NEUROCHEMISTRY OF THE RETINA

Proceedings of the International Symposium on the Neurochemistry of the Retina held in Athens, Greece, August 28 - September 1, 1979

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PERGAMON PRESS

OXFORD · NEW YORK · TORONTO · SYDNEY · PARIS · FRANKFURT

U.K.	Pergamon Press Ltd., Headington Hill Hall, Oxford OX3 0BW, England
U.S.A.	Pergamon Press Inc., Maxwell House, Fairview Park, Elmsford, New York 10523, U.S.A.
CANADA	Pergamon of Canada, Suite 104, 150 Consumers Road, Willowdale, Ontario M2J 1P9, Canada
AUSTRALIA	Pergamon Press (Aust.) Pty. Ltd., P.O. Box 544, Potts Point, N.S.W. 2011, Australia
FRANCE	Pergamon Press SARL, 24 rue des Ecoles, 75240 Paris, Cedex 05, France
FEDERAL REPUBLIC OF GERMANY	Pergamon Press GmbH, 6242 Kronberg-Taunus, Hammerweg 6, Federal Republic of Germany

First edition 1980

British Library Cataloguing in Publication Data

International Symposium on the Neurochemistry of the Retina, *Athens, 1979*
Neurochemistry of the retina.
1. Retina - Congresses
2. Neurochemistry - Congresses
I. Title II. Bazan, Nicolas G
III. Lolley, Richard N
IV. 'Neurochemistry international'
V. International Society for Neurochemistry, *Meeting, 7th, Athens, 1979*
596'.01'823 QP479 80-40215

ISBN 0 08 025485 3

Published as a special issue of the journal *Neurochemistry International*, Volume 1, Numbers 1-4 and supplied to subscribers as part of their subscription. Also available to non-subscribers.

In order to make this volume available as economically and as rapidly as possible the authors' typescripts have been reproduced in their original forms. This method unfortunately has its typographical limitations but it is hoped that they in no way distract the reader.

Printed in Great Britain by A. Wheaton & Co. Ltd., Exeter

Neurochemistry Vol. 1, p. 1-2.
Pergamon Press Ltd. 1980. Printed in Great Britain.

PREFACE

Neurochemistry as a discipline began about a century ago with the pioneering work of Thudichum who catalogued the chemical constituents of the brain. At about the same time, Kühne identified a visual pigment in the retina of the eye. From this discovery has developed a body of knowledge that is associated specifically with visual research but which holds truths and principles applicable to neurochemistry in general. The International Symposium on the Neurochemistry of the Retina held in Athens, Greece, was dedicated to bringing neurochemists together with visual scientists so that cross-fertilization between the two disciplines could occur and, hopefully, the fruits of this interaction would bring conceptual advances, profiting both disciplines.

In some species, the retina acts as a tiny brain telling the animal whether to strike at food or retreat from a predator. In all vertebrates, the retina is derived embryonically from an out-pocketing of the central nervous system, and the mature retina and brain share many features in common. For example, the retina lies within a protected blood-retinal barrier, and associative neurons of the retina are involved in the complicated process of information transfer. The retina differs from the brain, however, in that during differentiation, a population of retinal neurons become specialized for the absorption of light and for the ability to transduce the light-triggered signal into a change in membrane polarization.

All visual cells are not identical in the retina, and they can be subclassified morphologically and functionally into rods and cones. Rod visual cells have morphologically unique outer segments which are composed of stacks of membranous disks in which the visual pigment, rhodopsin, is embedded; they respond to low levels of illumination and interact with many second-order neurons of the retina. Cone visual cells possess outer segments which are shorter than those of rod photoreceptors and which contain a visual pigment (not rhodopsin) in closely opposed folds of the receptor plasma membrane; they respond to high intensity light and interact with fewer second-order neurons than do the rod photoreceptors. The rod system functions at night or in subdued lighting, and the images perceived by the rod system are gray or black and white. The cone system is functional in daylight, and the images are perceived in color.

We now know that rods and cones both are active continuously in the dark and that both are inhibited by light. This reality is not perhaps what one would expect; however, it is only one of the amazing discoveries that have recently emerged. The cessation of neurotransmitter release by photoreceptor cells upon exposure to light is the signal which alters the potential of second-order neurons and which initiates a series of cell: cell interactions within a discrete visual field circuitry.

Associative neurons and bipolar cells provide the ganglion cells with processed information that is transmitted to the brain. The cell: cell interactions which apparently control and define information processing in the retina are probably

identical to those of the brain. Recent advances in visual research suggest that it may be possible to identify and characterize the entire retinal circuitry.

It is intriguing how a neuron which cannot renew itself by cell division can remain vigorous and healthy throughout a lifetime. Photoreceptor cells remain biochemically young by continuously renewing their cellular constituents; this manner of rejuvenation may be universal among non-replicating cells. Membrane renewal has been documented beautifully in rod visual cells where the membranes of the outer segment are produced within the cell body, assembled at the base of the outer segment, pushed toward the tip of the outer segment by the assembly of new membranes and, finally, shed from the tip of the outer segment, only to be ingested and phagocytized by the adjacent pigment epithelium cells. The renewal of cone membranes differs slightly from that of rods due to the morphological organization of their outer segments.

Visual cell renewal mechanisms are attuned to a daily rhythm, with rods shedding membranes at dawn and cones shedding membranes at dusk. Overall, the renewal process provides for a visual cell that is continuously in good repair. Hopefully, an analogous process in the brain keeps the neurons in a state of youthful vigor.

An inability to renew the biochemical machinery of the cell or an inherited defect in the metabolism or function of visual cells can lead to pathological changes that result in cell degeneration and blindness. Rod visual cells seem to be especially susceptible to abnormal levels of cyclic GMP and of specific amino acids. Cyclic GMP appears to be an important regulatory component of rod photoreceptors but its role in visual cell degeneration has yet to be defined. Similarly, it is unclear why low systemic levels of taurine or high systemic levels of ornithine lead to visual cell degeneration and blindness.

Much has been discovered in the past decade about the morphology, physiology and biochemistry of visual cells and the capabilities of second-order neurons to process visual information. It is a pleasure to share some of these findings with neurochemists who have not kept abreast of the vision literature. In order to facilitate the flow of ideas and information between individuals, the Symposium in Greece was designed so that free and open discussion occurred in the formal sessions and during leisure time. The Symposium was a rewarding experience for speakers and participants alike. Perhaps, this volume will convey the spirit of the meeting to those who were unable to attend.

We gratefully acknowledge financial support from Fidia Pharm. Research Laboratories, Abano Terme, Padua, Italy; Sandoz Ltd., Basel, Switzerland; The Ministry of Culture and Sciences of Greece and The National Foundation March of Dimes, White Plains, New York. We are very grateful to the Instituto de Investigaciones Bioquímicas (INIBIBB), Universidad Nacional del Sur-Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) of Bahía Blanca, Argentina, where the organization and planning of the Symposium was done. Pergamon Press has made possible an early publication . We are indebted to Drs. Norma M. Giusto and Marta I. Aveldaño de Caldironi for their invaluable help. We are particularly thankful to Mrs. María Haydée Salaberry de Saint-Lary; Lic. Antonio P. Marcattili and Mrs. Lucía Mabel Fernandez de Ljutek for their very able editorial asistance.

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Neurochemistry Vol. 1, pp. 3-16.
Pergamon Press Ltd. 1980. Printed in Great Britain.

TRANSVERSE DISTRIBUTION OF PHOSPHOLIPIDS IN THE VERTEBRATE PHOTORECEPTOR MEMBRANE

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ABSTRACT

A study has been made of the distribution of the three major phospholipids of the bovine photoreceptor membrane over the two faces of the lipid bilayer.

Three different approaches have been used: a. Treatment of isolated intact outer segments and disk vesicles (lysed outer segments) with three different phospholipases and determination of the degradation pattern. As randomized control preparations a retinal lipid suspension and detergent solubilized disks were used. b. Treatment of intact and lysed outer segments with the amino group reagent trinitrobenzenesulfonic acid (with and without treatment by phospholipase D) and determination of modification of phosphatidylethanolamine and phosphatidylserine. c. Determination of the fatty acid composition of the membrane phospholipids during treatment by phospholipase D or TNBS.

The results consistently indicate a nearly symmetric distribution of phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine and their fatty acids over the two membrane faces, with a slight preponderance of the first phospholipid on the inner side and possibly a slight excess of the second one on the outer side.

KEYWORDS

Asymmetry, fatty acids, phospholipases, phospholipids, photoreceptor membrane, trinitrobenzenesulfonate.

INTRODUCTION

The importance of the phospholipid environment for proper functioning of membrane proteins is by now well documented (see e.g. Sanderman, 1978). Membrane bound enzymes commonly require phospholipids for activity, in some cases like Ca^{2+}-Mg^{2+} activated ATPase an annulus of about 30 moles of phospholipids per mole of enzyme (Warren and others, 1975), in others like Na^{+}-K^{+} activated ATPase a larger cloud of at least 90 moles of phospholipids per mole of enzyme (De Pont and others, 1978). In the case of the photoreceptor membrane removal of 90 % or more of the 65 moles of phospholipid normally present per mole rhodopsin severely lowers the thermal stabil-

ity and the regeneration capacity of the visual pigment and blocks its photolytic sequence after the formation of metarhodopsin I (Van Breugel and others, 1978) in parallel with lateral aggregation of the rhodopsin molecules (Olive and others, 1978), which changes are completely reversible upon detergent-mediated reconstitution with phospholipids. There appears to be little specificity for a particular phospholipid in these three cases (see e.g. De Pont and others, 1978).

It is generally agreed that the phospholipids in cell membranes are arranged in large part as a bilayer (Singer, 1974). For the photoreceptor membrane this has recently been confirmed by means of ^{31}P-NMR studies (De Grip and others, 1979b). This membrane has as its three major phospholipids phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine, comprising 36, 45 and 16 % of total phospholipid, respectively (Anderson and others, 1975).

In recent years studies have been made to determine how the various phospholipids are distributed between the two faces of the bilayer, since this could obviously be of significance for our understanding of the role of phospholipids in membrane function. It has been claimed that asymmetric distribution of phospholipids occurs in various biological membranes (Rothman and Lenard, 1977), although there is controversy in a number of cases especially with regard to intracellular membranes (see e.g. Van den Besselaar and others, 1978). It is clear that different independent methods need to be used with appropriate caution before reliable conclusions can be drawn.

In this paper we describe the methods used by us and the results obtained for the rod photoreceptor membrane, which can be considered to be an intracellular membrane.

EXPERIMENTAL APPROACHES

The methods that can be used include the application of phospholipases, of group specific reagents, of phospholipid exchange proteins and of NMR spectroscopy (Bergelson and Barsukov, 1977). The first two methods have been applied by us so far.

Three phospholipases, phospholipase A_2, C and D, have been employed to determine the distribution of the three major phospholipids of the photoreceptor membrane. Phospholipase A_2 removes the fatty acid from the glycerol-C-2 position with the formation of a lysophospholipid. Phospholipase C removes the phosphate ester, leaving a diglyceride. Phospholipase D removes the base group, leaving phosphatidic acid. In all cases two-dimensional thin layer chromatography with phosphate determinations of the spots is used to determine the phospholipids and their water-insoluble hydrolysis products (Broekhuyse, 1968). In addition, phosphate analysis of the aqueous layers is performed to allow determining a complete balance of the phospholipid breakdown.

This approach is based on the assumption that these enzymes will not penetrate an intact membrane, and thus will only attack the phospholipids in the outer membrane face. This requires the use of intact outer segments with stacked disks to ensure right-side-out photoreceptor membrane orientation, and the observation of the early phase of enzymatic degradation to minimize the risk of membrane and phospholipid inversion. Fresh preparations must be used, since freezing and thawing may invert the photoreceptor membrane (Adams and others, 1979). In addition, the specificity of the phospholipases toward different phospholipids should be taken into account. Randomized control preparations are used for this purpose.

The other approach involves the use of trinitrobenzenesulfonate (TNBS), which reacts with the free amino groups of phosphatidylethanolamine and phosphatidylserine yield-

ing their trinitrophenyl derivatives. These derivatives appear as discrete yellow spots on the two-dimensional thin layer chromatogram (Gordesky and others, 1975). The approach is again based on the assumption that the reagent will not penetrate the membrane or reverse the membrane orientation or cause phospholipid inversion. This requires selection of suitable reaction conditions and comparison of the effects fects with those on randomized control preparations. In addition to reaction with TNBS alone, the treatment can also be combined with phospholipase treatment before or after reaction with TNBS, which allows a direct comparison of the effects of the two agents.

Finally, the distribution of fatty acids in the phospholipids on either side of the membrane can be determined by gaschromatographic analysis of the methylated fatty acids (Morrison, 1964) present in the TLC spots before and after treatment of the membrane preparation with phospholipase D or TNBS.

EXPERIMENTAL DETAILS

Photoreceptor Membrane Preparations

The intact outer segment preparation ("stacked disks") consists of bovine rods isolated by density gradient centrifugation is a sucrose-Ficoll 400 medium (Schnetkamp and others, 1979). Electromicroscopic observation reveals stacked disks, surrounded by plasma membrane, closely resembling outer segment structure in situ, which ensures right-side-out orientation of the photoreceptor membrane in this preparation.

For comparison water-lysed outer segments isolated by sucrose density gradient centrifugation (De Grip and others, 1972) are used. Electromicroscopic observation shows globular unilamellar vesicles ("disk vesicles"), in which membrane inversion may have taken place to some extent.

As a randomized control preparation for the determination of phospholipase specificity a "retinal lipid suspension" is employed. The lipids of whole cattle retina are extracted (Bligh and Dyer, 1959) and after solvent evaporation suspended and sonicated in 0.16 M Tris-HCl buffer (pH 7.4). The phospholipid composition of this preparation sufficiently resembles that of rod outer segment to allow its use as a control preparation for determining the enzyme specificity. Phospholipase D requires for optimal activity 40 mM Ca^{2+}, which causes flocculation of the phospholipid suspension. Hence, for this enzyme a detergent solubilized disk preparation ("solubilized disks") is used, prepared by dissolving disk vesicles in 20 mM β-1-nonylglucose in 0.16 M Tris-HCl, pH 6.0 (De Grip and Bovee Geurts, 1979a).

Phospholipase A_2 Treatment

Phospholipase A_2 (E.C. 3.1.1.4) from pig pancreas is a gift of Prof. G.H. de Haas, Dept. of Biochemistry, University of Utrecht, The Netherlands. The stacked disks are resuspended in a medium containing 600 mM sucrose, 5% (w/w) Ficoll 400, 20 mM Tris-HCl (pH 7.4). Disk vesicles (and retinal lipid suspension) are resuspended in 0.16 M Tris-HCl (pH 7.4). Incubation is carried out at 20 °C in the presence of 10 mM $CaCl_2$ in darkness, and is started by adding an appropriate amount of enzyme solubilized in water. The reaction is stopped by adding an excess of icecold buffer containing 10 mM EDTA, but in the case of retinal lipid suspension by adding the chloroform-methanol extracting mixture.

During phospholipase A_2 treatment the decrease for each phospholipid is completely accounted for by the increase in the corresponding lysocompound, except that a

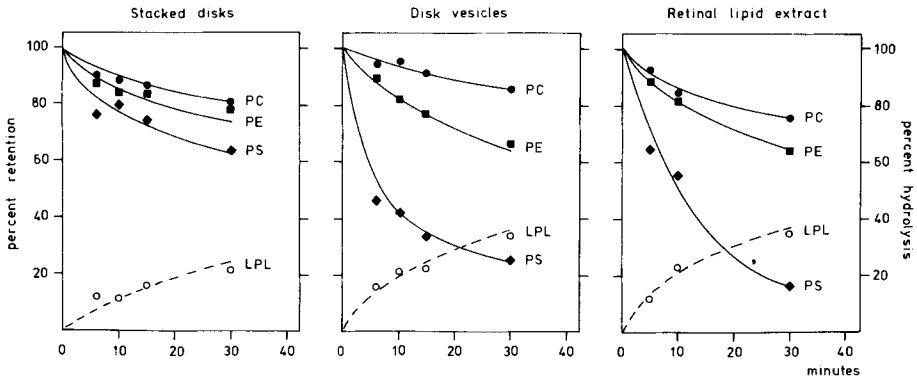

Fig. 1. Treatment with phospholipase A_2. Percent retention of individual phospholipids (closed symbols) and percent overall hydrolysis (open symbols) upon treatment of stacked disks, disk vesicles and retinal lipid suspension are shown. PC= phosphatidylcholine, PE= phosphatidylethanolamine, PS= phosphatidylserine, LPL= lysophospholipid.

small amount of lysophosphatidylserine is lost during washing.

The observed order of preference of phospholipase A_2 for retinal lipid suspension is: phosphatidylserine > phosphatidylethanolamine > phosphatidylcholine. The same order of preference is found with stacked disks and disk vesicles (Fig. 1).

At high enzyme concentrations all phospholipids in these two preparations are completely converted to their lysoproducts (Table 1), suggesting that ultimately this enzyme penetrates the disk membrane or causes membrane inversion. The initial rate of hydrolysis (first 10 min) is lower for stacked disks than for disk vesicles and retinal lipid suspension (Table 2). The preference for negatively charged phospholipids is in agreement with earlier observations (De Haas and others, 1968).

TABLE 1. Final Phospholipid Hydrolysis

Enzyme	Stacked disks	Disk vesicles	Reference
Phospholipase A_2	100	100	100
Phospholipase C	98	97	99
Phospholipase D	40	53	77

Results are expressed as percent of total phospholipids initially present hydrolyzed by the indicated enzyme in up to 3 hours under conditions described in text. Reference is retinal lipid suspension in the case of phospholipases A_2 and C, and solubilized disks in the case of phospholipase D.

TABLE 2. Initial Phospholipid Hydrolysis

Phospholipid	Stacked disks	Reference	Ratio
	Phospholipase A_2		
Total phospholipids	14	23	
Phosphatidylcholine	30	38	0.79
Phosphatidylethanolamine	45	36	1.25
Phosphatidylserine	25	26	0.96
	Phospholipase C		
Total phospholipids	33	59	
Phosphatidylcholine	38	49	0.75
Phosphatidylethanolamine	47	48	0.98
Phosphatidylserine	15	3	(5)
	Phospholipase D		
Total phospholipids	9	32	
Phosphatidylcholine	54	61	0.89
Phosphatidylethanolamine	44	37	1.20
Phosphatidylserine	2	2	1

Total phospholipids: percent of initially present phospholipid hydrolyzed in first 10 min of incubation. Results for disk vesicles are in all cases intermediate between those for Stacked disks and Reference (explanation under Table 1).
Results for individual phospholipids are expressed as percent of total phospholipids hydrolyzed in first 10 min of incubation.

Phospholipase C Treatment

Phospholipase C (E.C. 3.1.4.3) is isolated from cultures of B.cereus (Otnaess and others, 1972) and stored in 50% glycerol, 1 mM $ZnCl_2$. The incubation conditions are the same as those for phospholipase A_2, except that no $CaCl_2$ is added.

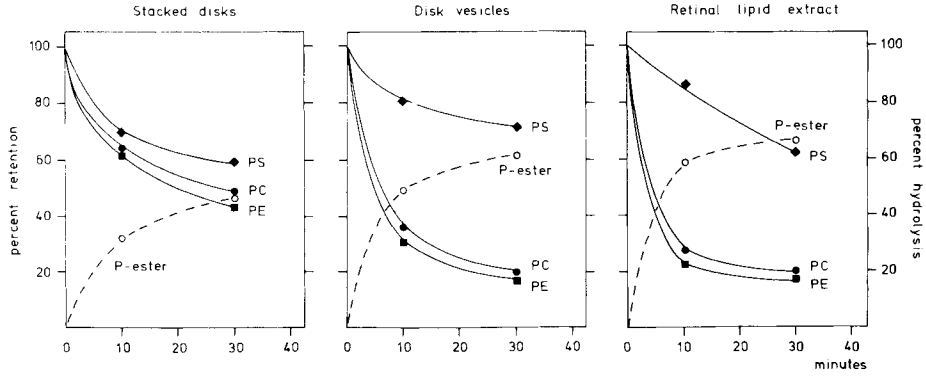

Fig. 2. Treatment with phospholipase C. Percent retention of individual phospholipids (closed symbols) and percent overall hydrolysis (open symbols) upon treatment of stacked disks, disk vesicles and retinal lipid suspension are shown. Abbreviations as in Fig. 1.

During phospholipase C treatment there is quantitative agreement between the decrease of phospholipids and the amount of phosphate ester appearing in the aqueous supernatant (measured as inorganic phosphate after acid hydrolysis).

The substrate preference for retinal lipid suspension is: phosphatidylethanolamine > phosphatidylcholine > phosphatidylserine, in agreement with an earlier report of Roberts and others (1978). The same order of preference is observed for stacked disks and disk vesicles (Fig. 2). The initial rate of hydrolysis is again lowest for stacked disks (Table 2).

Exhaustive treatment of all three preparations with phospholipase C results in nearly complete hydrolysis of the phospholipid, the residual phospholipids consisting almost exclusively of phosphatidylserine (Table 1).

Phospholipase D Treatment

Phospholipase D (E.C. 3.1.4.4) is isolated from Savoy cabbage (Davidson and Long, 1958) or obtained from Boehringer (Mannheim), both preparations giving the same results. Stacked disks are resuspended in a medium containing 600 mM sucrose, 5% (w/w) Ficoll 400, 20 mM Tris-maleate (pH 6.0). Disk vesicles and solubilized disks are resuspended in 0.16 M Tris-maleate (pH 6.0). Incubation is carried out at 30 °C at pH 6.0 in the presence of 40 mM $CaCl_2$ in darkness (optimal conditions). The reaction is started by the addition of the enzyme in aqueous solution, and is stopped by the addition of excess icecold buffer containing 10 mM EDTA for stacked disks and disk vesicles and of chloroform-methanol extraction mixture for solubilized disks.

Under the conditions specified here no transferase activity of phospholipase D is observed. There is, however, in the phospholipase D preparations phosphatidate phosphohydrolase (E.C. 3.1.3.4) activity, which hydrolyses phosphatidic acid to diglyceride and inorganic phosphate (Davidson and Long, 1958). This explains why we always find less phosphatidic acid formed than expected from the decrease in phospholipids. The sum of phosphatidic acid in the lipid phase and inorganic phosphate in the aqueous phase fully accounts for the amount of phospholipids hydrolysed.

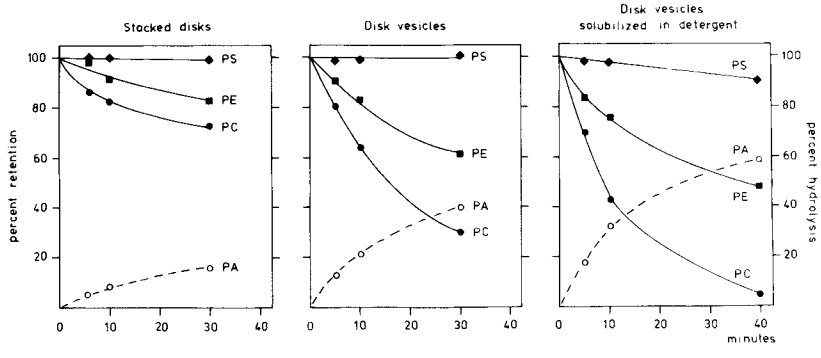

Fig. 3. Treatment with phospholipase D. Percent retention of individual phospholipids (closed symbols) and percent hydrolysis (open symbols) upon treatment of stacked disks, disk vesicles and solubilized disks (in nonylglucose). Abbreviations as in Fig. 1. PA: phosphatidic acid.

The substrate preference in solubilized disks is: phosphatidylcholine > phosphatidylethanolamine > phosphatidylserine. The same order of preference is found for stacked disks and disk vesicles (Fig. 3). The initial rate of hydrolysis decreases

in the order solubilized disks > disk vesicles > stacked disks, indicating a decreasing availability of the phospholipids in this order (Table 2).

