E. S., Zoology, Columbia Univ., New York, USA.
HOLL, A. Natural Resources, Univ. Michigan, USA.
HOSHINO, H., Dept. of Physiology, School of Medicine, Keio Univ., Japan.
HOSHISHIMA, KEIICHIRO, Hygine, Fukushima Medical College, Japan.
HUGHES, J. R., Neurophysiology, Northwestern Univ., Chicago, USA.
ICHIOKA, MASAMICHI, Oral Physiology, Medical and Dental Univ., Tokyo, Japan.
IGGO, A., Dept. of Veterinary, Univ. of Edinburgh, Scotland, UK.
ISHIKAWA, SHIGEO, Sericultural Experiment Station, Suginami-ku, Tokyo, Japan.
ISHIKO, NOBUSADA, Dept. of Physiology, Kumamoto Univ., Medical School Kumamoto, Japan.
IZUMI, K. Hygine, Fukushima Med. College, Japan.
JOHNSTON, J. W. Jr Georgetown Univ., Washington, D.C., USA.
KAPUR, K. K., Univ. of Detroit, Dentistry, Michigan, USA.
KAWAMOTO, NOBUYUKI, Fisheries, Agricultural and Veterinary Science, Nihon Univ., Setagaya-ku, Tokyo, Japan.
KAWAMURA, YOJIRO, Dept. of Oral Physiology, Dental School, Osaka Univ. Osaka, Japan.
KITCHELL, R. L., Veterinary; Medicine, Kansas Univ., USA.
KLEEREKOPER, HERMAN, Stazione Zoologica, Napoli, Italy.
KONISHI, JIHEI, Fisheries, Mie Prefectural Univ., Tsu, Japan.
KUWABARA, MASUTARO, Biology, School of Physics, Kyushu Univ., Fukuoka, Japan.
LEEK, B. F., Dept. of Veterinary Physiology Univ. of Edinburgh, UK.
LIVINGSTON, A. Jr, The Pavlovian Laboratories, The Johns Hopkins Univ., Medical School, Maryland, USA.
MACKENZIE, J. S., Dept. of Physiology, Univ. of Melborne, Victoria, Australia.
MANCIA, D., Clinica dell Malattie Newvese e Mentali, Università di Parma, Umana-Università, Italy.
MANCIA, MAURO, Physiology, Univ. of Milano, Via Mangiagalli, Italy.
MATHEWS, D. F., Biological Science, Florida State Univ., Tallahassee, USA.
MORITA, HIROMICHI, Biology, Faculty of Science, Kyushu Univ. Fukuoka Japan.
MOULTON, D. G., Biological Science, Florida State Univ. Tallahasse, USA.
MOZELL, MAXWELL M., Physiology, State Univ. of N.Y., Upstate Medical Center, New York, Syracuse, USA.
MUKHOPADHYAYA, B. K., Dept. of Anatomy, All India Institute of Medical Sciences, Ansari Nagar, New Delhi, India.
NAIDU, K. J. R., Dept. of Anatomy, All India Institute of Medical Sciences, Ansari Nagar, New Delhi, India.
OAKLEY, B., Zoology, Univ. of California, Los Angeles, USA.
OGAWA, HISASHI, Physiology, Kumamoto Univ. Japan.
OGURA, MITSUO, Physiology, Mie Prefectural Univ., Tsu, Japan.
OKANO, MASAOMI, Dept. of Cooperative Anatomy, Nihon Univ. Setagaya, Tokyo, Japan.
OOTSUKA, TOSHIRO, Physiology, Keio Univ., Tokyo, Japan.
PANGBORN, R. M., Food and Science Technology, Univ. of California, USA.
PFAFFMANN, C., Psychology, Brown Univ., Providence, USA.
ROGERS, V. P., Dept. of Anatomy, College of Veterinary Medicine, Kansas State Univ., Manhattan, USA.
SATO, MASAYASU, Physiology, Kumamoto Univ., Japan.
SATO, YOSHIHIKO *ibid.*
SAXENA, K. N., Zoology, Univ. of New Delhi, India.
SEKI, SONOKO, Physiology, Kanagawa Dental College, Yokosuka, Japan.

SEN GUPTA, P., Dept. of Anatomy, All India Institute of Medical Science, Ansari Nagar, New Delhi-16, India.
SHANNON, I. L., Dept. of Experimental Dentistry, School of Aerospace Medicine, USAF, Brooks Air Force Base, Texas, USA.
SHIBUYA, TATSUAKI, Dept. of Physiology, Gumma Univ., Maebashi, Japan.
SHIMIZU, WATARU, Fishery, Agriculture and Veterinary Medicine, Nihon Univ., Setagaya-ku, Japan.
STEINHARDT, R, A., Columbia Univ., New York,., N. Y., USA.
STEVENSON, J. A. F., Physiology, Univ. of Western Ontario, London, Canada.
STONE, H., Life Science, Stanford Research Institute, California, USA.
STRÖM, L., Otorhinological Clinic, Umeå and the Dept. of Physiology, Sweden.
STÜRCKOW, B., Entomology Research Division, Agr. Res. Serv., USDA, Beltsville, Md., USA.
SUGAYA, EIICHI, Physiology, Kanagawa Dental College, Yokosuka, Japan.
SUHARA, ROKURO, Physiology, Nihon Univ., School of Dentistry, Japan.
TAKAGI, SADAYUKI Physiology, Gumma Univ., Maebashi, Japan.
TAKASHITA, HIROO, Physiology, Nihon Univ., School of Dentistry, Japan.
TAKEUCHI, HIROSHI I. N. P., 2 Laboratoire de Neurophysiologique Cellulaire, 31 Chemin Joseph, Aiguire, Marseille, France.
TANYOLAC, N. N., Robert College Research Center, Bebek, Istanbul, Turkey.
TATEDA, HIDEKI, Dept. of Biology, Faculty of Science, Kyushu Univ., Japan.
TUCKER, DON, Biological Science, Florida State Univ., Tallahassee, USA.
VENSTROM, D., Western Regional Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Albany, California, USA.
WATERS, I. W., Dept. of Physiology, Univ. of Florida, College of Medicine, Gainesville, USA.
WELLER, S., Howard Florey Laboratories of Experimental Physiology, Univ. of Melbourne, Parkville, N.2, Australia.
WENZEL, BERNICE M., Physiology, Univ. of California, Los Angeles, USA.
WILLIAMS, C. H., The Pavlovian Laboratories, The Johns Hopkins Univ., Medical School, Maryland, USA.
WOLBARSHT, MYRON L., Naval Medical Research Institute, Bethesda, USA.
WRIGHT, R. H., British Columbia Research Council, Vancouver, Canada.
YAMADA, KASUHIRO, Dept. of Physiology, Kumamoto Univ., Medical School Kumamoto, Japan.
YAMASHITA, SATORU, Dept. of Physiology, Kumamoto Univ., Medical School, Japan.
YAZAKI, SHINOBU, Dept. of Physiology, Nihon Univ., School of Dentistry, Tokyo, Japan.
ZOTTERMAN, Y., Physiology, Kungl Veterinärhögskolan, Stockholm, Sweden.