In contrast to the situation for phospholipase A_2 and C, there is a limited final level of hydrolysis upon treatment of stacked disks and disk vesicles with phospholipase D (Table 1). About 50% of phosphatidylcholine and phosphatidylethanolamine and nearly all phosphatidylserine are resistant under our conditions. No additional hydrolysis is found upon removal of the water-soluble hydrolysis products by centrifugation or dialysis and addition of fresh enzyme. So it seems that in this case no penetration of the enzyme or membrane inversion occurs. Hence treatment with phospholipase D has been used in connection with TNBS treatment and in determining fatty acid distribution.

Trinitrobenzenesulfonate Treatment

Treatment of preparations (3-4 µM in rhodopsin) with 2,4,6-trinitrobenzenesulfonate (TNBS, BDH Chemicals Ltd., England) is carried out at 20 °C in darkness in 0.16 M Tris-HCl (pH 7.4) or in 40 mM 3-morpholinopropanesulfonate (Merck, W.-Germany), 2 mM $CaCl_2$, 3 mM $MgCl_2$, 140 mM NaCl (pH 7.4). For experiments at pH 8.5 0.12M $NaHCO_3$, 40 mM NaCl is used. Overall modification is determined by adding 1 N HCl (to final pH 2-3) and Triton X-100 (to final concentration of 1 %) and reading the 340 nm absorbance with suitable corrections for rhodopsin absorbance, light scattering and extraneous TNBS hydrolysis. Modification of each phospholipid is determined by stopping the reaction with 0.2 M acetate buffer (pH 5.5), followed by centrifugation and extraction of the pellet with chloroform-methanol and subjecting the extract to two-dimensional thin layer chromatography.

Disk vesicles have been used to determine optimal conditions for the reaction with TNBS, both with regard to speed and to impermeability of the membrane for the reagent. Two pH values (pH 7.4 and 8.5) have been compared. At the higher pH of 8.5 nearly 100% modification of phosphatidylethanolamine and 50% modification of phosphatidylserine is reached after 3 hrs with 1 mM TNBS. At pH 7.4 the modification reaches 50-60% for phosphatidylethanolamine and 40-50% for phosphatidylserine after 3 hrs, while after 1 hr their modification is only slightly less. Varying the TNBS concentration from 0.5 to 8 mM at pH 7.4, it is found that a 1 mM concentration is most suitable. This concentration represents a 5-fold excess with respect to the total number of free amino groups (52 per rhodopsin molecule, consisting of 27 phosphatidylethanolamine, 9 phosphatidylserine and 16 lysine; De Grip and others, 1973). These free amino groups are fully modified by TNBS at pH 7.4 in the presence of 1% Triton X-100. These findings suggest that treatment with 1 mM TNBS for 1 hr at pH 7.4 and 20 °C is optimal for our purpose. The minor additional modification upon further treatment is probably due either to reagent penetration or to reagent-induced membrane inversion. The latter process appears unlikely in view of the results obtained with combined treatment with TNBS and phospholipase.

Stacked disks treated with 1 mM TNBS at pH 7.4 show the reaction profile presented in Fig. 4. Within 1 hr 55% phosphatidylethanolamine and 40% phosphatidylserine has been modified, whereas in the next 2 hrs less than 10% of each phospholipid is additionally modified as also observed for the disk vesicles. Since the determination of trinitrophenyl-phosphatidylserine is hampered by some loss of this compound during extraction and by its rather weak colour, the profile for this substance has been omitted from Fig. 4.

Experiments involving successive treatments with phospholipase D and TNBS have also been carried out. After 0, 0.5 and 3 hrs incubation of stacked disks with phospholipase D (as described before), the reaction is stopped by addition of EDTA-contain-

ing buffer, the pellet obtained after centrifugation is suspended in the TNBS-medium. After incubation for 0, 0.7, 1, 2 and 3 hrs the reaction is stopped by adding acetate buffer (pH 5.5) and phospholipid analysis is carried out. After phospholipase D treatment for 3 hrs nearly 50% phosphatidylethanolamine is hydrolysed and during 3 hrs TNBS treatment there is only little further modification of this phospholipid (Fig. 5). On the other hand, after phospholipase D treatment only a few percent of phosphatidylserine is hydrolysed, but it is modified up to about 50% in less than 1 hr by subsequent TNBS treatment. Similar results are obtained when the reaction sequence is reversed.

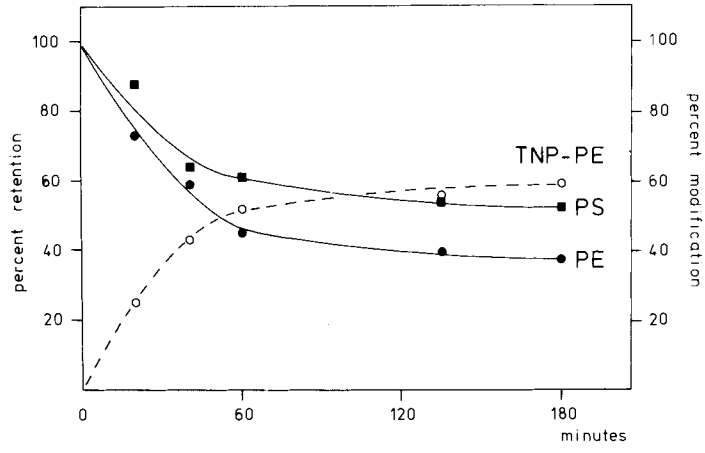

Fig. 4. Treatment of stacked disks with TNBS. Percent retention (closed symbols) and percent modification (open symbols) of individual aminophospholipids upon treatment of stacked disks is shown. Abbreviations as in Fig. 1. TNP-PE: trinitrophenyl-PE.

These results suggest that neither of the two treatments causes significant reagent penetration or membrane inversion, and that phosphatidylethanolamine and phosphatidylserine are about equally distributed over the two membrane faces.

Fatty Acid Distribution

The fatty acid composition of the phospholipids before and after treatment with phospholipase D and of the resulting phosphatidic acid has been determined by means of gaschromatography (Morrison, 1964). After separation of the phospholipids by two-dimensional thin layer chromatography (Merck Silicagel 60 HR plates), the spots are visualized by means of a 0.001 % aqueous solution of 1-amino-2-hydroxynaphthalenesulfonate-4 (Merck, W.-Germany). The spots are scraped off and transferred to 1 ml methanol-acetylchloride (9:1 v/v). Heating for 15 min at 100 °C gives phospholipid hydrolysis and methylation of the released fatty acids. The methylated fatty acids are extracted by addition of pentane and water. The pentane extract, after drying with $Na_2 SO_4$, is evaporated and the residue is taken up in iso-octanol. The resulting samples are analysed in a Pye-Unicam 204 chromatograph with flame ionization detector. Chromatography takes place at 210°C on a glass column with 10% SP-2330 in 100/120 Supelcoport (Supelco Inc., Bellefonte, PA, USA).

The fatty acid composition of outer segments and of the three major phospholipids is shown in Table 3. There are no great differences in fatty acid composition of the phospholipids, except the high C 16:0 content in phosphatidylcholine, which is com-

pensated by higher C 18:0 and C 22:6 contents in phosphatidylethanolamine and a high C 24 content in phosphatidylserine. The results are in good agreement with those recently published by Miljanich and others (1979) and Stone and others (1979), while they show a higher C 22:6 content than earlier studies (e.g. Anderson and others, 1975).

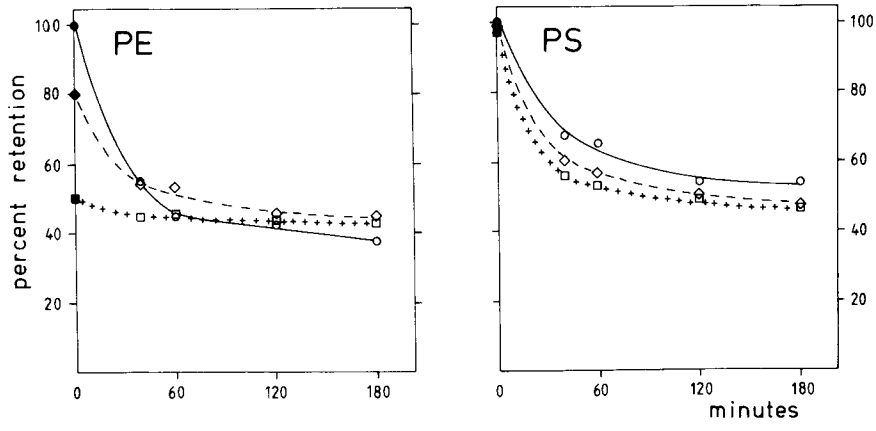

Fig. 5. Successive treatment of stacked disks with phospholipase D and TNBS. Percent retention (i.e. left unmodified by either agent) of phosphatidylethanolamine (PE) and phosphatidylserine (PS) upon treatment of stacked disks with phospholipase D for 0 hr (●), 0,5 hr (◆ : 20% PE, 1% PS, 29% PC hydrolysis) and 3 hrs (■ : 50% PE, 3% PS, 55% PC hydrolysis), followed by treatment with TNBS (open symbols).

When stacked disks are incubated with phospholipase D and samples taken after 10, 30, and 60 min are analyzed, the fatty acid contents of the remaining phospholipids and the resulting phosphatidic acid do not show any significant changes during enzymatic hydrolysis (Fig. 6). The same was true for blank incubations without phospholipase D. This indicates that there is no asymmetric distribution of phospholipid-bound fatty acids between the two faces of the photoreceptor membrane. Nearly identical results are obtained with TNBS modification.

DISCUSSION

Conditions for Valid Conclusions

As set forth before under Experimental Approaches, four requirements must be fulfilled in order to allow valid conclusions on the distribution of the phospholipids over the two faces of the photoreceptor membrane to be drawn.

First, a preparation must be used in which the membrane has the right-side-out orientation and retains it during treatment. This condition has been met by using freshly isolated outer segments with a stacked disk structure. Their morphology in phase-contrast microscopy and electronmicroscopy resembles that of rod outer segments in situ so closely that membrane inversion during isolation can be ruled out. During the initial stage of phospholipase treatment (10 min, up to 1/3 overall phospholipid hydrolysis) their morphology remains unchanged. Hence, we may conclude that no inversion occurs during the initial stage of phospholipase treatment.

TABLE 3. Fatty Acid Content of Outer Segments and of the Major Phospholipids

fatty acid	outer segments	PC	PE	PS
C 16:0	19.9	30.6	12.6	4.1
C 18:0	22.1	19.4	25.0	21.0
C 18:1	3.3	4.5	4.2	1.5
C 18:2	-	0.9	0.9	-
C 20:4	4.8	2.7	2.4	4.3
C 22:4	1.6	0.4	0.8	3.0
C 22:5 ω6	2.3	0.9	1.5	1.6
C 22:5 ω3	1.9	1.4	1.4	3.3
C 22:6	43.0	35.9	50.2	48.1
C 24:4	1.2	-	-	3.9
C 24:5		-	-	9.3

Phospholipids in percent of total phospholipids : 36% phosphatidylcholine (PC), 44% phosphatidylethanolamine (PE), 15% phosphatidylserine (PS). All values are expressed as mole percent.

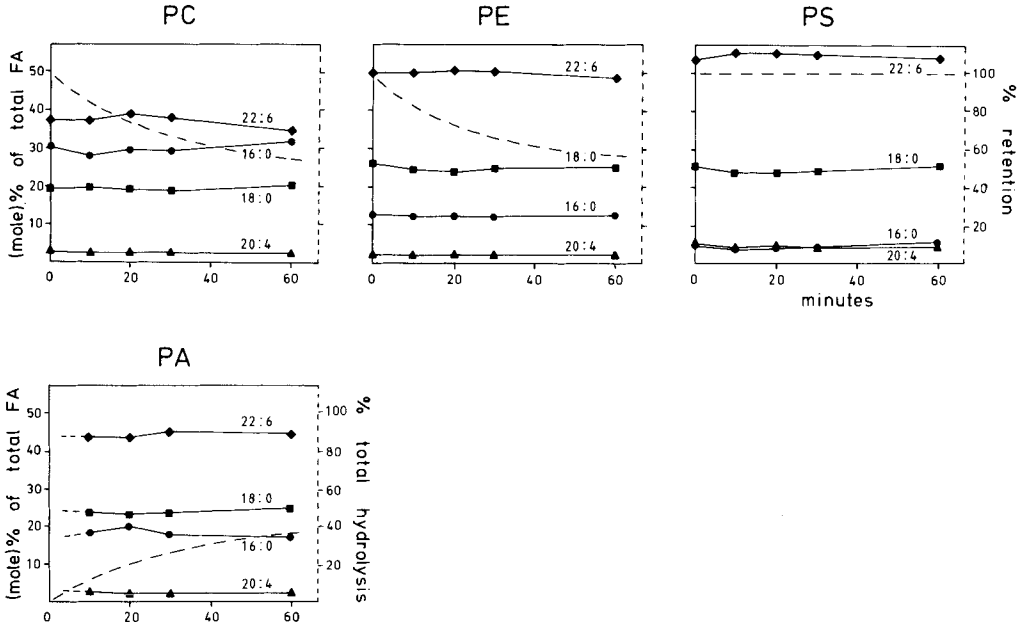

Fig. 6. Effect of phospholipase D treatment on fatty acid composition of stacked disks. Closed symbols and solid lines represent content of fatty acid indicated. Broken lines represent change in content of the phospholipid analyzed. Abbreviations as in Fig. 1.

Secondly, the phospholipases and TNBS must not penetrate the membrane during treatment. In the stacked disk preparation the rod sacs are intact and remain intact. Penetration into the rod sacs of the phospholipases A_2, C and D, which are water-soluble proteins of molecular weights over 14,000, is unlikely. Penetration of the water-soluble reagent TNBS, especially at pH 7.4 for up to 1 hr, also appears unlikely. Only when after prolonged treatment a substantial part (50% or more) of the phospholipids is hydrolyzed or modified, must we expect changes in the membrane structure, such as membrane inversion, and/or penetration of the enzyme or TNBS. This clearly happens with phospholipases A_2 and C, which give nearly complete hydrolysis of the phospholipids after 1-3 hrs incubation. Hence, it is necessary to draw our conclusions from the effects of the initial stage of treatment with phospholipases A_2 and C (ca 10 min) and TNBS (up to 1 hr at pH 7.4). For phospholipase D even extensive treatment (up to 3 hrs) does not give more than 40% overall hydrolysis of phospholipids in stacked disks. It is also worth noticing that the presence of residual plasma membrane in stacked disks does not seem to present a significant barrier to phospholipase or TNBS action, since no initial lag period in their action is observed and their overall effects are far too large to be restricted to the outer face of the plasma membrane.

Thirdly, the specificity of the agents towards the individual phospholipids should be taken into account. This has been done by comparing the effects of the phospholipases on the stacked disks with those on a reference preparation with randomized phospholipid orientation. The retinal lipid suspension used for this purpose also shows hexagonal and inverted micellar phases (De Grip and others, 1979b), indicating that the phospholipids in this preparation are randomly available. Disks solubilized in 20 mM nonylglucose, which is above its critical micelle concentration (6.5 mM; De Grip and Bovee-Geurts, 1979a), must also have a random phospholipid orientation.

Fourthly, the results obtained with the different agents should lead to the same conclusion. In our experiments this appears to be the case for the three phospholipases, which finding receives additional weight from the fact that each phospholipase preferentially attacks a different one of the three major phospholipids. In addition, the results obtained with the amino group reagent TNBS for phosphatidylethanolamine and phosphatidylserine completely confirm the conclusions reached with the phospholipases.

Conclusions

Confining ourselves to the initial effects of the phospholipases, the results are summarized in Table 2. For phosphatidylcholine we find for the three phospholipases an average hydrolysis ratio between stacked disks and a randomized reference preparation of 0.81 ± 0.042, which is significantly below one. This suggests a slight asymmetry for this phospholipid with about 41% on the outer face of the disk membrane. For phosphatidylethanolamine the average ratio is 1.14 ± 0.083, which is not significantly higher than one. This suggests a questionable asymmetry with possibly some 57% of the phospholipid on the outer face of the disk membrane. For phosphatidylserine only phospholipase A_2 gives sufficient hydrolysis with a ratio of 0.96, indicating symmetric distribution over the two membrane faces.

The results obtained with treatment with TNBS, alone and in combination with phospholipase D confirm these conclusions for the two amino group containing phospholipids. TNBS can modify phosphatidylethanolamine only to the extent to which it can be hydrolyzed by phospholipase D, while it can readily modify phosphatidylserine (which is hardly hydrolysed by the enzyme) to 50%. Essentially the same results are obtained whether phospholipase treatment preceeds or follows treatment with TNBS. In particular, these combined experiments strongly indicate that neither treatment causes significant membrane or phospholipid inversion.

Our conclusions do not agree with those obtained in three earlier studies with amino group reagents, where predominant (70-100%) location of both phosphatidylethanolamine and phosphatidylserine (Raubach and others, 1974; Smith and others, 1977) or of phosphatidylethanolamine alone (Crain and others, 1978) on the external face of the disk membrane is claimed. However, in all these studies disk vesicles prepared from frozen retina have been used, and hence the results are open to serious doubt.

Comparison of the fatty acid composition of the phospholipids before and after treatment with phospholipase D and with that in the resulting phosphatidic acid, or before and after treatment with TNBS, indicate that the fatty acids present in the phospholipids are symmetrically distributed over the two membrane faces. The good agreement found between the results from the various approaches used in our study strongly indicates a nearly symmetrical distribution of the three major phospholipids and their fatty acids over the two sides of the photoreceptor membrane, with only a slight preponderance of phosphatidylcholine on the inner side and possibly a slight preponderance of phosphatidylethanolamine on the outer side.

ACKNOWLEDGEMENTS

The excellent technical assistance of Mr. A.A. Klompmakers is gratefully acknowledged. The authors are much indebted to Dr. J. Olive, Laboratoire de Microscopie Electronique, Université de Paris VII, France, for carrying out thin section electronmicroscopy.

This investigation has been carried out with financial support from the Netherlands Organization for Basic Scientific Research (ZWO) through the Netherlands Foundation for Chemical Research.

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Neurochemistry Vol. 1, pp. 17-28.
Pergamon Press Ltd. 1980. Printed in Great Britain.

BIOSYNTHESIS OF MEMBRANE LIPIDS IN THE RETINA: SUBCELLULAR DISTRIBUTION AND PROPRANOLOL ACTION ON PHOSPHATIDIC ACID, PHOSPHATIDYLSERINE AND PHOSPHATIDYLETHANOLAMINE

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ABSTRACT

The de novo biosynthesis of phospholipids and glycerides was followed in subcellular fractions from bovine retinas incubated during short periods with radioactive glycerol. The labeling of lipid classes was determined in the following fractions: rod outer segments, P_1, P_2, microsomes and soluble. A highly labeled phosphatidic acid was found in microsomal fractions with a maximum at 5 min relative to other lipids labeling. The labeling of phospholipids and glycerides from microsomes as well as from other fractions was rapid. The soluble contained minor quantities of phospholipids with high radioactivity.

Using radioactive glycerol and amphiphilic drugs the rates of phosphatidic acid synthesis in microsomal membranes were assessed. Moreover, in agreement with previous studies from this laboratory showing that phosphatidic acid contains a high proportion of docosahexaenoate, it is demonstrated that this fatty acid is acylated in the biosynthetic route leading to phosphatidic acid formation. Phosphatidylserine is synthesized from radioactive serine and also through a Ca^{2+} independent pathway not involving phosphatidylethanolamine. Phosphatidylethanolamine is also formed by serine decarboxylation. Amphiphilic drugs greatly stimulate phosphatidylserine synthesis. Extracellular Ca^{2+} stimulates the synthesis of phosphatidylserine and its decarboxylation. Besides, Ca^{2+} potentiates the phospholipid effect exerted by propranolol. The synthesis of phosphatidylinositol as well as that of other minor acidic phospholipids seems to operate in a coordinated manner under different experimental conditions. The microsomal system of the retina generates a docosahexaenoyl enriched phosphatidic acid at high rates. In addition to the turnover of different phospholipid moieties there are active pathways for the de novo biosynthesis of membrane lipids in the retina.

KEYWORDS

Phosphatidic acid, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, propranolol action on membrane lipids, docosahexaenoate in phosphatidic acid, microsomes, calcium on membrane lipids, phosphatidylserine decarboxylase.

INTRODUCTION

The phospholipids enriched in highly unsaturated fatty acids from photoreceptor membranes as well as from neuronal membranes conform an appropriate millieu for lateral diffusion and conformational changes of membrane proteins. Although acidic lipids (e.g. phosphatidic acid -PA-, phosphatidylserine -PS-, and phosphoinositides) are minor components they may play important roles by interacting with other molecules in membrane functions. However, scarce information is available about the metabolic steps where long-chain polyenoic fatty acids of the docosahexaenoic acid type (22:6 ω 3) are acylated and on the biosynthesis of acidic phospholipids.

This chapter summarizes recent studies devised to answer the following questions: a) Is docosahexaenoic acid introduced during the de novo synthesis or through a deacylation-acylation cycle?; b) How are newly synthesized phospholipids distributed among retinal subcellular fractions?; c) Through which pathways does the biosynthesis of phosphatidylserine and of phosphatidylethanolamine take place?; How do Ca^{2+} and/or drugs modify the biosynthetic pathway of acidic membrane lipids?.

The followed approach was based upon the use of radioactive glycerol as precursor for membrane lipids biosynthesis during short-term incubations of the entire retina (Bazán and coworkers, 1976; Bazán and Bazán, 1976; Giusto and Bazán, 1979). Thus, glycerol uptake and metabolic flow took place in the intact tissue from where subcellular fractions were then isolated and measurements performed. The action of Ca^{2+} and/or propranolol and the study on docosahexaenoate of phosphatidic acid were also conducted following a similar outline.

The phospholipid effect elicited by dl-propranolol or phentolamine is unrelated to the adrenergic blocking properties of these drugs. (Bazán and coworkers, 1976a, 1976b; Bazán, Ilincheta de Boschero and Giusto, 1977). Since this effect is characterized by the redirection of biosynthetic pathways yielding a stimulated formation of acidic phospholipids in the retina (Bazán and coworkers, 1976a; Bazán, Ilincheta de Boschero and Giusto, 1977) it was used as a tool to investigate several of the questions presented above. Apart from this, it was also interesting to follow up these studies from the point of view of the mechanism of the drug action itself.

DOCOSAHEXAENOATE IS PRESENT IN HIGH CONCENTRATIONS IN MICROSOMAL PHOSPHATIDIC ACID AND IS INTRODUCED DURING THE DE NOVO BIOSYNTHESIS FROM GLYCEROL-3-PHOSPHATE

Retinal phospholipids, particularly phosphatidylserine, phosphatidylethanolamine (PE) and phosphatidylcholine, are rich in long-chain highly unsaturated fatty acyl groups (Anderson 1970; Aveldaño and Bazán, 1972; Anderson, Maude and Zimmerman, 1975; Aveldaño de Caldironi and Bazán 1977). Since docosahexaenoate (22:6 ω 3) is the major unsaturated acyl chain questions arise about the metabolism of this fatty acid. Is docosahexaenoate introduced into phospholipids by an acylation-deacylation cycle such as the one described by Lands in other tissues (Thompson, 1973) or is it alternatively acylated during the early stages of de novo biosynthesis of glycerolipids prior to phosphatidic acid formation? Since the presence of phospholipids with this polyenoic fatty acyl group in membranes yields a highly fluid environment, membrane functioning may be modified by increasing or by decreasing the concentration of 22:6. Moreover a) the de novo biosynthesis of retinal phospholipids from precursors such as glycerol (Bazán and Bazán, 1975; Giusto and Bazán, 1979) or glucose (Bazán, Giusto and Bazán, 1977) is rapid and b) PA from whole retinas (Aveldaño de Caldironi and Bazán, 1977) as well as from microsomes (Giusto and Bazán, 1979) contains high proportions of 22:6. Thus, the hypothesis that these acyl groups are acylated during the formation of PA is presented.

The determination of the PA fatty acid composition in microsomes from retinas incubated during short periods of time with and without propranolol was carried out. Radioactive glycerol was also employed in some of these experiments as a control of the de novo synthesis. Docosahexaenoate comprises about 22% of the PA fatty acyl groups in microsomes and upon incubation of retinas with propranolol an increased content of PA is observed and docosahexaenoate as well as other fatty acids are greatly increased (Table 1). At the same time if 2-^{3}H-glycerol is used as a marker of the synthesis there is an increase in PA formation (Fig. 1 and Table 1). Thus, the increase a) in the mass assessed by lipid P, b) in the radioactivity from glycerol and c) in the fatty acyl chains, especially 22:6, suggests that the retina is able to synthetize highly unsaturated PA in its endoplasmic reticulum at very fast rates.

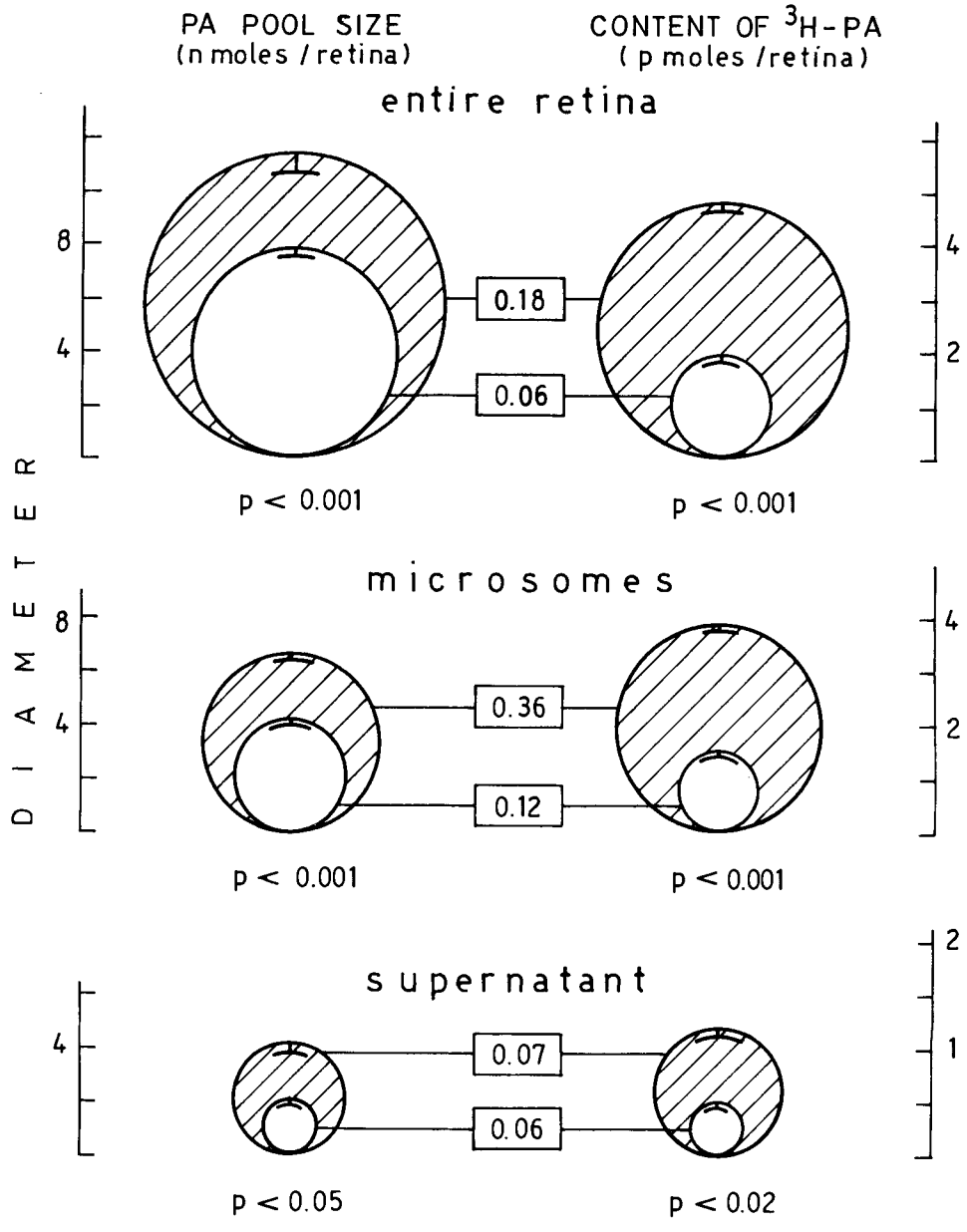
TABLE 1 Ca^{2+} and/or dl-Propranolol Effect on the Content of Acyl Chains in Phosphatidic Acid from Retinal Endoplasmic Reticulum

Acyl Chain	Controls		500 µM dl-Propranolol	
	No Calcium	5 mM Calcium	No Calcium	5 mM Calcium
	nmol of methyl esters/100 mg of protein			
16:0	84.2 ± 26.6	104.2 ± 4.4	554.8 ± 96.9	554.5 ± 102.5
18:0	135.8 ± 51.4	183.4 ± 19.5	334.7 ± 98.0	518.4 ± 86.8*
22:6 ω3	87.7 ± 14.5	114.7 ± 13.2**	320.2 ± 74.3	442.6 ± 96.9
Total Content	462.8 ± 125.1	583.4 ± 32.7	1681.8 ± 228.4	2105.8 ± 362.8

Values are averages ± S.D. from at least five independent samples made by four bovine retinas each (about 2 g fresh tissue weight) * $p<0.05$; ** $p<0.025$. Retinas were preincubated in an ionic medium (Ames and Hastings, 1956) during 10 min, then retinas were incubated for additional 30 min. 5mM of calcium ions were present with or without 500 µM propranolol during both periods. Lipids were extracted (Folch, Lees and Sloane-Stanley, 1957), and isolated by TLC (Rodriguez de Turco and Bazán, 1977). PA was analysed by gas-liquid chromatography (Bazán and Bazán, 1975). Proteins were measured by the method of Lowry and coworkers (1951) using crystalline serum albumin as standard.

The newly formed PA contains a high proportion of 22.6 although the basal level of this acyl group is about the same percentage in controls. This means that 22:6 is introduced into PA in one of the two enzymatic steps after glycerol-3-phosphate.

Whether propranolol is actually stimulating PA synthesis or inhibiting its further metabolization is not clear yet. However, the drug seems to act at the level of the endoplasmic reticulum by stacking itself among membrane constituents and in turn altering enzymatic steps of glycerolipid metabolism. The relationship between 22:6 enriched PA in the retina and membrane fluidity has been discussed elsewhere (Bazán and Giusto, 1979).



Fig, 1. Pool size and newly synthetized phosphatidic acid from 2 | ^{3}H | glycerol in the retina. Circle areas are proportional to the units given at the top part. Empty circles stand for controls and hatched circles correspond to propranolol treated samples. Data on content and labeling were obtained from 4 samples of entire retinas, from 5 samples of microsomes and from 3 samples of postmicrosomal supernatants. S.D. as well as p values are indicated in each circle. Experimental conditions are as in Table 1. Numbers between the circles are ratios of (^{3}H)-phosphatidic acid/pool size of intrinsic phosphatidic acid. Lipid P was determined according to Rouser, Fleicher and Yamamoto, (1970).

SUBCELLULAR DISTRIBUTION OF NEWLY SYNTHETIZED PHOSPHOLIPIDS AND GLYCERIDES FROM 2-^{3}H-GLYCEROL IN ROD OUTER SEGMENTS, MICROSOMES AND OTHER SUBCELLULAR FRACTIONS OF THE RETINA

So far the metabolism of phospholipids has been studied only in rod outer segments, though these studies included only certain aspects. Consequently, it seemed of interest to survey the de novo biosynthesis of phospholipids and of glycerides at the subcellular level including the rod outer segments. Thus, 2-^{3}H-glycerol was employed to examine the de novo biosynthesis of glycerolipids in subcellular fractions after incubating the entire retina during short periods of time. Then, the retina was subjected to subcellular fractionation. At early periods of time most of the radioactive lipids were confined to the microsomal fraction (Fig. 2 and Table 2). Here again the labeling sequence clearly shows that PA is the precursor for the other lipids. In addition all the label under this condition is restricted to the glycerol backbone. However, several kinds of lipids appear very rapidly in all the fractions studied. Rod outer segments also display significant amounts of labeled PA (Fig. 2). Whether this represents newly synthetized PA in ROS, microsomal contamination or a PA pool rapidly being transferred from the inner segment remains to be elucidated.

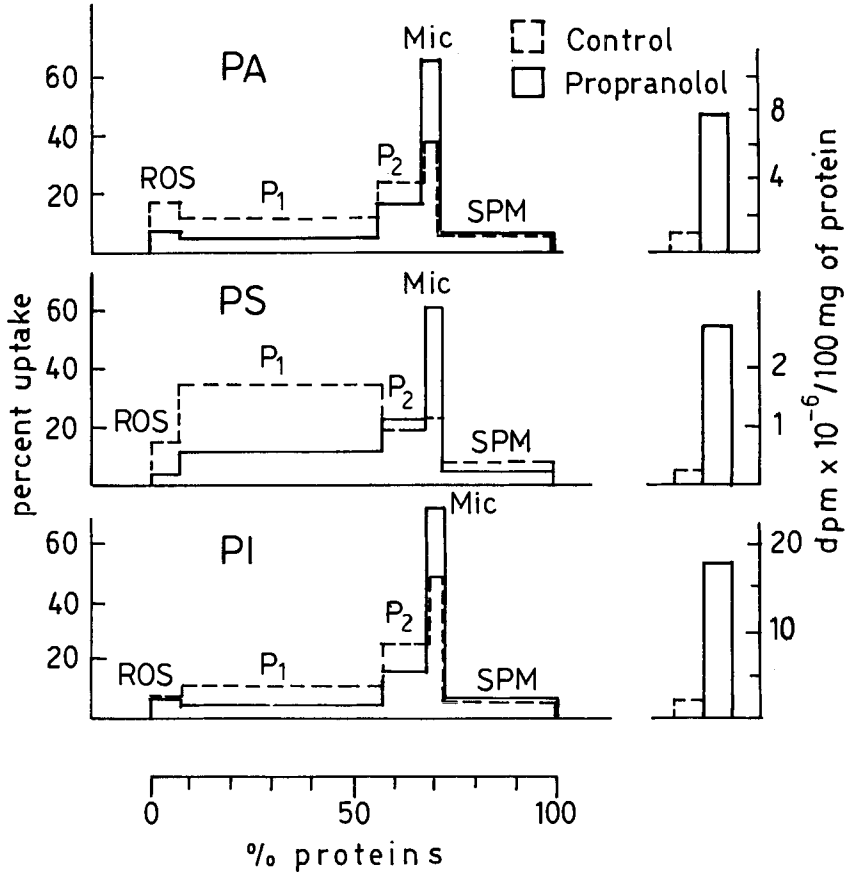


Fig. 2. Subcellular distribution of acidic phospholipids in the bovine retina. On the right, total incorporation in all fractions for each lipid is shown. Experimental details as in Table 2.

TABLE 2 Incorporation of 2-^{3}H glycerol in Neutral Glycerides from Subcellular Fractions of Bovine Retina. Effect of dl-propranolol

	ROS	P_1	P_2	Mic	SPM
	(dpm/100 mg of protein x 10^{-3})				
Monoacylglycerol	113 (0.97)	71 (1.21)	129 (1.27)	155 (2.16)	54 (0.48)
Diacylglycerol	790 (1.2)	439 (1.46)	946 (1.73)	2093 (2.34)	455 (1.87)
Triacylglycerol	2554 (0.05)	984 (0.11)	3121 (0.10)	6195 (0.21)	818 (0.28)

Figures between parenthesis indicate changes due to dl-propranolol being 1.00 controls. Retinas were preincubated in an ionic medium (Ames and Hastings, 1956) during 20 min. Then 5 µCi/retina of [^{3}H]glycerol (200 µCi/mmol, New England Nuclear Corp., Boston, Mass.) was added. After a further incubation period of 30 min retinas were subjected to subcellular fractionation. 500 µM dl-propranolol were present throughout preincubation and incubation. ROS were isolated (Krishna and co-workers, 1976) in 67 mM phosphate buffer-40% sucrose. The remainder of the retina was homogenized in 50mM Tris-HCl-0.32 M Sucrose containing 10^{-4} EDTA-pH 7.4. P_1, P_2 and microsomal fraction (Mic) were isolated by differential centrifugation at 1000 xg for 15 min; 11,500 xg for 20 min and 140,000 xg for 50 min. Lipids were isolated by gradient-thickness TLC (Bazán and Bazán, 1975).

PHOSPHATIDYLSERINE BIOSYNTHESIS IN MICROSOMES

Radioactive serine is rapidly incorporated into phosphatidylserine in the entire bovine retina and in retinal homogenates amounting to about 50 times higher values than those found in the incorporation from labeled glycerol (Ilincheta de Boschero and Bazán, submitted for publication). In Figures 3 and 4 we present results about the synthesis of free amino containing phospholipids from glycerol or serine as precursors. These studies were performed in microsomal preparations obtained after incubation of the entire retina with the precursors in the presence or absence of Ca^{2+} and/or dl-propranolol. Without Ca^{2+} the labeling of phosphatidylserine is 8-10 times higher than that by glycerol. 5mM Ca^{2+} slightly inhibits the synthesis of this phospholipid from glycerol whereas the incorporation of serine is stimulated in about an 80%. The high ratio of ^{3}H-serine/^{3}H-glycerol in PS suggests that in the retina there is an active base exchange reaction. However we have previously suggested that, in addition to this route, there is a pathway of PS synthesis from PA which is stimulated by propranolol in the retina (Bazán and coworkers, 1976b).

The main pathway for PS synthesis in mammalian tissues is the base exchange reaction (Porcellatti and coworkers, 1971; Kanfer, 1972). Procariotes, instead, synthesize this lipid through CDP-diacylglycerol (Kanfer and Kennedy, 1964). In cultured neural cells PS seems to be formed through two routes. One of them is the Ca^{2+} -dependent energy-independent base exchange reaction and the other has opposite characteristics (Yavin and Ziegler, 1977). In spite of this and other observations (Kiss, 1977) there is not yet a conclusive evidence for the existence of an alternate pathway for PS synthesis in higher organisms.

DECARBOXYLATION OF PHOSPHATIDYLSERINE YIELDING PHOSPHATIDYLETHANOLAMINE TAKES PLACE IN RETINAL MICROSOMES

In the entire retina as well as in retinal homogenates only PS and PE are the lipids labeled after short-time incubations with radioactive serine. The labeling patterns suggest a product-precursor relationship (Ilincheta de Boschero and Bazán, submitted for publication).

In Fig. 3 it can be observed that PE is labeled by serine. This means that a decarboxylation of the aminoacid has taken place and that PE can be synthetized from serine. Thus, a PS decarboxylase is likely to be present as discussed in conclusions. Ca^{2+} stimulates PE synthesis from serine greatly. However, when glycerol is used as a precursor, Ca^{2+} inhibits the de novo biosynthesis of PE slightly although only 1% of the serine-labeled PS corresponds to PE in the retina under the present experimental conditions (Table 3). This reaction may underline metabolic steps involved in membrane fluidity changes (Hirata, Axelrod and Crews, 1979).

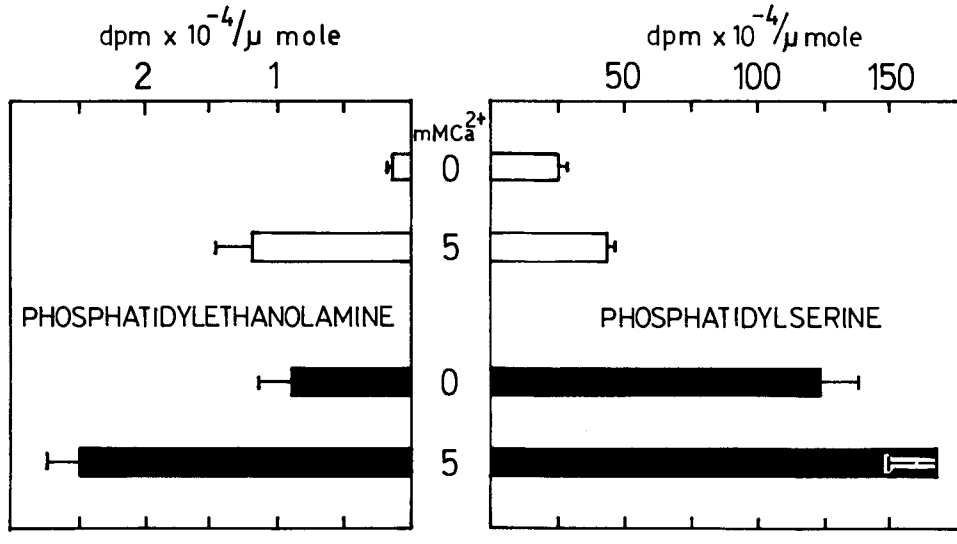


Fig. 3. Labeling of microsomal phosphatidylethanolamine and phosphatidylserine by ^{3}H-serine in retinas incubated with high Ca^{2+} concentration and with propranolol. Each bar is the average ± S.D. of five individual samples. Black bars are from propranolol-treated retinas. Retinas were preincubated in an ionic medium (Ames and Hastings, 1956) during 10 min, then 3.56 μCi/retina of |^{3}H-(G)|-serine (2.76 Ci/mmol, New England Nuclear Corp., Boston, Mass.) was added. After a further incubation period of 30 min retinas were subjected to subcellular fractionation. Dl-propranolol (500 μM) was present throughout preincubation and incubation. Other details as in Table 1 and 2.

TABLE 3 Incorporation of ^{3}H-serine in Microsomal Phosphatidylserine and Phosphatidylethanolamine

	Ca^{2+}		dl-Propranolol	
	0	5mM	Without Ca^{2+}	5mM Ca^{2+}
	(pmoles of ^{3}H-serine / mg of protein)			
PS	289 ± 49	390 ± 34	1,651 ± 207	2,228 ± 228
PE	3 ± 0.5	20 ± 4	18 ± 6	50 ± 5
(PE/PS) x 100	1.0	5.1	1.1	2.2

Values represent the mean ± S.D. of five different samples. For other details see Fig. 3.

PROPRANOLOL STIMULATES PHOSPHATIDYLSERINE SYNTHESIS AND ITS DECARBOXYLATION TO PHOSPHATIDYLETHANOLAMINE

The effect of dl-propranolol shown in Figures 3 and 4 gives further support to the possibility that PS is also synthetized by a route other than the base exchange. PS labeling, either from serine or glycerol is stimulated by dl-propranolol. However, only the entrance of serine is potentiated by Ca^{2+}. Moreover, the PS labeling stimulated by serine is also highly significant even in the absence of extracellular Ca^{2+}.

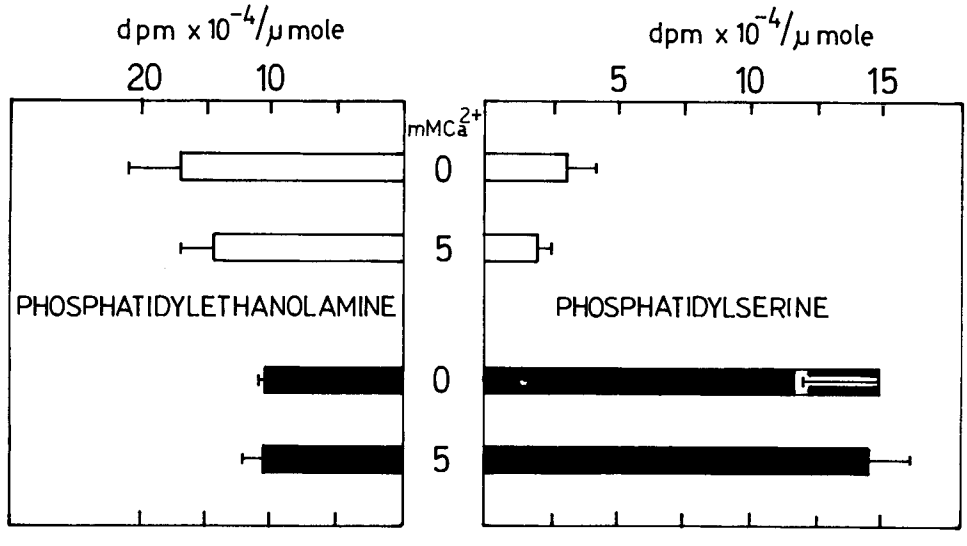


Fig. 4. Synthesis of free-amino-containing phospholipids from 2-^{3}H -glycerol in microsomes from retinas incubated with high Ca^{2+} concentration and/or propranolol. Black bars are propranolol-treated samples. Details as in Table 2 and Fig. 3.

In the absence of Ca^{2+} dl-propranolol stimulates six times the specific activity of PS and PE using serine as precursor (Table 3). If the same is studied in a medium with 5mM Ca^{2+} there is a further 30% increase in PS and about 3 times more in PE.

PS labeled from glycerol is enhanced 5-6 times in the presence of the drug without Ca^{2+} and PE is inhibited in a 30%. Ca^{2+} does not modify these effects.

It is of interest to point out that the propranolol-induced stimulation in the synthesis of PS from glycerol matches the enhanced labeling by serine. This supports the idea that the PS de novo synthesis parallels PA and PI stimulated formation (Bazán and coworkers, 1976b). Moreover, PS may derive, at least in part, from PA without involving the base exchange reaction.

CONCLUSIONS

PA biosynthesis from labeled glycerol predominates in the endoplasmic reticulum of the retina. A very rapid flow of radioactivity directed from PA to other glycerolipids in microsomes as well as from other subcellular fractions of the retina has been found (Giusto and Bazán, submitted for publication; Bazán and Giusto, 1979). These observations suggest that there are rapid mechanisms redistributing newly synthetized glycerolipids among subcellular organelles in agreement with the recent description of a phospholipid transfer protein in the retina (Dudley and Anderson, 1978).

Propranolol promotes an accumulation of the microsomal pool of PA and stimulates even more their synthesis de novo. This may be a reflection of a slowing down in PA further metabolism and simultaneously a stimulation of its synthesis.

One interesting feature of microsomal PA in the retina is the relatively high proportion of 22:6 ω3 (Giusto and Bazán, 1979). Since this acyl group is enhanced when the PA pool size is increased due to propranolol, we suggest that docosahexaenoate may, at least in part, be introduced in retinal lipids through the de novo biosynthetic pathway.

PS biosynthesis in the retina takes place at least through two routes. One of them is Ca^{2+}-dependent and may be a base exchange reaction (Mizuno, 1976). An outline summarizing the effects of Ca^{2+} or dl-propranolol and the possible reactions involved is given in Fig. 5.

For PE synthesis, PS decarboxylation seems also to be a route besides the ethanolamine phosphotransferase one. When Ca^{2+} is omitted the decarboxylation amounts to about 3% of the total synthesis from glycerol. This reaction is Ca^{2+}-dependent and unaffected by propranolol. The stimulation observed by the drug is likely to be an indirect one and a consequence of the stimulated entrance of serine in PS. Since PS and PE enhancements are very similar when serine is utilized as precursor, the decarboxylation is likely to be of PS rather than of free serine followed by labeling from ethanolamine (Fig. 5).

However, our data on the stimulation by Ca^{2+} with propranolol do not rule out this later possibility. Although these studies were made on retinal microsomes, the complexity of the effects should be still kept in mind. Both Ca^{2+} and drug action were actually exerted on the entire retina and were then fractionated. In spite of the fact that glycerolipids synthesis in the entire retina uses glycerol actively as soon as a cell free preparation is made, an inefficient utilization of the pre-

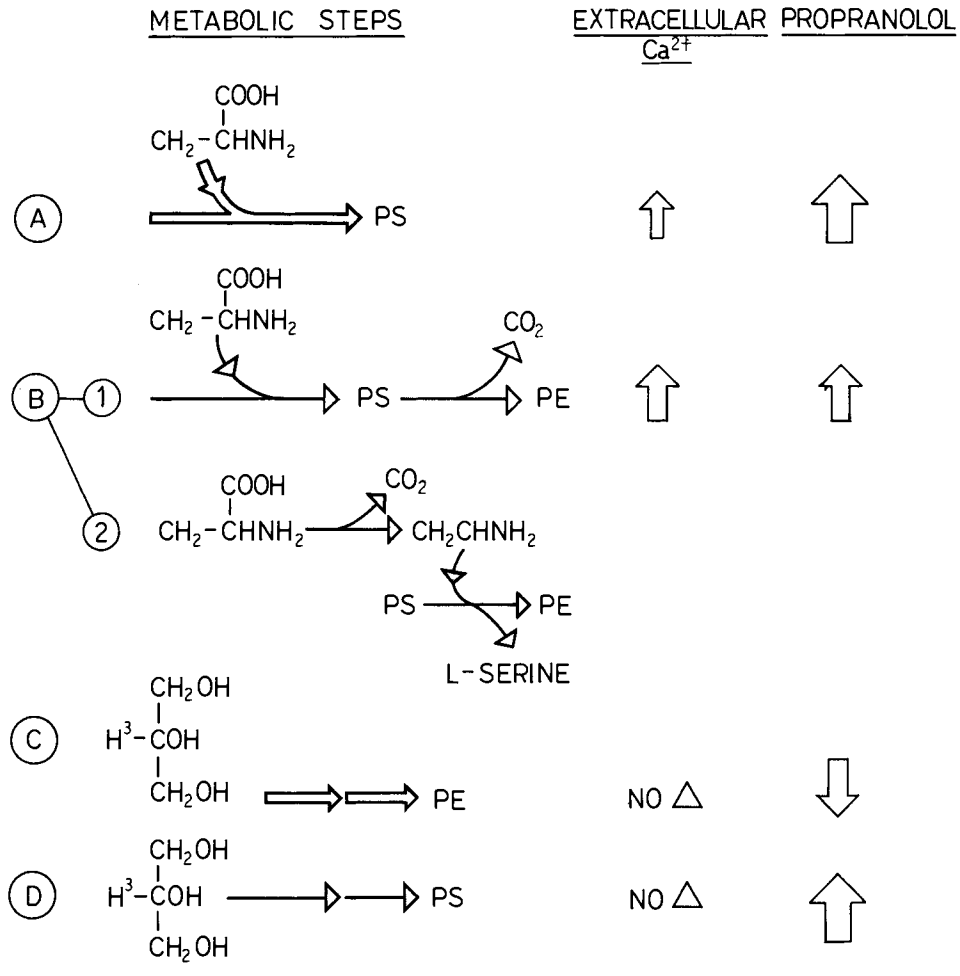


Fig. 5. Drug-induced modifications in the biosynthesis of phosphatidylethanolamine and phosphatidylserine in the endoplasmic reticulum of the retina. Arrows indicate changes and their magnitude. Ca^{2+} and propranolol refer to the results presented in Figures 3 and 4. The Ca^{2+} is that observed at 5 mM concentration compared to the condition without Ca^{2+}. The propranolol effect is that seen in the absence of Ca^{2+} as compared to the corresponding controls

cursor for lipid synthesis occur (Ilincheta de Boschero, M. and Bazán, N. G., submitted for publication). Thus, at this stage it is not possible to use simpler approaches such as the direct study of the action of drugs and of Ca^{2+} in the isolated microsomes.

Propranolol elicits the described effects by its interaction with the endoplasmic reticulum membrane and then, by affecting in turn membrane-bound enzymes of the biosynthetic pathway of phospholipids.

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SYNTHESIS AND TURNOVER OF LIPID AND PROTEIN COMPONENTS OF FROG RETINAL ROD OUTER SEGMENTS

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ABSTRACT

The incorporation and turnover of several lipid precursors in frog retina lipid and protein were studied using biochemical and autoradiographic techniques. Lipid and protein are synthesized on the photoreceptor microsomes from where some migrate to the base of the rod outer segments (ROS) and are incorporated into basal discs. Most of the labeled protein is confined to a discrete group of discs which is displaced apically as newly synthesized unlabeled discs are added. The specific radioactivity of ROS protein is constant throughout this period, which in the present study was determined to be 39 days. Conversely, lipid, once incorporated into the basal discs, diffuses throughout the entire ROS as evidenced by an exponential decline in specific radioactivity. The half-life of ROS lipid is shorter than the renewal rate for protein, suggesting that lipid turns over faster than protein in the ROS.

KEY WORDS

Phosphatidyl serine; phosphatidyl ethanolamine; rod outer segments; lipid synthesis; lipid turnover; membranes; renewal.

INTRODUCTION

Over a decade ago Young (1967) demonstrated that the proteins of rod outer segments (ROS) are constantly being renewed. Following injection of radioactive amino acids, a band of silver grains representative of newly synthesized proteins was observed at the base of the ROS. With time, these labeled proteins were displaced apically until they reached the tips of the ROS where they were shed and phagocytized by the pigment epithelium (reviewed by Young, 1973 and 1976; and O'Brien, 1978). In an elegant study involving biochemistry and electron microscopic autoradiography, Hall, Bok and Bacharach (1969) followed the fate of injected ^{3}H-leucine over a 9-week period. They showed by autoradiography that the label was first incorporated into proteins of the ribosomes of the inner segment, from which it moved to the Golgi body and finally percolated through the mitochondrial-dense ellipsoid region to the base of the ROS. Biochemical analysis of the radioactive products showed that most of the label was in rhodopsin. By carefully monitoring the temporal relationship between autoradiographic pattern and the specific radioactivity of

rhodopsin in isolated ROS, they demonstrated that a molecule of rhodopsin, once incorporated into a disc, remained with that disc until it was shed.

Other studies on the biosynthesis of ROS proteins have come from the laboratory of Papermaster and co-workers (1975). Following intravitreal injection of ^{3}H-leucine, the specific activity of frog opsin peaked in two non-ROS membrane fractions of retina prior to reaching maximum activity in ROS. Since opsin was never found in a soluble form, delivery of newly synthesized opsin to the growing ROS was indicated to occur via a membrane-bound intermediate.

Renewal of lipids in rod outer segments is not so well understood. The autoradiographic studies of Bibb and Young (1974a) following the fate of injected tritiated palmitic, stearic, or arachidonic acids in frogs showed some early banding in the basal discs which quickly diffused throughout the entire ROS. In another autoradiographic study (Bibb and Young, 1974b), glycerol was rapidly incorporated into lipid in the rod inner segment which subsequently migrated to the ROS. As observed in the fatty acid study, the lipid was diffusely spread throughout the ROS. Some sustained banding observed in the outer segments in the glycerol study was shown to be due to protein. They concluded that ROS lipids are renewed by both molecular and membrane replacement, and that ROS lipids are renewed more rapidly than protein. Biochemical studies by Hall, Basinger and Bok (1973) on the fate of injected $^{33}PO_4$ and ^{3}H-choline in frog ROS phospholipids showed that, with the exception of phosphatidyl inositol in which a rapid incorporation and turnover was observed, there was a slow incorporation of both labels into phospholipids. Maximum specific activity of ^{33}P-phospholipids was not achieved until 30 days after injection and remained constant through day 63, and only a slight decline in tritiated phosphatidyl choline (PC) specific activity was noted by day 73. There was no indication from these biochemical studies that ROS phospholipids were turning over within the time frame of protein renewal, in contrast to Bibb and Young's (1974b) glycerol autoradiography data.

In an attempt to gain a greater insight into the renewal of lipid in ROS, we have carried out a series of biochemical and autoradiographic experiments in which the biosynthesis and turnover of retinal lipids were studied by following the fate of several radioactive lipid precursors. In this paper we discuss the incorporation of $^{32}PO_4$, $^{33}PO_4$, 3-^{3}H-serine, and 2-^{3}H-glycerol into protein and phosphatidyl serine of the frog retina.

METHODOLOGY

The details of the methodology will be presented in subsequent publications; only a brief outline will be given here.

Frogs acclimated to a body temperature of 24.5^{o} in a metabolic chamber under a light cycle of 14L:10D were injected in the dorsal lymph sac with either $^{33}PO_4$, $^{32}PO_4$, 2-^{3}H-glycerol or 3-^{3}H-serine. Eyes were removed at various times and processed for biochemistry or autoradiography. For biochemistry, retinas were gently homogenized in sucrose (1.17 gm/cc) containing 10 mM Tris and 2 mM $MgCl_2$ (pH 7.35) and overlaid with 1.15, 1.13 and 1.11 gm/cc sucrose in Tris and $MgCl_2$. After centrifugation at 82,000 x g x 60 min., ROS were collected at the 1.11/1.13 g/cc interface. The remaining volume of sucrose was diluted 1:1 with buffer without sucrose and centrifuged at 18,800 x g x 15 min. The resulting supernatant was centrifuged at 96,000 x g x 90 min. to obtain a microsomal pellet.

Lipids from the ROS and microsomal fractions were extracted with chloroform: methanol (2:1, v/v) and separated into classes by preparative thin-layer chromatography. Individual lipid classes visualized after exposure to iodine vapors were assayed for lipid phosphorus and an aliquot counted for the determination of

specific radioactivity. Phospholipid classes identified after spraying with water were methylated and the esters analyzed by gas chromatography.

In some instances, intact retinas were homogenized in chloroform:methanol (2:1) and again after the addition of 0.2 vols of 0.9% NaCl. Aliquots of the upper water: methanol phase were taken for determination of water soluble phosphorus and radioactivity. The specific radioactivity of water soluble precursors per micromole of phosphorus was determined. Protein was determined by the standard Lowry procedure.

For autoradiography of retinal lipids, the procedure of Gould and Dawson (1976) was followed with some modifications which will be described in detail in a subsequent publication. Several experiments showed that all of the radioactive lipid was extracted from the tissue designated "lipid out", while less than 8% was removed during the "lipid in" procedures.

BIOSYNTHESIS OF RETINAL PROTEIN FROM SERINE AND GLYCEROL

Serine and glycerol were incorporated into both protein and lipid in the frog retina. Figures 1 and 2 are the specific activities of microsomal and ROS protein following injection of ^{3}H-serine and ^{3}H-glycerol, respectively. In the microsomes, maximum specific radioactivity of protein was reached on the first day and decreased exponentially with time. This was different from the labeling pattern of ROS protein where maximum labeling occurred between days one and three and remained fairly constant until some time between days 24 and 40.

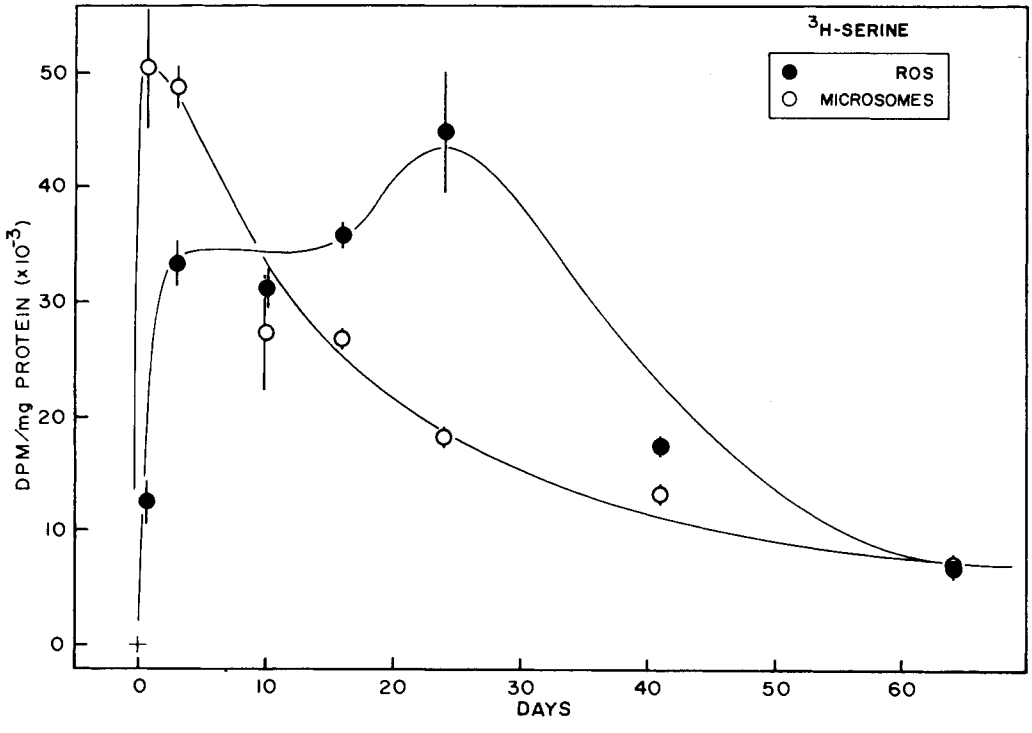

Fig. 1. Specific radioactivity of microsomal and ROS protein following injection of 3-^{3}H-serine.

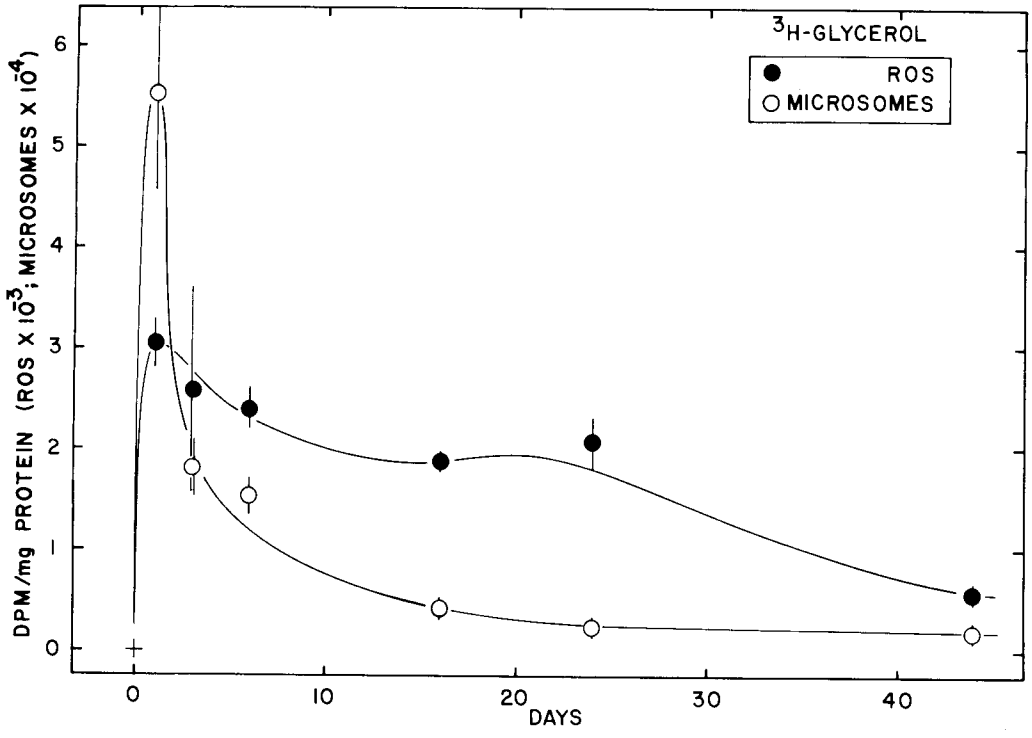

Fig. 2. Specific radioactivity of microsomal and ROS protein following injection of 2-^{3}H-glycerol.

These data are consistent with previous demonstrations that ROS proteins are synthesized in the inner segments of rod visual cells and migrate to the base of the ROS where they are incorporated into newly forming discs (Hall and co-workers, 1969). Maintenance of constant specific radioactivity in this dynamic membrane system where protein is daily being added at the base of the ROS and shed at the apical tips indicates that the radioactive proteins, once incorporated into ROS discs, do not diffuse throughout the ROS. If they did, an exponential decline in specific radioactivity similar to that observed for microsomal protein would also be evident for ROS protein. Such is not the case.

Autoradiograms from retinas of animals sacrificed one or 17 days after the injection of ^{3}H-serine are shown in Figs. 3A-D. Several features are evident in these figures: (1) A band of radioactivity indicative of the assembly of newly synthesized membrane components is present at the base of the rods but not the cones at day one. These bands persist in the delipidated tissue suggesting that most of the banded material is protein. The band has been displaced nearly half the length of the ROS by day 17, consistent with a 39-day turnover for integral disc proteins at 24.5°C. (2) At day one, the paraboloid regions of the accessory members of the double cones are highly labeled relative to the other cells (see arrows). This labeling pattern was observed by Bibb and Young (1974b) following tritiated glycerol injection, as well as by Basinger (personal communication) with $^{32}PO_4$, ^{3}H-inositol, and ^{3}H-glycerol. Since the paraboloid contains large amounts of glycogen, Bibb and Young (1974b) suggested that the label was in glucose derived by gluconeogenesis.

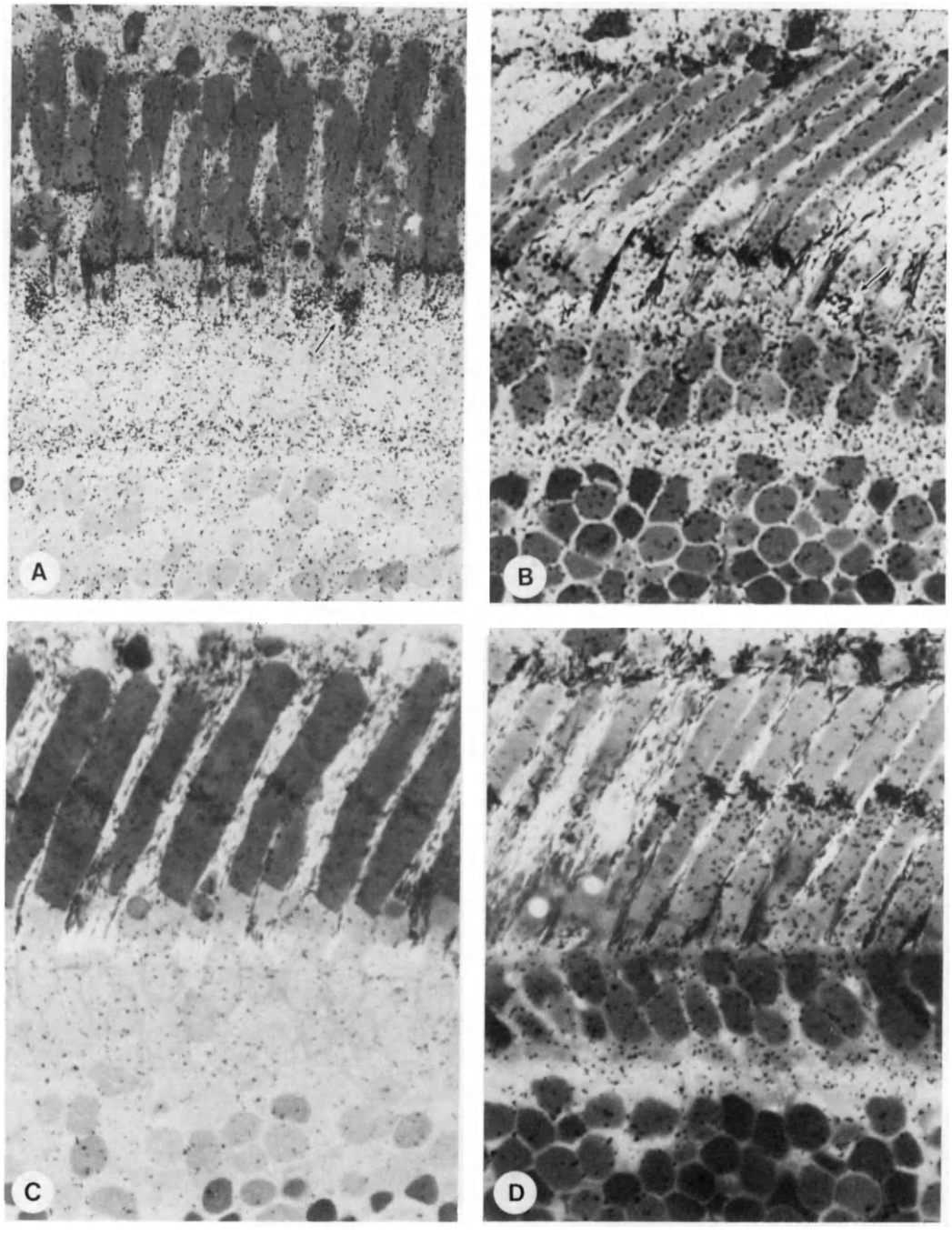

Fig. 3. Autoradiograms of frog retinas following the injection of 3-^{3}H-serine. (A) Day 1, lipid in; (B) Day 1, lipid out; (C) Day 17, lipid in; (D) Day 17, lipid out.

However, label must also be in other components since lipid precursors not readily converted to glucose are also concentrated in this area. Determination of grain densities over the paraboloid region relative to the protein bands of adjacent ROS at day one for "lipid in" and "lipid out" tissue revealed that the densities in the paraboloid region were the same for both treatments, suggesting that the label was in protein. This labeling pattern was not observed following leucine injection, and its significance is not known. (3) Radioactive proteins accumulated in the synaptic region of the frog retina on the first day and were still seen, although somewhat diminished in intensity, at day 17. (4) Although lipid accounted for about half of the tissue radioactivity at both day one and day 17, unique labeling patterns were not seen for lipid. Rather, lipid seems to be diffusely incorporated into all of the cellular membranes, including the ROS. This point will be discussed in following sections.

BIOSYNTHESIS OF RETINAL PHOSPHOLIPIDS

Tritiated serine is incorporated into phospholipids of the frog retina. A precursor-product relationship is indicated by the higher specific activity of PS in the microsomes than in the ROS at the early time points, followed by a reversal in this pattern at day 2 (Fig. 4).

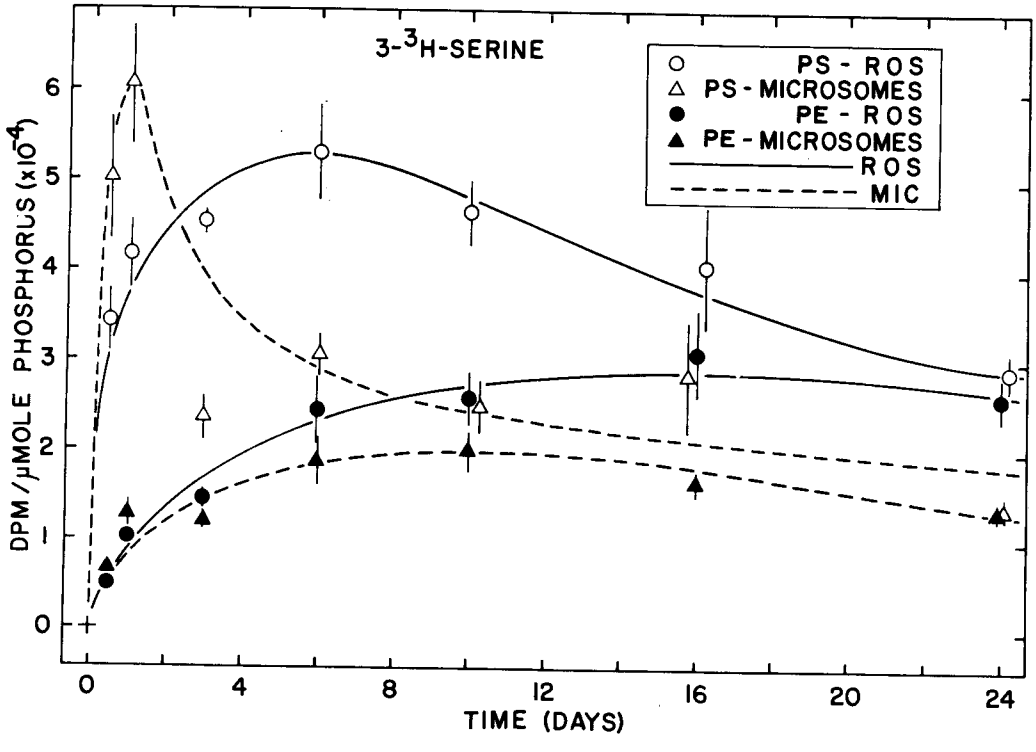

Fig. 4. Biosynthesis of phosphatidyl serine and phosphatidyl ethanolamine in microsomes and ROS following the injection of 3-^{3}H-serine.

This is consistent with the synthesis of phospholipid on retinal microsomes and the subsequent incorporation of this lipid into ROS disc membranes. A similar labeling pattern for both the microsomal and ROS PS was observed following the injection of tritiated glycerol (data not shown). Whether the serine is incorporated during *de novo* synthesis of phospholipids or by base exchange reactions remains to be determined. However, preliminary data from our laboratory have shown an active serine base exchange system in bovine retinal microsomes.

DECARBOXYLATION OF PHOSPHATIDYL SERINE TO PHOSPHATIDYL ETHANOLAMINE

One of the pathways for the formation of phosphatidyl ethanolamine in some tissues is the enzymatic decarboxylation of PS. The present study demonstrated an active decarboxylase in the frog retina. Although the site of decarboxylation cannot be unequivocally established from these *in vivo* studies, it is likely to be both the microsomes and the ROS. Evidence for this is as follows: (1) The specific activities of ROS or microsomal phosphatidyl serine and phosphatidyl ethanolamine are nearly the same 24 days after injection of labeled serine (Fig. 4), but these 24-day values are different for the two subcellular fractions indicating that decarboxylation occurred in each fraction. (2) Although a precursor-product relationship exists in both fractions for the decarboxylation reaction, and for the synthesis of phosphatidyl serine, one does not exist for transfer of microsomal phosphatidyl ethanolamine to ROS. In unpublished studies, following the injection of labeled ethanolamine, the specific activity of microsomal phosphatidyl ethanolamine was several times that of ROS phosphatidyl ethanolamine at early time points. Thus, if a precursor-product relationship existed between microsomal and ROS phosphatidyl ethanolamine formed by the decarboxylation of microsomal phosphatidyl serine, it should have been easily identified.

Although not as pronounced as the data obtained following injection of labeled serine, the conversion of phosphatidyl serine to phosphatidyl ethanolamine in ROS was also demonstrated following injection of other lipid precursors. As shown in Fig. 5 and 6, the specific activity of phosphatidyl serine in the ROS following the injection of $^{32}PO_4$ or 2-^{3}H-glycerol was clearly higher than that of phosphatidyl ethanolamine at the early time points, and only after several weeks did the specific activities of these two lipids become the same.

The fatty acid composition of phosphatidyl ethanolamine and phosphatidyl serine given in Table 1 is also consistent with an interconversion of these two lipid classes. The phosphatidyl serine contains slightly less long-chain polyunsaturated fatty acids than the phosphatidyl ethanolamine while the phosphatidyl ethanolamine has a slightly higher percentage of palmitic acid. However, a remarkable similarity in fatty acid composition does exist.

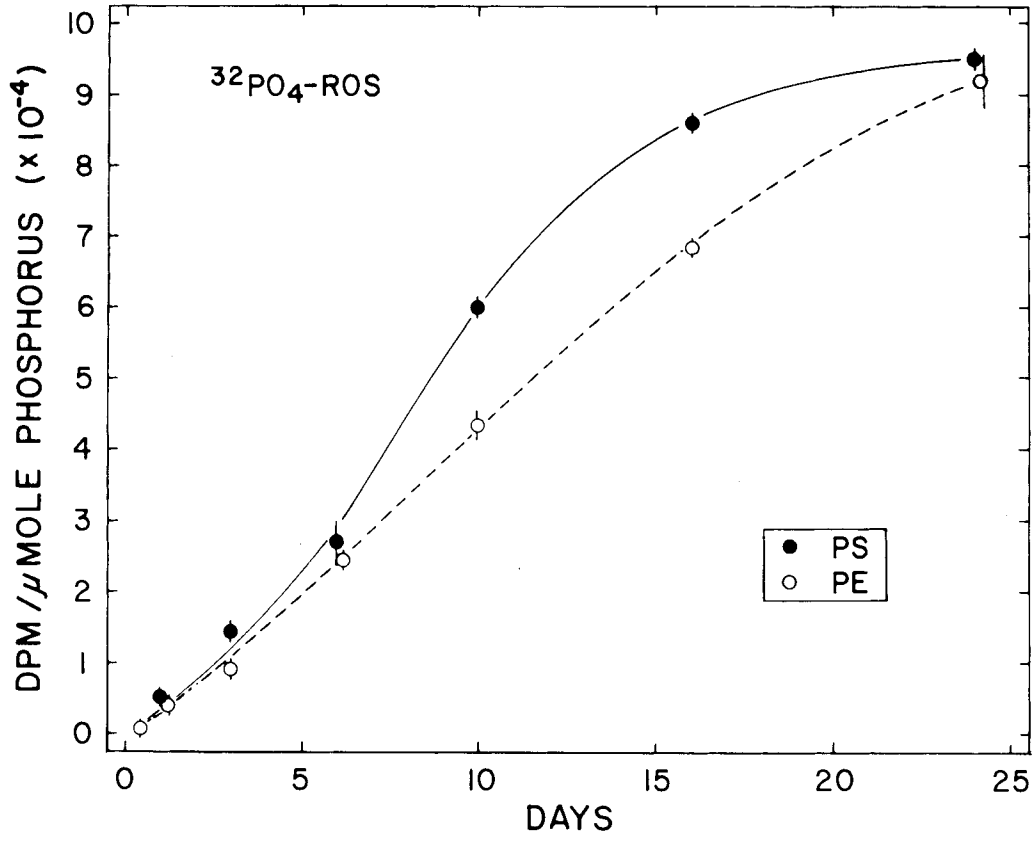

Fig. 5. Specific activities of phosphatidyl serine and phosphatidyl ethanolamine in ROS following the injection of $^{32}PO_4$.

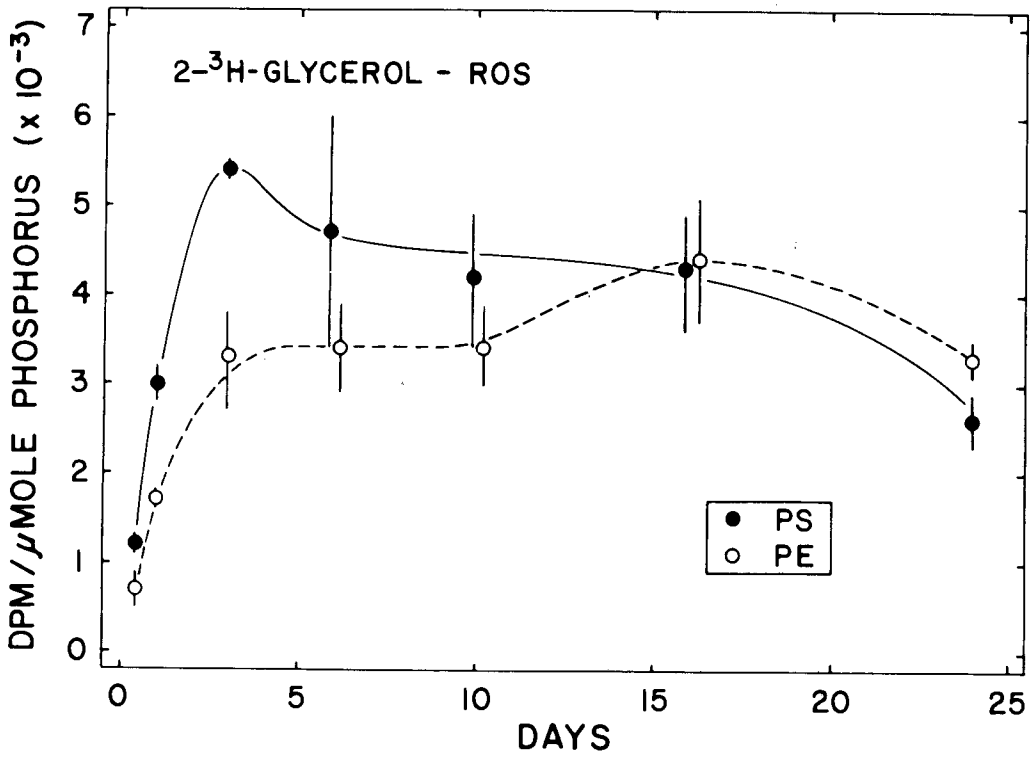

Fig. 6. Specific activities of phosphatidyl serine and phosphatidyl ethanolamine in ROS following the injection of 2-^{3}H-glycerol.

TABLE 1 Composition of the Major Fatty Acids of Phosphatidyl Ethanolamine and Phosphatidyl Serine from Frog ROS.

Acid	PS	PE
16:0	4	6
18:0	19	13
18:1	3	6
20:4ω6	2	7
22:4ω6	7	7
22:5ω3 + ω6	8	5
22:6ω6	46	51

(Anderson and Risk, 1974)

TURNOVER OF PHOSPHATIDYL SERINE IN ROD OUTER SEGMENTS

The incorporation and turnover of 2-^{3}H-glycerol, $^{33}PO_4$, and 3-^{3}H-serine in phosphatidyl serine in rod outer segments are shown in Fig. 7.

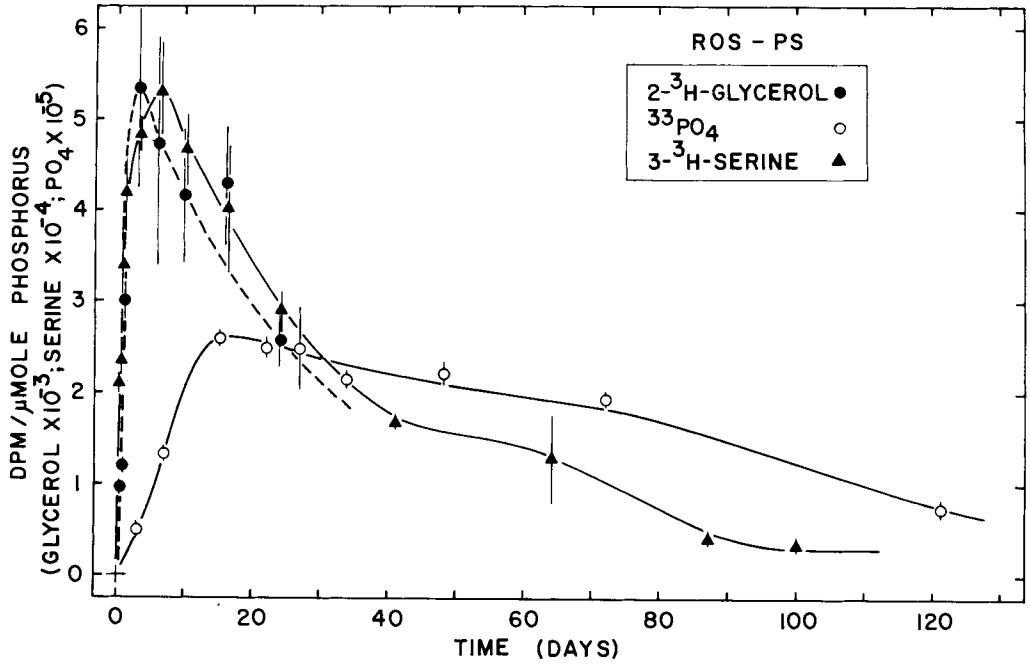

Fig. 7. Turnover of phosphatidyl serine in ROS following the injection of 2-^{3}H-glycerol, $^{33}PO_4$, or 3-^{3}H-serine.

As previously observed by Hall and co-workers (1973), the incorporation of labeled phosphorus into ROS phosphatidyl serine was quite slow, requiring 20 days to achieve maximum specific activity, and the loss of label due to turnover was equally slow. It was impossible to calculate turnover times for the membrane phospholipids from the labeled phosphorus data. On the other hand, both glyserol and serine rapidly labeled the ROS phospholipids with glycerol achieving maximum specific activity three days and serine six days after injection of the isotopes. The reason for the distinctive labeling pattern of phosphate is evident from the data in Fig. 8 where the percent of maximum specific activity of water soluble radioactivity from frog retinas (DPM per micromole phosphorus) is plotted against time. Although the chemical identities of the labeled water soluble products were not established, it is clear that glycerol and serine are rapidly catabolized in the retina and thus are true "pulses" of radioactive lipid precursors whereas labeled phosphate remains available for lipid synthesis for weeks. Thus, the rapid labeling observed autoradiographically by Bibb and Young (1974b) following tritiated glycerol injection was the result of a pulse of radioactive precursor being presented to the tissue, whereas the slow labeling following phosphorus administration resulted from the presence of a long-lived retinal pool of radio-

active phosphate.

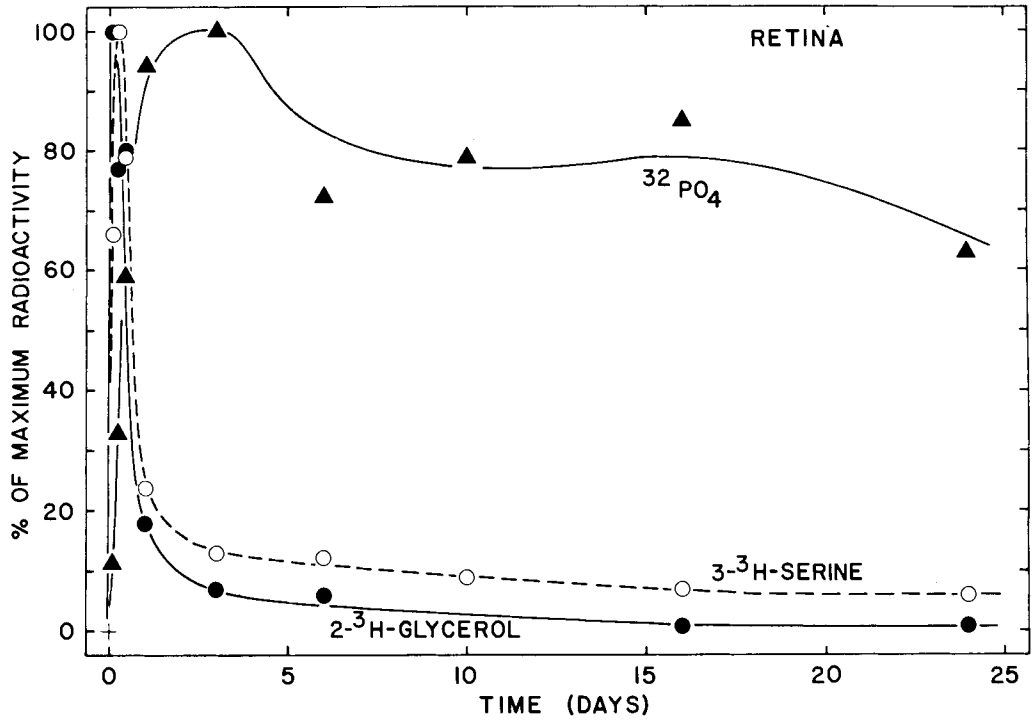

Fig. 8. Time course of labeling of water soluble precursors of retinal lipids.

Returning to the data in Fig. 7, the specific activity of phosphatidyl serine from both glycerol and serine declined from their maximum value in an exponential manner. Linear regression analysis of the log of specific activity versus time for each precursor gave identical 23-day half-lives for phosphatidyl serine in the ROS. A similar analysis for phosphatidyl ethanolamine derived from phosphatidyl serine gave a half-life of 38.5 days. The different half-lives of phosphatidyl serine and phosphatidyl ethanolamine are probably due to the fact that phosphatidyl serine is lost through decarboxylation and disc shedding while phosphatidyl ethanolamine is lost through disc shedding, but is replaced by decarboxylation.

DISCUSSION

Protein and lipid are synthesized on the photoreceptor microsomes and migrate to the base of the ROS where they are incorporated into the basal infoldings of the

plasma membrane, which eventually pinch off to become free-floating discs. From this time on, the protein is "trapped" and remains with the same disc throughout the 5½ week period of apical displacement. Lipid, on the other hand, is not confined to any particular disc. The exponential decline in specific radioactivity of ROS phospholipids is consistent with a diffuse rather than a discrete labeling of ROS by lipid and supports the earlier reports of Bibb and Young (1974a and 1974b).

Since the labeled lipid diffuses throughout the ROS, one-half of the label should be lost in one complete turnover of outer segment protein if disc shedding is the only means of losing lipid, and calculations of $t_{\frac{1}{2}}$ for lipid should give a value comparable to the ROS turnover rate. We calculated a value of 39 days for turnover of ROS discs, which is longer than the half-life determined for phosphatidylserine (23 days), phosphatidyl choline (24 days, unpublished) and phosphatidyl inositol (< 5 days, unpublished). (The value of 38.5 days calculated for phosphatidyl ethanolamine is longer than the actual value, since phosphatidyl serine is slowly converted to phosphatidyl ethanolamine, and thus is not derived from a "pulse" of radioactive precursor.) It appears then that ROS lipid is turning over at a rate faster than protein, a conclusion also reached by Bibb and Young (1974b).

Lipid and protein are probably delivered and inserted into the growing membrane as a package, since newly synthesized frog opsin is not found in a soluble form (Papermaster and co-workers, 1975). How the lipid diffuses throughout the ROS is not known, but it may be mediated by phospholipid exchange proteins, which we recently described in bovine retina (Dudley and Anderson, 1978). Whether or not the diffusion involves only interdisc transfer of lipid originally delivered to the growing membrane as a lipoprotein complex, as opposed to lipid exchange with other rod cellular organelles, remains to be determined. However, based on half-life determinations of ROS phospholipids discussed above, the latter possibility seems reasonable.

An unexpected observation in these *in vivo* studies was the decarboxylation of ROS phosphatidyl serine, which suggests an active lipid metabolism in ROS. In studies to be reported elsewhere, phosphatidyl ethanolamine was methylated to phosphatidyl choline, apparently in the ROS, while the entire molecule of phosphatidyl inositol completely turned over in less than seven days. Enzymes of lipid metabolism have not previously been reported in ROS (Swartz and Mitchell, 1973, 1974; Mizuno, 1976). However, these investigations utilized vigorous homogenization and extensive washing procedures during the isolation of ROS which may have led to losses of some loosely bound proteins. We are currently searching for the subcellular localization of the enzymes of lipid metabolism responsible for the various interconversions demonstrated in these *in vivo* experiments.

ACKNOWLEDGEMENTS

This research was supported in part by a grant from the Retina Research Foundation (Houston), grants EY 00871 and EY 07001 from the National Eye Institute, and departmental grants from Research to Prevent Blindness, Inc. and The Brown Foundation (Houston).

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THE GANGLIOSIDES OF THE CHICKEN RETINA AND OPTIC TECTUM. THE INFLUENCE OF LIGHT ON THEIR LABELLING AFTER AN INJECTION OF LABELLED PRECURSORS

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ABSTRACT

Cultures of retinas from 8-day-old embryonic chicken in the presence of [^{3}H]-glucosamine showed that GD3 and GM3 are the gangliosides with highest labelling. From this age onward, and apparently responding to the appearance of the required enzymes, the labelling of GM1, GD1a and GT increases and at hatching day GD1a becomes the highest labelled ganglioside. The main site of synthesis of neuronal gangliosides was found in the neuronal perikarya, in a Golgi membrane enriched fraction. In this fraction the nascent gangliosides are protected from the neuraminidase attack. Experiments in the visual system of chicks indicated that the gangliosides synthesized in the soma of retinal ganglion cells are transported axonally to their endings in the contralateral optic tectum. After 3, 5 and 8 h of injection of N[^{3}H] acetylmannosamine into one eye of the chick, the gangliosides in the contralateral optic tectum of animals exposed to 1000 lux were more labelled than in their controls in dark. The glycoproteins followed a pattern of labelling similar to that of gangliosides. Most of the difference in labelling appears in the same subcellular fraction in which normally is found the maximal deposition of gangliosides. The increase of labelling was not due to a greater turnover of gangliosides.

KEYWORDS

Retinal gangliosides; visual system sialosylglycoconjugates; ganglioside subcellular site of synthesis; light on ganglioside biosynthesis.

INTRODUCTION

The hypothesis of the existence of two pools of gangliosides was formulated around ten years ago from findings of experiments with rat brain homogenates and with rats in vivo (Arce and others, 1971; Maccioni and others, 1971; Caputto and others, 1974). This hypothesis stated that there is in brain a small pool of transient or precursor gangliosides which are utilized for the synthesis of more complex gangliosides and a relatively large pool of end product gangliosides. Both pools are chemically similar, but the pool of end products can neither be utilized for further synthesis nor is able to undergo renewal of parts of the ganglioside molecule at least in any appreciable measure (Maccioni and others, 1971; see also Holm and Svennerholm, 1972; Ledeen and others, 1976). The hypothesis carried with it the connotation that

the pool of transient gangliosides was in contact with glycosyltransferases (probably in the Golgi apparatus) which build the carbohydrate chain of the gangliosides, whereas the large pool of end products, probably located in the plasma membranes of the neuronal and glial cells, has totally or partially lost contact with the synthesizing system. If this hypothesis is correct it should be a main point to consider when any problem of renewal of gangliosides is studied. Conceptually it is in opposition to the several hypotheses which attribute to the plasma membrane capability for the building or renewal of the carbohydrate chain of gangliosides (Roseman, 1970), even if it is possible that on quantitative grounds there may be room for both hypotheses, one describing the general events, the other the exceptions.

In the optic system, the neuronal perikarya of the ganglion cells of the retina are widely separated from their nerve endings which are located in the brain and specifically in the chicken, in the optic tecta. This make an almost ideal situation to test the hypothesis of the two pools of gangliosides. It was first shown that the gangliosides, similarly to what had been found for other lipids, are moved by axonal transport from the retina to the superior colliculus and lateral geniculate body in the rabbit (Ledeen and others, 1976) and to optic tecta in the goldfish (Ledeen and others, 1976) and in the chicken (Landa and others, 1979). These later authors also showed that only a minor part, if any at all, of the gangliosides were synthesized in the optic nerve endings.

Considering the effect of light on the labelling of the optic tectum gangliosides from the point of view of the two pools hypothesis it becomes clear that changes in the biosynthesis processes in the retina or changes in the axonal transport from retina to the brain may have interest in explaining the mechanisms by which a sensorial organ may influence the functioning of the central nervous system, specially if such mechanisms can be extended to other substances such as the glycoproteins.

THE SUBCELLULAR SITE OF SYNTHESIS

Assuming that the bulk of gangliosides is representative of the pool of end products (G) whereas the endogenous ganglioside acceptors (EGA) of sialosyl groups and the hematoside synthetase (HS) are linked to the pool of transient gangliosides, a separation of the two pools from the brain was obtained by washing the conventional crude mitochondrial fraction before preparation of synaptosomal and mitochondrial fractions (Table 1). The ratio EGA/G was 43.9 in the synaptosomal fraction obtained from the washed pellet and 411 in that obtained from the supernatant. For the HS/G ratios the corresponding values were 306 and 2244 respectively. Similar wide differences were found between the precipitate and supernatant after washing the conventional mitochondrial fraction. Ng and Dain (1977) also reported that the highest activity of sialosyltransferases was not associated with the synaptosomes.

A further step in characterizing the subcellular site of synthesis of gangliosides was the separation of the neuronal perikarya from the synaptosomes (Table 2) which showed that the sialosylating activity was concentrated in the neuronal perikarya with respect to the synaptosomes about 100 to 150-fold. Similar results were obtained when the galactosyltransferase which catalyzes the synthesis of GM1 from GM2 was assayed, or when the acceptors of the galactosyl groups were the EGA.

In the chicken optic system the distribution of radioactivity in retina and in the optic tecta was determined after an intraocular injection of [^{3}H] N-acetylmannosamine. In this animal the fibers of the optic nerve decusate completely at the optic chiasma, and consequently any labelled material transported from the retina to the optic tectum by axonal transport as opposed to any material transported systemically can be determined by the difference between the labelling in the contralateral and the ipsilateral tectum. Figure 1 shows that whereas the acid soluble material

TABLE 1 Ganglioside NeuAc, Endogenous Ganglioside Acceptors and Hematoside Synthetase in Synaptosomes and Mitochondria from the Washed Pellet and from the Washing Fluid of crude Mitochondrial Fraction

	Synaptosomes from		Mitochondria from	
	Washed Pellet	Washing Fluid	Washed Pellet	Washing fluid
Ganglioside NeuAc (nmol/mg protein)	23	21	10	9
Endogenous ganglioside* acceptors	1010	8600	188	1200
Hematoside synthetase*	7040	46920	4186	8391

From Landa and others (1977).
* Figures are counts/min/mg of protein of [^{3}H]NeuAc transferred from CMP-[^{3}H]NeuAc to endogenous gangliosides (in the absence of detergents) or to exogenous lactosylceramide (in the presence of detergents).

TABLE 2 Endogenous Ganglioside Acceptors of Sialosyl Groups and Sialosyltransferase Activity in Neuronal Perikarya and Synaptosomal Fractions from Rat Brain Homogenate

Fraction	Endogenous Ganglioside acceptors (EGA)	Transfer of sialosyl groups to*	
		Hematoside	Lactosylceramide
Neuronal perikarya	2166	13892	6948
Synaptosomes	13	140	62

From Maccioni and others (1978)
*Results obtained in optimal detergent (Triton CF54:Tween 80, 2:1)/protein ratio. For the determination of EGA no detergent was used.
Figures are counts/min/nmol of ganglioside NeuAc.

reaches the tectum by systemic transport and in such amounts or conditions that in 8 h does not significantly originate gangliosides in the tectum, the gangliosides reach the contralateral tectum through axonal transport.

The specific radioactivity of gangliosides of the neuronal perikarya and of the synaptosomal fraction from the contralateral tectum of animals which received an intraocular injection of [^{3}H]ManNAc were compared with the specific radioactivity of the gangliosides from the same fractions from animals which received an intracranial injection of [^{3}H]ManNAc. Results showed that 5 h after the injection the gangliosides from the synaptosomes have higher specific radioactivity than the gangliosides from the neuronal perikarya after the intraocular injection whereas the opposite occurred after the intracranial injection (Table 3).

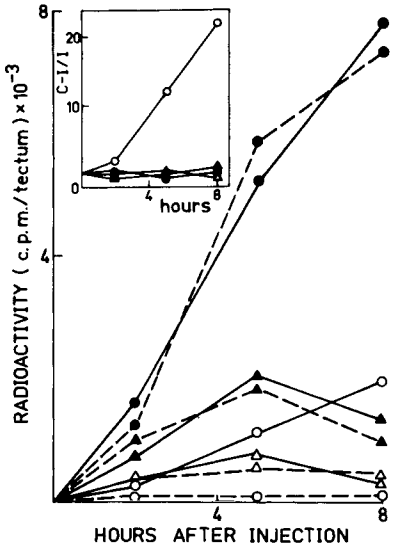

Fig. 1. Radioactivity of gangliosides (O) and acid soluble material (evaporable under vacuum (●), retained (Δ) and not retained (▲) by Dowex-1 in the contralateral (——) and in the ipsilateral (---) optic tecta of the chick after an intraocular injection of [^{3}H]-ManNAc. In the inset the values C-I/I (C= contralateral; I = ipsilateral) show the excess of the radioactivity in the contralateral with respect to the ipsilateral tectum.

TABLE 3 Specific Radioactivity of Gangliosides from Neuronal Perikarya and Synaptosomal Fractions from Optic Tecta after an Intraocular or an Intracranial Injection of [^{3}H]-ManNAc

Injection	Hours after the injection	Neuronal perikarya	Synaptosomes
Intracranial	2.5	211	42
Intracranial	5.0	210	100
Intraocular	5.0	18*	37*

From Landa and others (1979)
*Difference between the corresponding values in the contralateral and ipsilateral tecta.
Figures are counts/min/nmol of ganglioside NeuAc

The radioactivity patterns of the gangliosides labelled after an intraocular or an intracranial injection of [^{3}H]ManNAc afforded another evidence that after the intraocular injection the labelled tectum gangliosides were synthesized in retina. The

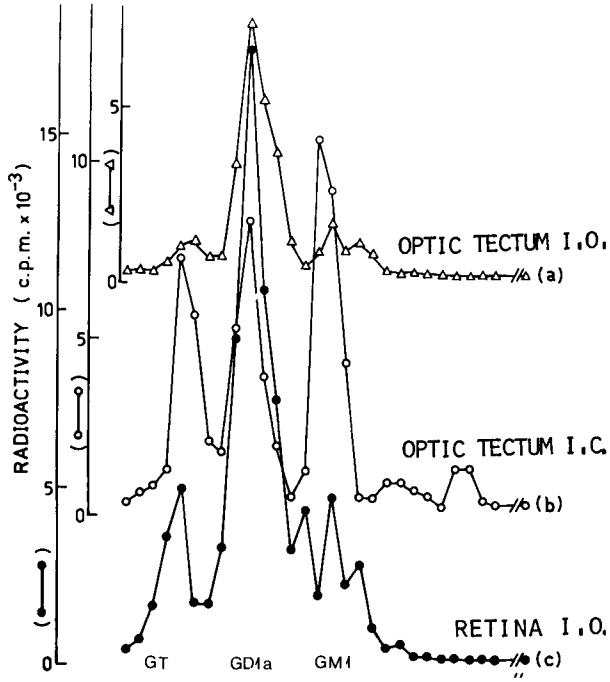

Fig. 2. Thin-layer chromatography of retina and optic tectum gangliosides labelled after an intraocular (I.O.) or intracranial (I.C.) injection of [^{3}H]ManNAc. The position of standard gangliosides is indicated.

pattern of the labelling of the gangliosides from tectum after thin layer chromatography was similar to the color pattern after staining gangliosides from retina run in the same TLC system and also similar to the pattern of labelling of these same gangliosides (Fig. 2). On the other hand after the intracranial injection the pattern of labelling of the gangliosides of the optic tectum was similar to the labelling and to the color pattern after staining the optic tectum gangliosides. In the optic tectum the three prominent gangliosides were GT1, GD1a and GM1 whereas in retina GD1a prevails ostensibly over all other gangliosides in the 1-day-old chick.

All these results show that the main site of synthesis of gangliosides in the rat whole brain and in chick optic system is the neuronal perikarya, where the transient pool of gangliosides and the glycosylating enzymes needed for the synthesis are located. From that site of synthesis the gangliosides are translocated to their places of deposit, the most important of which are probably the plasma membranes of the cell. Whether or not there is synthesis of gangliosides also in the synaptosomal fraction is certainly a more difficult assertion to make. The glycosylating enzymes distribute almost evenly in most fractions. Does this mean that they are intrinsic components of all fractions or simply that they belong to membranes of such densities that have tendency to scatter all over the different density fractions in the centrifugation procedure so far devised? Direct evidence of renewal of parts of the ganglioside molecules of the large pool of deposit are certainly non existing. Still the stronger evidence against this hypothesis is the observation that after labelling in vivo the two galactoses of the tetraose chain of complex gangliosides and the neuraminidase labile and stable sialosyl groups of

the di- and polysialogangliosides have the same specific radioactivity, indicating that neither the two galactoses between them nor the sialosyl groups between them are subjected to different dilutions during their incorporations (Maccioni and others, 1971). Indeed, this observation is compatible only with the assumption that the carbohydrate chain of gangliosides is synthesized from a very small pool of ganglioside precursors and that once the carbohydrate chain of the gangliosides starts to be synthesized it is swiftly finished and if it is degraded to be rebuilt it has to go at least to the stage of glucosylceramide.

Subfractionation of neuronal perikarya by centrifugation on sucrose density gradient yielded a membrane fraction in which the ganglioside glycosyltransferases were enriched between 10- and 15-fold over the whole homogenate (Table 4).

TABLE 4 Ganglioside Glycosyltransferases in Subfractions from Rat Brain Neuronal Perikarya

Transferase	Relative Specific Activity		
	Homogenate	Neuronal Perikarya	Golgi Membrane Fraction
CMP-NeuAc: lactosylceramide	1	4.7	12
CMP-NeuAc:GM3	1	5.7	13
UDP-Gal:GM2	1	7.9	12

The morphology under the electron microscope and the enrichments in glycosyltransferases indicate that this fraction is enriched in membranes from the Golgi apparatus (Fig. 3).

TOPOGRAPHIC DISPOSITION OF THE GANGLIOSIDES SYNTHESIZED IN THE NEURONAL SOMA

Incubation of neuronal soma homogenates with labelled CMP-NeuAc resulted in the incorporation of labelled NeuAc into endogenous gangliosides. Subfractionation of the labelled membranes showed that the labelled gangliosides cosedimented with the membrane fraction enriched in Golgi membranes, where they appeared concentrated about 15-fold over the homogenate.

The disposition of the labelled gangliosides with respect to the transversal plane of these membranes was investigated on the basis of their accessibility to added neuraminidase. Table 5 shows that the labelled gangliosides of these membranes were protected from the action of neuraminidase. The protection was not abolished by treatments designed to release vesicle contents or to release or degrade possible masking proteins, but was abolished by treatments of these membranes with low deoxycholate concentration (0.05%). This low DOC concentration did not solubilize the ganglioside carrying membranes, but probably permeabilize them. Our tentative con-

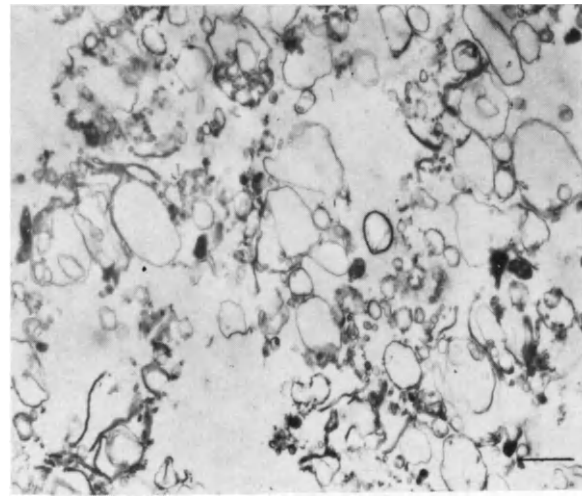

Fig. 3. Electron micrograph of the Golgi enriched membrane fraction showing numerous smooth-surfaced vesicles of varying sizes, some flattened membrane sacs and occasional rough-surfaced vesicles. Bar represents 0.5 μm

TABLE 5 Accessibility to Neuraminidase of Labelled Gangliosides in Golgi Membranes

Treatment	Radioactivity in gangliosides (counts/min)
None	2600
Neuraminidase	2300
Neuraminidase + Pronase	2300
Freezing and thawing + neuraminidase	2400
Deoxycholate + neuraminidase	400

From Landa and others (in preparation)

clusion of these experiments is that the gangliosides are built in the luminal (extracytoplasmic) face of the Golgi membranes of the neuronal soma. In their position in the plasma membranes of the synaptosomes they appeared accessible to neuraminidase facing the extracytoplasmic side (see also Hansson and others, 1977).

THE DEVELOPMENT OF GLYCOSYLATING ENZYMES IN RETINA AND ITS RELATION TO THE APPEARANCE OF GANGLIOSIDES

The chromatographic pattern of retina gangliosides undergoes important changes during the embryonic development of the chick from the 8th day of the embryo to the hatching day (Dreyfus and others, 1975 ;Panzetta and others, submitted). Table 6 shows that those changes are apparently determined by the appearance of the N-acetylgalactosaminyl- and galactosyl-transferases during that embryonic period.

TABLE 6 Ganglioside Glycosyltransferases and Ganglioside Labelling during Development of Chicken Retina

Embryonic age	Glycosyltransferase activities*					Ganglioside labelling in cultures#		
	Lac-Cer sialyl	GM1 sialyl	GM3 sialyl	GM3 galactosaminyl	GM2 galactosyl	GD3	GD1a	GT1
8 days	3.5	3.3	1.5	3.0	0.8	68	18	3
10 "	2.4	2.5	0.3	9.2	2.2	---	---	-
13 "	3.2	3.2	0.4	15.0	4.0	15	47	15
16 "	1.7	1.0	0.8	6.0	7.4	10	45	13
1 day post-hatching	1.6	0.9	0.3	5.0	15.4	5	70	10

From Panzetta and others (Submitted)
*Values are given in counts/min/μg DNA x 10^{-2}
#Values are percents of the total ganglioside labelling

Experiments of labelling with [^{3}H] GlcNAc of cultures of intact retinas showed that at the 9th day around 75 % of the labelling of the gangliosides was distributed between the gangliosides GM3 and GD3. At this age the retina shows a poor activity of N-acetylgalactosaminyl- and galactosyl- transferases whereas the sialyltransferases have reached the maximal activity they will reach during development. After this age the galactosaminyl-and galactosyl- transferase activities start to increase rather sharply and so do the labelling of N-tetraose gangliosides GD1a and GT1 which increase to become around 80% of the ganglioside labelling in retina at hatching day (Table 6). The changes in labelling probably did not reflect merely changes in the relative rates of turnover between gangliosides with short and long carbohydrate chains since Dreyfus and others (1975) showed that GD1a increases in amounts with respect to the other gangliosides during retina development. It appears as if the appearance of the GM3:N-acetylgalactosaminyltransferase and GM2:galactosyltransferase simultaneously with the decrease in the activity of GM3:sialyltransferase determined that GM3 instead of being used for the synthesis of GD3 (reaction 1) is utilized as a substrate that initiates the synthesis of GD1a (reaction 2)

(1) GM3 + CMP-NeuAc ⟶ GD3
(2) GM3 + UDP-GalNAc + UDP-Gal + CMP-NeuAc ⟶ GD1a

THE INFLUENCE OF LIGHT IN THE LABELLING OF RETINA AND OPTIC TECTUM GANGLIOSIDES

Light influences the labelling of gangliosides in rat brain and chicken brain and retina when the given labelled precursor ([^{3}H] GlcNAc) reached the brain and the retina via systemic circulation (Maccioni A.H.R. and others 1971, 1974; Caputto B. and others, 1975). However, using this type of experiments the situation became confusing and the hopes for progress were not much. Results in rat and chicken brains were in the opposite direction: whereas light decreases the labelling of brain gangliosides in the rat it increased the labelling in the chick. In the chick, whereas the light increased the labelling in brain, decreased it in retina. We could not decide if the effect of light was direct on the labelling of brain gangliosides or indirect through the different influence that light may have on the activity of rat and chicken. In retina this question could be decided by labelling the gangliosides of chickens exposed to light with one eye occluded,whereby it was observed that the occluded eye was more labelled than the exposed eye. More revealing experiments were possible with the awareness that the gangliosides of the presynaptic membranes of ganglion cell terminals of the first station in the optic system were synthesized in the retina, in the perikarya of the ganglion cells from which the optic nerve originates (Landa and others, 1979). For this purpose chickens (Cobb Hardig Concord or Cobb Hardig sex linked) were maintained from hatching to the eighth day of age exposed to periods of 12 h each of darkness and light and then put in the dark for 48 h. The labelled precursor ([^{3}H]-ManNAc) was injected into one eye and the animals were alternatively incorporated into a group that remained in the dark or was exposed to 1000 lux. No differences were found in the total and soluble labelled materials or in the labelled sialic acid of the retina. This information if confirmed will indicate that either the amount of sialic acid in retina is very small and is rapidly and completely replaced reaching the highest possible specific radioactivity as soon as the ^{3}H ManNAc enters the cell or else that the processes by which [^{3}H]ManNAc is transformed into [^{3}H]NeuAc are not altered in the whole retina. Also no differences were found in the labelling of gangliosides (Table 7). This situation with the precursors and with the gangliosides of retina is not clearly understood at present and will not be until we can determine more precisely the influence that each layer of cells has on the

whole phenomenom. The gangliosides in tectum, which we will study immediately are presumably expression of the activity of the ganglion cells whereas the labelling of the components of retina we have studied to date express the activity of the whole retina. Since in the chick in no case we have been able to find any difference between the acid soluble precursors in the ipsi- and contra- lateral tecta to the eye that received the injection we conclude that precursors are not axonally transported. The axonal transport must be, consequently, of the gangliosides themselves as was established by Ledeen and others (1976) and confirmed by Maccioni and others (1977) who also established that most if not all of the ganglioside synthesis is carried out in the neuronal perikarya, in a subcellular fraction which by its morphological characteristics and by the set of transferases that carries with it is the equivalent to the Golgi apparatus in liver (Keenan and others, 1974) thyroid (Pacuszka and others, 1978) and other organs. The differences found in the optic tectum between the animals exposed to light or kept in the dark (Table 8) are indicative of either higher activity in the retina or higher axonal transport in the optic nerve of chickens exposed to light with respect to those kept in dark.

TABLE 7 Labelling of Retina Gangliosides after an Intraocular Injection of [^{3}H]ManNAc

Time after injection (Hours)	Dark	Light	p
	counts/min/mg protein		
1	(8) 587±42	(10) 543±71	N.S.
3	(7) 3716±442	(8) 3865±142	N.S.
5	(7) 5860±421	(8) 6704±447	N.S.

Figures are means ± S.E.M. of the number of determinations given in parentheses. N.S.: non significant.
From Caputto B. and others (1979)

TABLE 8 Labelling of Optic Tecta Gangliosides after an intraocular injection of [^{3}H]ManNAc

Time after injection (Hours)	Dark	Light	p
	counts/min/mg protein		
3	(8) 45±8	(8) 70±2	<0.01
5	(10) 128±10	(11) 164±8	<0.01
8*	(6) 253±43	(6) 355±21	<0.01

Figures are means ± S.E.M. of the number of determinations given in parentheses. p was calculated by Student's t test.
*animals from a different experiment
From Caputto B. and others (1979)

STUDIES ON THE GANGLIOSIDES THAT ARRIVE TO THE OPTIC TECTUM IN LIGHT AND DARK

It has not been possible up to now to establish qualitative differences between gangliosides that arrive to the optic tectum in light and dark. The chromatographic patterns of these gangliosides have shown in some cases a relative increase in the labelling of GD1a in light and this is currently under investigation. In retina, the determination of labelling of gangliosides at short time after the injection of [^{3}H] ManNAc is made difficult by the presence of very high amounts of precursors. However, previous separation of gangliosides and precursors in a DEAE-Sephadex column produced a relatively satisfactory preparation but no differences between the patterns of labelling of gangliosides in light and dark were found. An experiment was run in which the optic tectum gangliosides were allowed to reach a maximum of labelling after an intraocular injection of [^{3}H] ManNAc. Then, during the period in which the labelling was decaying the animals were divided into two groups, one exposed to light and the other maintained in the dark and the rate of decay of labelling was studied in both groups (Fig. 4). No differences were found in the decay of labelling suggesting that the differences in labelling during exposition to light or dark are not due to changes in turnover. In another experiment, we attempted to see whether the gangliosides which were labelled under the influence of light had a different destination in the tectum than those labelled in the dark. For this purpose the crude mitochondrial fraction of the tectum obtained by subcellular fractionation was subjected to hypoosmotic shock according to the procedure of Whittaker (1969). After centrifugation in a conventional density gradient different subsynaptosomal fractions were obtained. The highest labelling were found in the synaptosomal plasma membrane fraction in both preparations obtained from animals whose gangliosides were labelled in the dark or in the light. From these experiments it appears that light just accelerates a process occurring in dark and that probably the gangliosides produced in light are undistinguishable in their chemistry and in their site of deposit from those produced in dark. The difference established in light is probably not a difference in the quality of the gangliosides that arrive to the tectum. Any inference on the possibility that there is a difference in the quantity that arrives to the tectum will probably have to wait until the phenomenon that occurs in retina is understood.

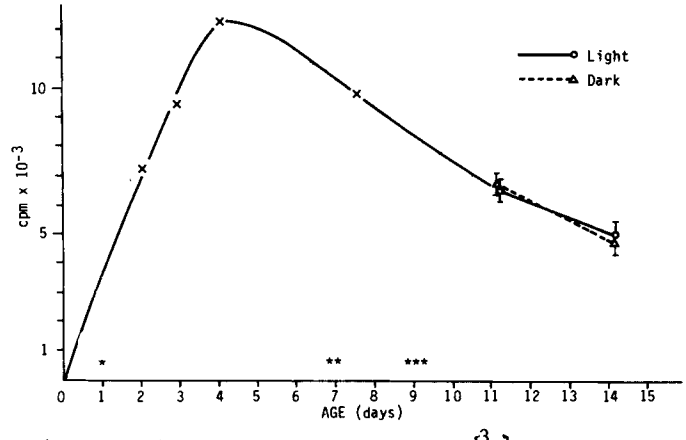

Fig. 4. * Animals were injected with [^{3}H] ManNAc.
** All animals were put in the dark.
*** Half of the animals were exposed to light (1000 lux) and the other half remained in the dark.
Results of animals in light and dark are means of ten determinations.

GLYCOPROTEINS LABELLING IN DARK AND LIGHT

The changes in the labelling of the sialic acid of glycoproteins of optic tecta from chickens maintained in the dark or subjected to 1000 lux after an intraocular injection of [^{3}H] ManNAc are very similar to those observed for gangliosides even if the differences were somewhat smaller (Table 9). In the retina no differences were observed between the two groups of animals.

TABLE 9 Labelling of the Sialic Acid of Glycoproteins from Optic Tecta of Chickens Injected Intraocularly with [^{3}H] ManNAc

Hours after injection	Contralateral Dark	Contralateral Light	p	Ipsilateral Dark	Ipsilateral Light
0.5	n.d.	n.d.		n.d.	n.d.
3.0	(10) 50±2	(10) 60±2	<0.001	(8) 5±0.5	(10) 5±0.5
5.0*	(11) 43±1	(11) 58±1	<0.001	(10) 19±2	(11) 16±2

Figures are given in counts/min/mg of protein and are means ± S.E.M. of the number of determinations given in parentheses. p was calculated by Student's t test.
n.d.: non detectable
* This experiment was carried out with a different batch of chickens than that used for the experiment at 3 h.
From Maccioni A.H.R. and others (submitted)

SURFACE PROPERTIES OF THE GANGLIOSIDE GD1a

Since at least in the chicken GD1a is the quantitatively main ganglioside that is conveyed by axonal transport from the retina to the optic tectum we deemed of interest to comment on some properties of this ganglioside found in our laboratories by Maggio and others (1978a, b). The polysialogangliosides GD3, GD1a and GT1 have a surface potential per molecule of ganglioside significantly higher than monosialogangliosides in monolayers of single components. In monolayers mixed with phosphatidylcholine the polysialogangliosides have the effect of decreasing the surface potential, thus probably increasing the ionic permeability of the membranes (Cumar F.A. and Maggio B., personal communication). This conclusion is in agreement with the finding that GD3, GD1a and GT1 are efficient fusogenic agents for erythrocytes (Maggio and others, 1978c) and stimulate both the uptake and the release of dopamine by synaptosomal preparations from the corpus striatum (Cumar and others, 1978). Other authors (Whatley and others, 1976) have reported the coincidence of GD1a appearance in myoblasts with their fusion into myotubes.

POSSIBLE FUNCTIONS OF GD1a IN THE OPTIC SYSTEM. POSSIBLE MECHANISM OF INFLUENCE OF RETINA IN THE FUNCTIONING OF THE CNS

The observations referred to above should be considered when it is attempted to relate the functioning of retina (and for this purpose perhaps of any other sensorial organ) with the functioning of the CNS. One is the importance to establish precisely the mechanism and the site of formation of any substance in the cell, specially of those substances which are components of the cell membrane. Since by now it appears that the quantitatively main subcellular site of biosynthesis of gangliosides and perhaps of glycoproteins is located in the neuronal perikarya it is important to establish whether the increase of the retina GD1a ganglioside from the

8th day of embryonic development to hatching is correlated with an increase in the development of the axonal processes. If such were the case the possibility that gangliosides are agents for recognition of the corresponding cells in the optic tectum becomes an assumption with distinct probabilities of being correct.
As for the increase in light of labelling of gangliosides and glycoproteins the suggestion is inescapable that this may be a part of the mechanism of memory or any other phenomenon by which a sensorial organ influences the structures of the brain. The subject is in the initial stage of investigation and consequently may be that fundamental changes in concepts will follow, but so far the extra labelling carrying material produced under light was found chromatographically similar to the material produced in the dark, the turnover period of both materials did not differ and they had a distribution in the subcellular fractions of the tectum that were indistinguishable for both materials. Every thing occurred as if the material was an addition to the normal structural in the brain. The concept of a reaction which stimulates the formation of a substance in the neuronal perikarya which by axonal transport is carried to the nerve endings may become a basic concept in the functioning of the nervous system.

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THE LIPID INTERMEDIATE PATHWAY IN THE RETINA FOR THE ACTIVATION OF CARBOHYDRATES INVOLVED IN THE GLYCOSYLATION OF RHODOPSIN

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ABSTRACT

The presence of and many of the properties of enzymes in the retina involved in the biosynthesis of key intermediates in the dolichol pathway of carbohydrate activation has been demonstrated. The formation of dolichol phosphate-mannose and of mannose-containing oligosaccharide-polyprenols, and their use as substrates in further biosynthetic reactions was shown. The biosynthesis of the core-region glucosamine-containing mono, di and tri-saccharide pyrophosphoryl polyprenols, and the novel function of GDP-mannose as a regulator in their biosynthesis, was described. The participation of the dolichol pathway in the glycosylation of rhodopsin was demonstrated by the inhibition of core-region glycosylation of this glycoprotein by the antibiotic, tunicamycin. Preliminary evidence was obtained that glycosylation of rhodopsin was not a requirement for its insertion into disc membranes.

KEY WORDS

Polyprenols; dolichol phosphate-mannose; carbohydrate-activation; rhodopsin; glycosylation; glucosaminyl-polyprenols; glycoprotein biosynthesis; retina; dolichol pathway.

INTRODUCTION

Studies from many laboratories over the past several years have revealed the important role played by lipid-activated carbohydrates in the form of their polyprenol derivatives in the biosynthesis of the core-region saccharide of the asparagine-linked glycoproteins (Waechter and Lennarz, 1976). The means by which this region of the carbohydrate chain of glycoproteins was synthesized resisted understanding until relatively recently. The process of sequential, stepwise transfer of monosaccharide residues from their nucleotide derivatives to acceptors catalyzed by specific glycosyl transferases, served to explain the attachment of peripheral sugars such as sialic acid and galactose (Roseman, 1970). However, the synthesis of the more internally located "core" region oligosaccharide, containing mannose and glucosamine, did not seem to proceed in this manner. During this past decade it has become abundantly clear that the polyprenol phosphate/pyrophosphate sugar derivatives are the crucial intermediates for this process. Derivatives containing

up to 15 sugars have been shown to participate in a complex series of reactions involving synthesis of the polyprenol-oligosaccharide, transfer to the apo-glycoprotein followed by excision of some of the sugar groups (Spiro, Spiro and Bhoyroo, 1976; Kornfeld, Li, and Tabas, 1978). Thus far, while the entire sequence of reactions has been demonstrated in only a limited number of tissues, various phases of the polyprenol-pathway have been demonstrated in over 15 animal tissues. While a considerable amount of information is thus available concerning this biosynthetic pathway, information is still limited concerning a variety of aspects of this process such as demonstrations of its participation in the biosynthesis of well characterized specific secretory and membranous glycoproteins, and information concerning mechanisms which regulate its functioning.

We have addressed ourselves to examining the characteristics of the lipid-intermediate pathway in the retina and its role in the biosynthesis of glycoproteins of this tissue with specific attention ultimately to the biosynthesis of the carbohydrate chains of the glycoprotein rhodopsin. This visual pigment is an integral major component of the membranes of the discs of the rod outer segment. Bovine rhodopsin was shown to contain 9 moles of mannose and 5 moles of glucosamine per mole of visual pigment (Plantner and Kean, 1976a). Recently the structure of the major oligosaccharide chain of rhodopsin was shown to be similar to that of other asparagine-linked glycoproteins, Liang, and co-workers, 1979; Papermaster, Fukuda, and Hargrave, 1979). Studies from our laboratory have investigated the presence in the retina of the pathway for the lipid-activation of carbohydrates and the involvement of this pathway in the glycosylation of rhodopsin (Kean and Plantner, 1976; Kean, 1977a, 1977b, 1977c, 1977d, Kean and Bruner, 1977; Kean, 1979). This report will summarize some of the salient features of these studies which have described many of the properties of this pathway primarily in the retina of the embryonic chick as well as in the retina of other species.

MANNOSYL TRANSFERASES OF THE RETINA

Incubations were performed and enzymatic activities were measured as described previously (Kean, 1977a; Kean, 1977b). In summary, reactions were stopped by the addition of a mixture of cold 6% trichloroacetic acid/0.5% phosphotungstic acid to the incubation mixture. From the pellet which was formed, three products were obtained: Lipid I, the material extracted with a mixture of chloroform/methanol (2:1), and recovered in the chloroform-rich phase after partitioning according to the procedure of Folch, Lees and Sloane-Stanley (1957); Lipid II, the material extracted from the delipidated pellet with a mixture of chloroform/methanol/water (10:10:3) (this fraction contained the oligosaccharide-lipids); Residue, the pellet remaining after Lipids I and II are removed (this fraction contained the endogenous glycoproteins). The radioactivity was measured in each of the fractions by scintillation spectrometry.

While the formation of each of these products was measured in every experiment, this report will deal, in the main, with the biosynthesis by the retina of the low molecular weight carbohydrate-lipid intermediates which are extractable by chloroform/methanol (2:1).

Endogenous Activities

Cell-free preparations obtained from retinas of several species of widely varying phylogeny catalyzed the transfer of mannose from the sugar nucleotide, GDP-(^{14}C)-mannose to material extractable into chloroform/methanol (C/M) (2:1) (Table I). The retina of the embryonic chick was the most active of the species which were examined. Most of the studies characterizing the products of this reaction and the properties of the enzyme systems involved were carried out with homogenates of the retina from the 15 day embryonic chick. When examined as a function of the age of

the embryo, there was little difference in this capacity in retinas obtained from eggs incubated from 9 days until hatching (21 days).

The incorporation of (^{14}C)-mannose into the 3 fractions as a function of time of incubation is seen in Fig. 1. The formation of Lipid I (dolichol phosphate-mannose) was rapid, being complete within 5 min, while the labeling of the other products (Lipid II, the oligosaccharide-lipid; Residue, the glycoproteins) was linear for longer periods of time.

TABLE I Mannosyl Transferases in Retinas from Animals of Different Species*

Retina Source	cpm/g retina/min Lipid
Embryonic chick	32900 ± 2210 (24)
Dogfish	10100 ± 1000 (9)
Squid	14200
Cattle	14900 ± 1890 (4)
Adult chicken	
Hen	16900
Rooster	13400

*Kean and Bruner (1977).

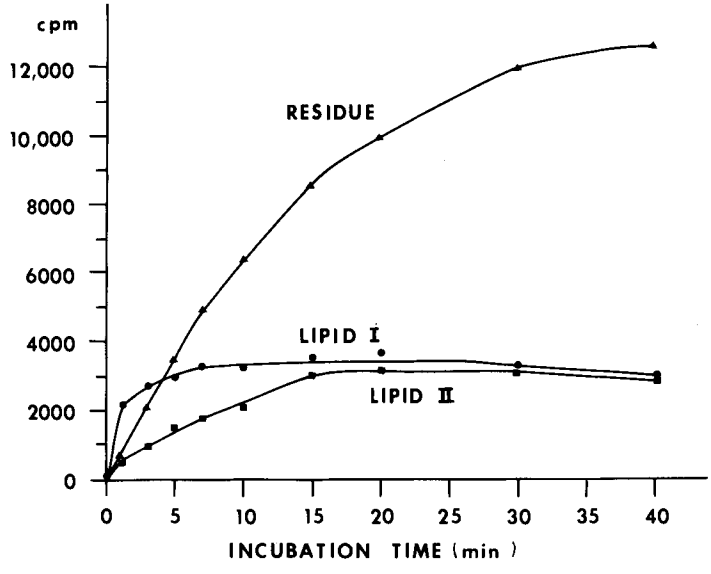

Fig. 1. Time course of the mannosyl transferase reactions (Kean, 1977a)

The only class of mannose-containing lipids that have been described in animal tissues are the polyprenol phosphate derivatives. The mannolipid synthesized by the retina utilizing the lipid acceptors endogenous in this tissue was shown to have the characteristics of dolichol phosphate-mannose, as discussed later.

In addition to mannose, polyprenol derivatives of N-acetylglucosamine and of glucose have also been described, the former being involved in the assembly of the core-region oligosaccharide, and the latter as part of the large oligosaccharide-lipid involved in the processing mechanism (Kornfeld, Li, and Tabas, 1978).

As seen in Table II, UDP-N-acetylglucosamine and UDP-glucose functioned as substrates for the formation of lipid extractable products, in addition to GDP-mannose, although the latter was 6 to 9 fold more active in this regard. The products formed by the retina using UDP-N-acetylglucosamine as the sugar donor have been extensively characterized, as described later, while the products formed from UDP-glucose have not been characterized.

When examined under optimal conditions of pH (0.2 M Tes buffer, pH 7.0) and metal ($MnCl_2$, 3.3 mM), and linearity with time of incubation and protein concentration an apparent K_m of 1.4 μM was obtained for GDP-mannose.

TABLE II Sugar Nucleotide Substrate Specificity (Kean, 1977c)

Radioactive Sugar Nucleotide	Conc. (μM)	pmol sugar incorporated per g wet wt.,/min Lipid
GDP-Mannose	2.6	122
GDP-Glucose	2.6	2.8
UDP-GalNAc	3.1	1.2
UDP-GlcNAc	3.2	22
UDP-Glucuronic acid	2.8	0.06
UDP-Glucose	2.9	14
UDP-Galactose	2.6	2.1
CMP-Sialic acid	3.0	1.8

Polyprenol Phosphate Specificity

As seen above, a relatively high degree of specificity for GDP-mannose as the sugar nucleotide substrate was shown by the retina. When the specificity of the mannosyl transferases of the retina for the lipid acceptor was investigated, an even greater degree of specificity for dolichol phosphate as an exogenous acceptor of radioactivity from GDP(^{14}C)-mannose was demonstrated. As seen in Table III, little or no stimulation of activity over the endogenous level was demonstrated by several long chain polyprenol phosphates or by short chain analogues under conditions in which the presence of dolichol phosphate resulted in about an 18 fold stimulation.

TABLE III Specificity for Polyprenyl Phosphates as Acceptors by Mannosyltransferases of Retina (Kean, 1977a)

Compound Added	Conc.	Radioactivity in Lipid-I	Lipid-II	Residue
	μM		cpm	
None		4,140	668	1,190
Dolichyl phosphate	19	76,800	1,850	3,360
Citronellyl phosphate	16	3,770	745	1,140
Ficaprenyl phosphate	21	5,130	790	1,310
Farnesyl phosphate	22	3,490	680	1,170
Geranyl phosphate	22	4,120	660	1,180
Phytyl phosphate	19	3,860	700	1,140
Phytanyl phosphate	21	4,290	882	993
Retinyl phosphate	22	2,980	596	758
Undecaprenyl phosphate	17	3,930	772	903

Of considerable interest was the lack of activity shown by retinol phosphate. Although the latter compound has been shown to act as an acceptor of mannose in liver (DeLuca, and co-workers, 1973), the role of the product, retinol phosphate-mannose, in glycoprotein biosynthesis is not clear.

The requirement by the retina for dolichol phosphate as exogenous acceptor was more rigorous than that displayed by other tissues, such as calf pancreas (Tkacz, and co-workers, 1974), calf thyroid (Adamany and Spiro, 1975) or pig liver (Richards and Hemming, 1972). When examined as a function of variation in concentration of dolichol phosphate, saturation kinetics were observed, as can be seen in Fig. 2. From these studies an apparent K_m for dolichol phosphate was calculated to be 7.3 μM (Kean, 1977a).

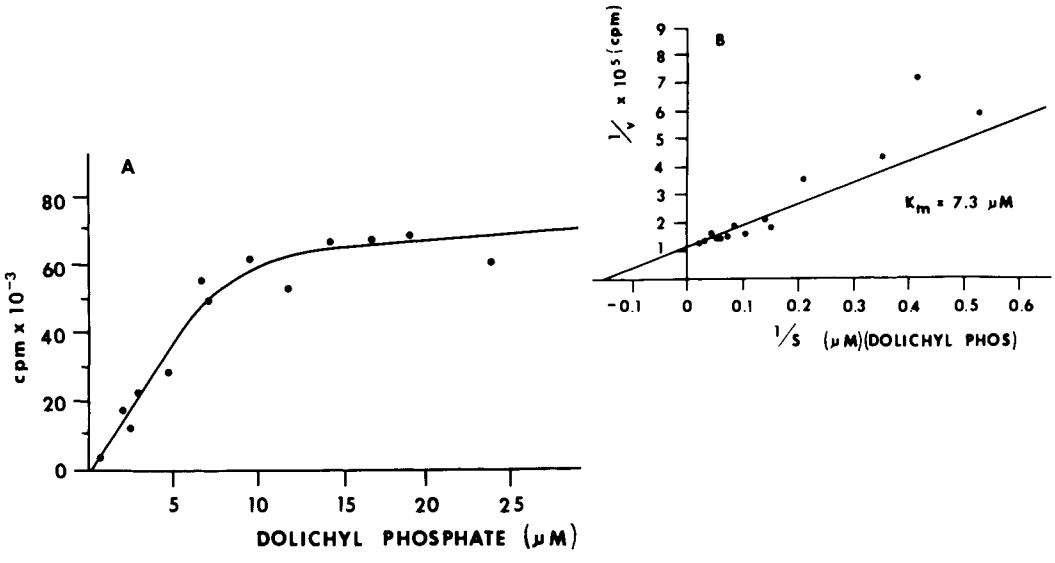

Fig. 2. Effect of the concentration of dolichyl phosphate on the rate of Lipid-I-mannosyltransferase of the retina.

In addition to stimulating the incorporation of mannose into the C/M (2:1) extractable fraction, or Lipid I, the presence of dolichol phosphate also resulted in a 2 to 3 fold stimulation of the labeling of the oligosaccharide-lipid fraction (Lipid II) and the glycoprotein-containing Residue.

Identification of Products

Dolichol phosphate-mannose. Large scale incubations were carried out in the presence and absence of exogenously added dolichol phosphate. The chloroform/methanol (2:1)/fraction from these preparations were treated in an identical manner. After purification by DEAE cellulose acetate chromatography, followed by silicic acid chromatography, the products formed from the endogenous lipid acceptors was identical to that formed in the presence of added dolichol phosphate although the latter was greatly increased in amount. The chromatographic properties on the above adsorbants as well as thin layer chromatography in several solvent systems in addition to acid and base stability characteristics of these products were similar to one another and to that of authentic dolichol phosphate-mannose. The anomeric configuration of the mannose linkage, investigated by chemical and enzymatic means, was shown to be β.

The latter characteristic, observed also in the product synthesized by thyroid (Adamany, and Spiro, 1975) and pancreas (Herscovics, Warren and Jeanloz, 1975) would be consistent with its role as a donor of α-mannose residues in the assembly

of the oligosaccharide-lipid, but not serving as a donor of the β-mannosyl unit of the trisaccharide-lipid core region. Although the lipid component has not been identified, all of these characteristics are consistent with the product synthesized by the retina being dolichol-β-D-mannosyl phosphate.

Since the characteristics of the product synthesized from the endogenous acceptor and from dolichol phosphate were identical, it is suggested that the endogenous lipid acceptor in the retina is in fact dolichol phosphate.

Mannose-containing oligosaccharide-lipids and glycolipids. The mannose-oligosaccharide-lipid, Lipid II, the product extracted from the delipidated pellet with C/M/water (10:10:3), had the chromatographic properties on DEAE cellulose acetate, paper chromatography and thin layer chromatography similar to the mannose-oligosaccharide-pyrophosphate-lipids described in other tissues (Adamany and Spiro, 1975; Lucas, Waechter, and Lennarz, 1975; Chambers and Elbein, 1975).

After Lipids I and II were removed, the radioactivity in the residual pellet was shown to be associated with glycoproteins. The only radioactive component was mannose, as revealed after strong acid hydrolysis. The mannose was shown to be present in large molecular weight material which was converted to radioactive glycopeptides by the action of pronase.

Polyprenol-mannose Derivatives as Substrates

Dolichol-(^{14}C)-β-mannosyl phosphate, either synthesized in situ or added exogenously served as substrates for the transfer of (^{14}C)-mannose to the oligosaccharide-lipids and to endogenous glycoproteins. Shown in Fig. 3 are the results of experiments in which dolichol phosphate-(^{14}C)-mannose, previously prepared by large scale incubations and extensively purified by DEAE cellulose chromatography and silicic acid chromatography, was the substrate for the mannosyl transferases of the retina. As a function of time of incubation, the radioactivity in this compound decreased accompanied by the increased labeling of the oligosaccharide-lipid and glycoprotein fractions.

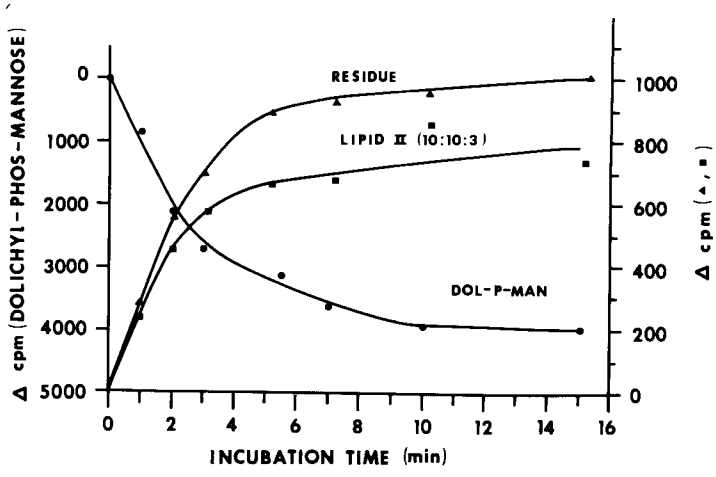

Fig. 3. Dolichyl phosphate (^{14}C)mannose as substrate (Kean, 1977a)

Similarly, when purified Lipid II (the oligosaccharide-lipid) was present as the sole source of radioactivity, the retina also catalyzed the transfer of (^{14}C)-mannose to endogenous glycoproteins. Thus, the retina contained the enzymatic capacity for the formation of the polyprenol-activated carbohydrates and the transferases required for their use in further biosynthetic reactions.

Subcellular Localization of the Lipid-mannosyltransferases

The distribution of the mannosyl transferases active with the endogenous lipid acceptors was examined in preparations of the retinas from the embryonic chick and from the dogfish. Essentially the same results were obtained with both species. Fractions were obtained based on the procedures of differential centrifugation described for rat liver by Schneider and Hogeboom, 1950, and are thus operational in nature.

Since one cannot distinguish whether lipid-acceptor or enzyme is limiting from studies of this type, the data obtained is qualitative in nature. Although the overall yields in enzymatic activities varied in different experiments, of the recovered mannolipid-transferase activity, about 70% was associated with the pellet sedimented at low centrifugal speed (750 x g) i.e., the fraction containing nuclei and cell debris. Less than one-third of this was associated with the microsomal fraction. This is in contrast to the microsomal location of these enzymes as reported by others (Waechter, Lucas, and Lennarz, 1973; Tkacz, and co-workers, 1974; Adamany and Spiro, 1975) and the rough endoplasmic reticulum (Czichi and Lennarz, 1977). In order to test the nuclear location of the mannosyltransferase further, we attempted to purify nuclei from the embryonic chick retina. However, techniques applicable to other tissues for this purpose when used with the embryonic retina did not result in nuclei of sufficient yield for these experiments. Recently, however, Richard, Tytgat and Louisot, 1978, described the presence of the lipid-mannosyl transferases in nuclei purified from rat liver. The important question of the subcellular location of these enzymatic reactions is still not resolved, however, since it is not clear from any of these studies what the distribution, in quantitative terms is of these enzymatic activities in the various subcellular compartments of the cell.

N-ACETYLGLUCOSAMINYL TRANSFERASES: BIOSYNTHESIS OF THE CORE REGION TRISACCHARIDE

The trisaccharide sequence, Man$\overset{\beta}{\text{---}}$GlcNAc$\overset{\beta}{\text{---}}$GlcNAc is a common feature of the asparagine-linked glycoproteins. As shown by several laboratories, GDP-mannose is used as a substrate for its formation, not dolichol phosphate-mannose (Chen and Lennarz, 1976; Heifetz and Elbein, 1977; Waechter and Harford, 1979; Levy and coworkers, 1974). Studies from this laboratory have described the biosynthesis by the retina of the embryonic chick of the lipid intermediates involved in the biosynthesis of the core-region trisaccharide (Kean, 1979). The biosynthesis of GlcNAc-P-P-polyprenol, GlcNAc-GlcNAc-P-P-polyprenol and Man$\overset{\beta}{\text{---}}$GlcNAc-GlcNAc-P-P-polyprenol were demonstrated. It is onto the trisaccharide-lipid unit that additional GlcNAc and mannose units are attached, as well as glucose before the assembled oligosaccharide-lipid is transferred to the acceptor nascent glycoprotein (Kornfeld, Li, and Tabas, 1978). A novel observation was made during these studies whereby the N-acetylglucosaminyl transferases concerned with the biosynthesis of the above GlcNAc-lipids was greatly stimulated by the presence of GDP-mannose (Kean, 1979 and manuscript submitted for publication). The stimulation was shown not to be due to GDP-mannose merely acting as a substrate for the formation of the mannose-containing product and pulling the reaction in the direction of its synthesis. Rather, the incorporation of (^{3}H)-GlcNAc into GlcNAc-lipid and GlcNAc-GlcNAc-lipid were enhanced 2 and 5 fold respectively, compared to the labeling of the trisaccharide-lipid. Thus, GDP-mannose acted as a modifier of the activities of the GlcNAc-lipid transferases, in addition to acting as a substrate in mannosyl transferase reac-

actions, an influence which may be an aspect of metabolic regulation of the biosynthesis of the core region oligosaccharide.

THE LIPID INTERMEDIATE PATHWAY AND THE GLYCOSYLATION OF RHODOPSIN

The experiments described above demonstrate the presence in the retina of key enzymes required for the pathway of lipid activation of mannose and glucosamine. While dolichol phosphate-mannose and the mannose-oligosaccharide lipid were synthesized by the retina and were shown to act as substrates in the transfer of mannose to endogenous glycoprotein acceptors, it is of importance to demonstrate the participation of the lipid-intermediate pathway in the glycosylation of specific glycoprotein by this tissue. The most well characterized glycoprotein of the retina is rhodopsin. Previous studies (Kean and Plantner, 1976) using cell-free preparations of bovine retina demonstrated the transfer of mannose from GDP(^{14}C)-mannose to a variety of endogenous acceptors. Little of the labeled sugar was found in visual pigment, however.

Using the intact retina, *in vitro*, the incorporation of glucosamine and of mannose into rhodopsin has been demonstrated (O'Brien and Mullenberg, 1973; O'Brien, 1977). Adapting this system, we have investigated the glycosylation of rhodopsin under conditions which inhibit the assembly of the lipid-oligosaccharide intermediate required for glycosylation of the asparagine-linked glycoproteins. The antibiotic, tunicamycin, has been shown to act in this manner (Tkacz and Lampen, 1975). In cell-free preparations of the retina, tunicamycin was shown to block completely the transfer of GlcNAc from the sugar nucleotide to glucosaminyl-lipids (Kean, 1979). Bovine retinas were incubated in the presence of either (^{3}H)-mannose or (^{3}H)-glucosamine, in the presence or absence of tunicamycin. Rod outer segments were purified from these retinas by sucrose floatation and sucrose density centrifugation as described by Papermaster and Dreyer (1974). Rhodopsin was extracted with Emulphogene BC 720 and purified by adsorption chromatography on columns of calcium phosphate-Celite, as described previously (Plantner and Kean, 1976a), followed by preparative isoelectric focusing (Plantner and Kean, 1976b). In retinas incubated in the presence of tunicamycin, the incorporation of mannose into purified rhodopsin was inhibited greater than 90%, and the incorporation of glucosamine was inhibited about 70% compared to the controls. Attachment of glucosamine to core-region sites, i.e., de novo glycosylation as contrasted to peripheral labeling, was completely blocked by the antibiotic (Plantner, Poncz and Kean, manuscript submitted for publication). These effects with this relatively specific inhibitor strongly implicate the pathway of lipid activation of carbohydrates as the means whereby the biosynthesis of the carbohydrate chains of rhodopsin occurs.

While tunicamycin extensively blocked the glycosylation of rhodopsin isolated from the rod outer segments, it had relatively little effect on protein synthesis. In experiments in which retinas were incubated in the presence of ^{35}S-methionine, there was about 30% inhibition of its incorporation into rhodopsin. Newly synthesized apo-rhodopsin molecules, i.e., those labeled with ^{35}S-methionine, were thus synthesized in the presence of tunicamycin and as described above, were essentially devoid of carbohydrate. From these preliminary studies we can infer that glycosylation of rhodopsin is not a requirement for its insertion into the disc membranes of the rod outer segment.

The role of the carbohydrate groups on this glycoprotein in the assembly and turnover of the disc membranes is under further investigation.

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Neurochemistry Vol. 1, pp. 69-80.
Pergamon Press Ltd. 1980. Printed in Great Britain.

ELECTROPHYSIOLOGICAL RESPONSES RELATED TO THE PIGMENT EPITHELIUM AND ITS INTERACTION WITH THE RECEPTOR LAYER

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ABSTRACT

The standing potential (SP) of the eye is generated mainly at the apical surface of the pigment epithelium, where tight junctions between adjacent cells create a high resistance membrane. It can be recorded indirectly by means of the electrooculogram (EOG) or directly by recording between electrodes connected to the cornea and the forehead. Changes in illumination provoke slow SP changes in the form of damped oscillations with a frequency of about 2/hour. These oscillations are abolished or substantially reduced in pigment epithelial diseases, such as retinitis pigmentosa and vitelline macular degeneration.

The c-wave of the electroretinogram (ERG) represents the hyperpolarization mainly of the apical membrane of the pigment epithelial cells that occurs in response to a decrease in potassium ion concentration in the extracellular space of the receptor layer induced by light stimulation. When repeatedly recorded the amplitude of the c-wave oscillated with time in the same way as the SP. The c-wave is abolished or markedly reduced in pigment epithelial disorders.

The negative h-wave of the off-ERG shows such extensive similarities to the c-wave of the on-ERG that it seems to represent the reversal of the processes in the pigment epithelium - receptor complex underlying the c-wave.

KEYWORDS

Retina; pigment epithelium; ocular electrophysiology; standing potential of the eye; electroretinography; c-wave; off-effects; animal experiments; clinical methods.

INTRODUCTION

When the retina is stimulated with light a series of biochemical and electrophysiological reactions are initiated. Some of the electrical responses are generated in the neuroretina, whereas others are also closely related to the pigment epithelium, which interdigitates through processes with the retinal photoreceptor outer segments. The study of these responses has contributed to the basic understanding of the function of the retina and the pigment epithelium and the interaction bet-

ween these two layers. Electrophysiological tests are also used in clinical ophthalmology as valuable aids in the diagnosis of retinal and pigment epithelial diseases.

THE STANDING POTENTIAL OF THE EYE

In vertebrates the cornea is positive in relation to the posterior pole of the eye. This potential difference is called the standing potential (SP) of the eye, discovered by du Bois-Reymond (1849). Experimental evidence indicated that the SP was generated mainly at the level of the pigment epithelium (Arden and Kelsey, 1962; Gouras, 1969; Heck and Papst, 1957; Noell, 1953, 1954). When advancing a microelectrode through the retina Brindley (1956) observed a layer of high electrical resistance, the R membrane. He proposed that the major part of the SP was generated across this membrane. The location of the R membrane was found to be behind the retina (Brindley and Hamasaki, 1963; Tomita, Murakami and Hashimoto, 1960), and Cohen (1965) proposed that the tight junctions (zonulae occludentes) between the pigment epithelial cells corresponded to the R membrane. It now seems generally accepted that the apical surface (facing the photoreceptors) of the pigment epithelial cells together with the tight junctions constitute the morphological basis of the R membrane. The passive and active transport mechanisms related to this barrier were studied extensively by Steinberg and Miller (1973) and by Miller and Steinberg (1977 a, b).

The Electrooculogram (EOG)

SP variations can be recorded indirectly in human as the electrooculogram (EOG) by using a rather uncomplicated technique (Arden, Barrada and Kelsey, 1962; François, Verriest and de Rouck, 1955). The eye is rotated voluntarily from side to side at a certain angle. The potential variations created when the eye, being a dipole, is rotated like this, are picked up by electrodes at each canthus (Fig. 1).

Recording is performed first in the dark and then in the light. Figure 2 shows that the potential variations decrease in the dark (dark trough) and increase in the light (light rise). The relation between the light rise and the dark trough (the 'Arden ratio') (Arden, Barrada and Kelsey, 1962), which is used in clinical tests, is pathologic in diseases affecting the pigment epithelium, e.g. in retinitis pigmentosa (Fig. 3) and vitelline macular degeneration. The increase and decrease in the SP during EOG registration, initiated by turning on or off the light, respectively, were found to be cyclic and slow, about 2/h (Kolder, 1959; Kris, 1958; Täumer, Hennig and Pernice, 1974).

Direct SP Recordings

A method for a more direct recording of the human SP between the cornea and the forehead, without using eye movements, was developed in our laboratory (Nilsson and Skoog, 1975; Skoog 1975). Very stable calomel electrodes were connected to a suction contact lens on the eye and to reference and ground chambers on the forehead by saline-agar bridges in polyethylene tubes (Fig. 4). The signals were fed into low-drift d.c. amplifiers.

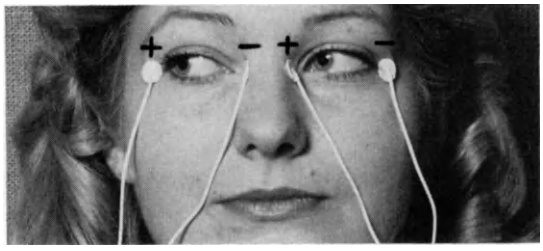

Fig. 1. Arrangement for EOG recording.

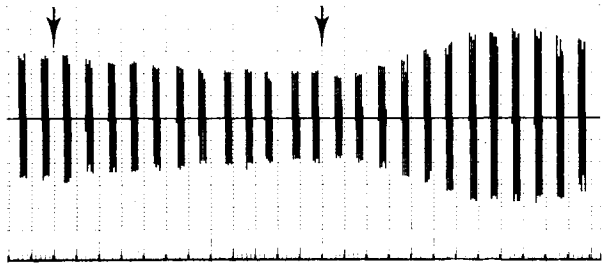

Fig. 2. Normal human EOG. Arrows indicate turning off and on the adapting light, respectively. Time calibration: 1 min intervals.

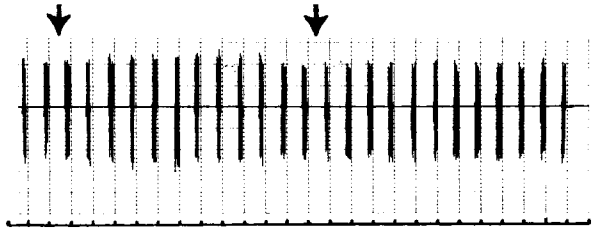

Fig. 3. 'Flat' EOG in retinitis pigmentosa.

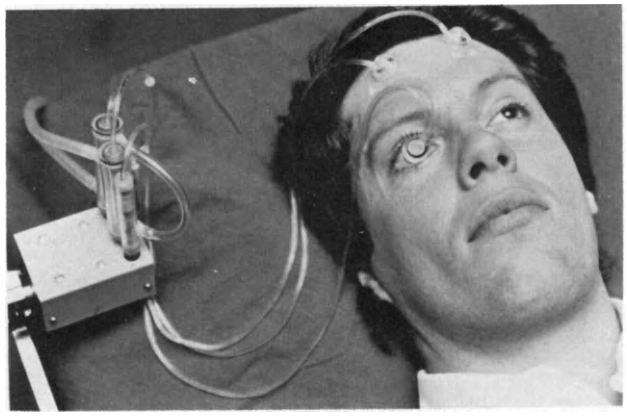

Fig 4. Arrangement for direct SP registration.

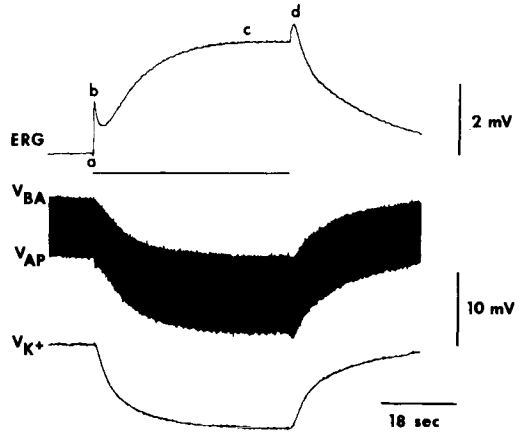

Fig. 8. Light evoked responses in the frog. Upper recording: ERG recorded across a retina - pigment epithelium - choroid preparation (vitreal - positive). Middle recording: the pigment epithelial cell membrane potentials, recorded between an intracellular microelectrode and either basal (V_{BA}) or apical V_{AP}) reference electrodes. Lower recording: differential potential from a double barrel K^+-specific microelectrode located approximately 10 µm vitreal to the pigment epithelium. Reproduced from Oakley and co-workers (1977), with permission.

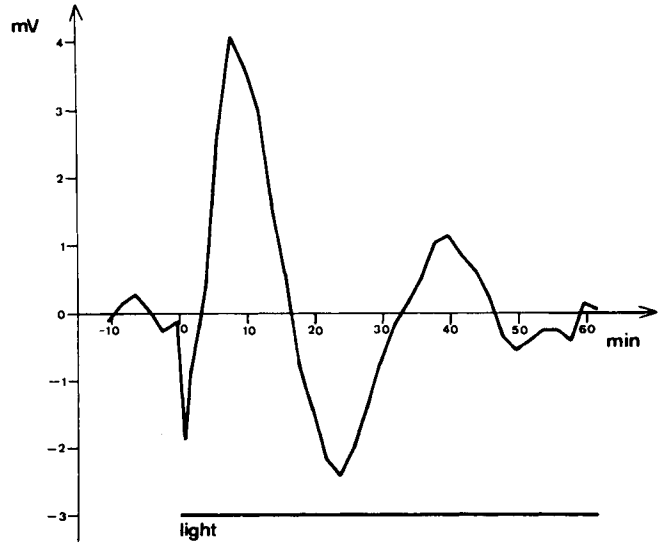

Fig. 5. SP oscillations in response to a change in illumination from darkness to 16 Lux. Reproduced from Skoog (1975), with permission.

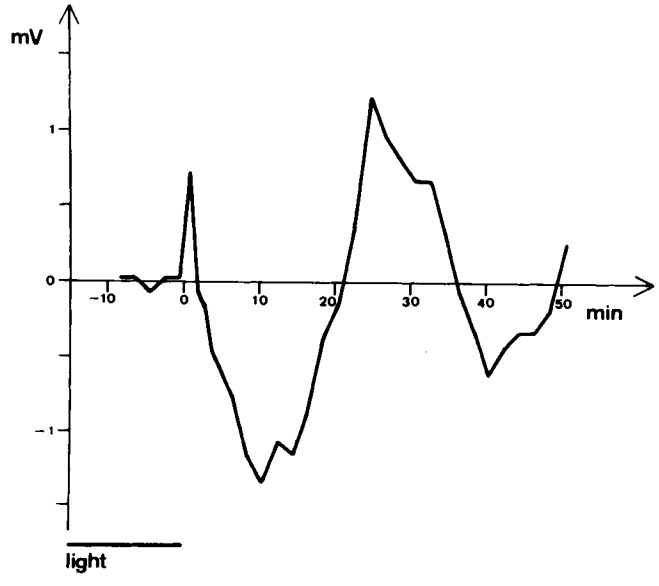

Fig. 6. SP oscillations in response to a change in illumination from 1100 Lux to darkness. Reproduced from Skoog (1975), with permission.

Turning on the light after dark adaptation initiates slow and damped SP oscillations with a frequency of about 2/h and with a maximum amplitude of approximately 5 mV (Skoog, 1975). A faster, negative transient precedes the slower changes, the first and major peak of which is positive (Fig. 5). A change from light to darkness provokes the same kind of SP oscillations, but with completely reversed polarity (Fig. 6). Like the slow EOG variations also these SP oscillations are markedly reduced or abolished in pigment epithelial diseases. Flashes of light (Nilsson and Skoog, 1975) and, unexpectedly, ethyl alcohol, taken orally (Skoog, Textorius and Nilsson, 1975), gave rise to the same type of response as turning on a steady light.

The mechanisms behind the SP oscillations are still unknown, but we suspect that metabolic processes in the pigment epithelium are involved. However, it seems that also factors outside the pigment epithelium influence upon the oscillations, since it has been shown (Textorius, Skoog and Nilsson, 1978) that experimental occlusion of the central retinal artery reduced markedly or abolished the light induced slow SP oscillations (Fig. 10).

THE c-WAVE OF THE ELECTRORETINOGRAM (ERG)

The electroretinogram (ERG) consists of a series of rather fast potential variations, the most prominent of which are the negative a-wave and the positive b- and c-waves (Fig. 7). These waves, which arise when the eye is stimulated with flashes of light, are superimposed upon the very much larger and slower SP variations. The a-wave reflects mainly the activity of the photoreceptor cells and the b-wave the computer part of the neuro-retina. The a- and b-waves are fast enough to be recorded with the ordinary a.c. technique, but for the slower c-wave it is necessary to employ the more complex d.c. technique (Nilsson and Skoog, 1975).

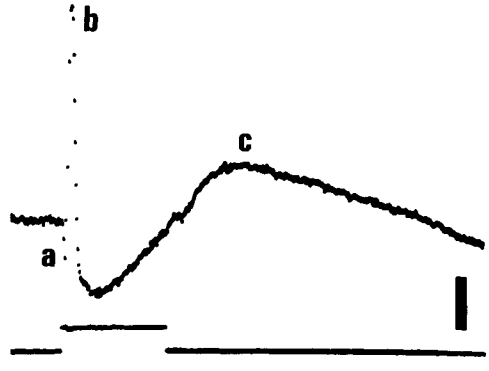

Fig. 7. The d.c. recorded human ERG in response to a 1 sec stimulus. Stimulus intensity 4.5 log rel. units above b-wave threshold. Amplitude calibration 100 μV.

Animal Experiments

The c-wave is generated primarily in the pigment epithelium and closely related also to the activity of the photoreceptor cells. Noell (1954) demonstrated that the c-wave was abolished by sodium iodate. Light (Noell, 1954) and electron (Grignolo,

Orzalesi and Calabria, 1966) microscopy showed that the pigment epithelium was damaged by sodium iodate more than and prior to the neuroretina. Nilsson, Knave and Persson (1977 a, b) found that after sodium iodate injection there was definitely a period of time, when the c-wave was abolished and the pigment epithelium severely damaged, but when the a- and b-waves were still unaffected and the neuroretina ultrastructurally normal. Brown and Wiesel (1961) demonstrated that the amplitude of the c-wave of the local ERG was maximal when the electrode was placed adjacent to the pigment epithelium.

Steinberg, Schmidt and Brown (1970) and Schmidt and Steinberg (1971) showed that the intracellular response from the pigment epithelium was rod-dependent and identical to the c-wave of the local ERG. It was found by Oakley and Green (1976) that light induced a decrease in extracellular potassium ion concentration in the receptor layer, and that this decrease had the same time course as the c-wave of the ERG. In experiments on the frog Oakley and co-workers (1977) and Oakley (1977) could demonstrate that simultaneously with the c-wave and the decrease in extracellular potassium the apical as well as the basal membrane of the pigment epithelial cells hyperpolarized, but the apical one significantly more than the basal one, and that this difference in hyperpolarization between the two membranes corresponded in amplitude to the c-wave of the ERG (Fig. 8).

It thus appears that absorption of photons by the rods leads to a movement of potassium ions from the extracellular space, probably into the rods, and that this decrease in potassium causes a hyperpolarization mainly of the apical membrane of the pigment epithelial cells, which is recorded as the c-wave of the ERG.

Other slow potentials, simultaneous with the c-wave, such as slow P III (Granit, 1947; Noell, 1953, 1954), generated proximal to the receptor layer (Hanitsch, 1973; Murakami and Kaneko, 1966; Murakami and Sasaki, 1968; Pautler, Murakami and Nosaki, 1968), most likely by a Müller cell response to the earlier mentioned decrease in extracellular potassium ion concentration (Oakley, 1977; Witkovsky, Dudek and Ripps, 1975) may modify the positive c-wave, however (Faber, 1969; Oakley 1977; Rodieck, 1972). In studies by Textorius (1978), Textorius, Nilsson and Skoog (1978) and by Textorius, Skoog and Nilsson (1978) it was shown that occlusion of the central retinal artery, supplying the inner retina, caused a decrease in c-wave amplitude, slight but significant at the early stage, and markedly at the late stages (Fig. 10). These findings were surprising and further complicate the full understanding of the c-wave. The ion fluxes through the retina may be altered.

The c-wave of the Human ERG

The human c-wave (Fig. 7) has been studied in detail in our laboratory as to methodological development (Nilsson and Knave, 1974; Nilsson and Skoog, 1975), amplitude and stimulus intensity relations (Skoog and Nilsson, 1974 a) and disturbances caused by superimposed off-potentials (Textorius, 1977). Furthermore, it was shown that the amplitude of the human c-wave, when repeatedly recorded, oscillated with time in the same way as the SP, i.e. with a frequency of approximately 2/h (Fig. 9) (Nilsson and Skoog, 1975, 1976; Skoog and Nilsson, 1974 b). These c-wave oscillations were earlier observed also in animal experiments (Calissendorff, Knave and Persson, 1974; Knave and co-workers, 1973). Thus, one c-wave cannot be compared meaningfully to another c-wave, unless both are taken from identical phases of the oscillations. Many of the contradictory results in earlier reports may be explained by the fact that these amplitude oscillations were unknown (Textorius, Skoog and Nilsson, 1978).

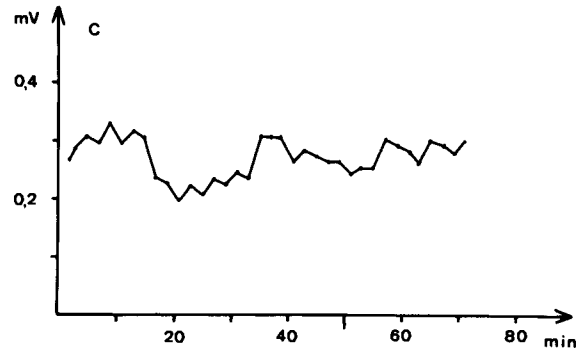

Fig. 9. Slow cyclic changes of the amplitude of the c-wave of the human ERG on repeated stimulations and registrations. Reproduced from Skoog and Nilsson (1974), with permission.

Like the SP oscillations also the c-wave amplitude oscillations were abolished in experimental occlusion of the central retinal artery (Textorius, Skoog and Nilsson, 1978) (Fig. 10). Ingestion of ethyl alcohol provoked oscillations of the c-wave amplitude of the same type as those induced by light flashes, although larger in

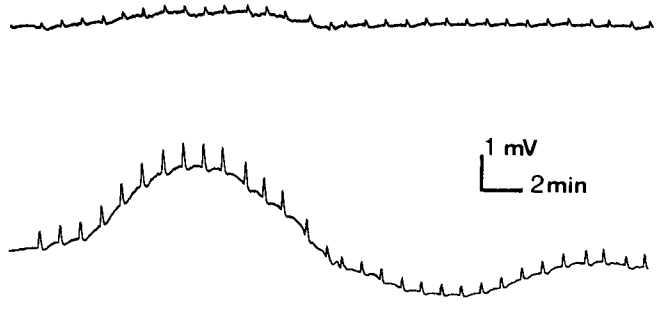

Fig. 10. Variations in the SP and the c-wave amplitude with time in response to repeated light stimuli (1 stimulus per min) at a late stage (35 days) of occlusion of the central retinal artery (upper trace) and in the healthy control eye (lower trace). ERG:s mainly seen as c-waves, superimposed upon the SP traces. Stimulus duration 1 sec. Stimulus intensity 45 cd/m^2. Reproduced from Textorius, Skoog and Nilsson (1978), with permission.

magnitude (Skoog, 1974). Thus, many of the characteristics of the c-wave are also typical of the SP, which shows that the two potentials are closely related to each other and to the pigment epithelium. Furthermore, it has been shown in animal experiments that c-wave amplitude oscillations were elicited also by a number of drugs with melanin affinity, such as chloroquine (Calissendorff, 1976; Knave and co-workers, 1973).

Fig. 11. The d.c. registered human ERG in vitelline macular degeneration. Stimulus duration 4 sec. Stimulus intensity 4.0 log rel. units above b-wave threshold. Amplitude calibration 100 μV. Time calibration 1 sec.

In d.c. registrations of the ERG in clinical cases the c-wave is found to be markedly reduced or abolished in for example retinitis pigmentosa and vitelline macular degeneration (Fig. 11). In the former case the a- and b-waves are not seen or are very small, whereas in the latter case they are normal or slightly reduced. The described electrophysiological tests: EOG, direct SP registration and d.c. ERG, have proved to be most valuable in the diagnosis of many diseases concerning the retina and the pigment epithelium, above all in children and in hereditary cases, where the ophthalmoscopic picture may be difficult to interpret.

THE h-WAVE OF THE OFF-ERG

The potential variations occurring in the human ERG after cessation of an adapting light (with a duration of 20 sec or more) were studied by Skoog, Welinder and Nilsson (1977). A series of waves appeared at 'off': a fast positive d-wave and a fast negative f-wave were followed by a slower positive g-wave and a slow negative h-wave. The h-wave at 'off' showed the same time course as the c-wave at 'on'. The h-wave amplitude also oscillated with time when repeatedly recorded as responses to 'dark flashes' (Welinder, 1979) and after ingestion of ethyl alcohol (Nilsson, Skoog and Welinder, 1978; Skoog, Welinder and Nilsson, 1978). It is therefore suggested as a working hypothesis that the h-wave of the 'off-ERG' represents the reversal of the processes generating the c-wave of the 'on-ERG', i.e. an increase of the potassium ion concentration in the extracellular space of the receptor layer upon cessation of light, in turn causing a depolarisation mainly of the apical membrane of the pigment epithelial cells. More basic work is needed before the h-wave can be tried in routine clinical tests, however.

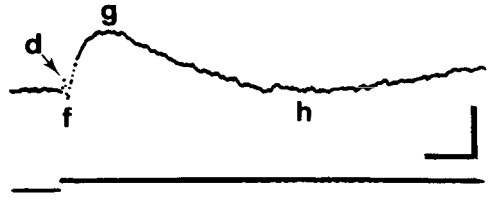

Fig. 12. Off-responses of the human d.c. registered ERG after the termination of an illumination of 60 Lux. Average of two recordings. Amplitude calibration 100 μV. Time calibration 1 sec. Reproduced from Skoog, Welinder and Nilsson (1978), with permission.

The present paper described three potentials that are related to the activity of the pigment epithelium and its interaction with the retinal photoreceptors: the standing potential of the eye, the c-wave of the on-ERG and the h-wave of the off-ERG.

ACKNOWLEDGEMENT

These investigations were supported by grants from the Swedish Medical Research Council, Project No. 12X-734.

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Neurochemistry Vol. 1, pp. 81-92.
Pergamon Press Ltd. 1980. Printed in Great Britain.

CONTROL OF ROD SHEDDING IN THE FROG RETINA

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ABSTRACT

In all vertebrate species examined thus far, rod outer segment shedding follows a cyclic pattern in which the outer segment tips are shed shortly after the onset of light. Work in the rat retina suggests that rod shedding may follow a circadian rhythm which is controlled by one or more circadian oscillators. Our results in the frog retina are significantly different in that: rod shedding can be driven by the onset of light or other environmental cues; shedding does not persist in constant darkness; shedding is unaffected in frogs with chronic unilateral or bilateral optic nerve section; and shedding will rapidly phase shift to the time of light onset on a wide variety of diurnal cycles. Thus, rod shedding in the frog retina does not appear to be a classical circadian rhythm.

KEY WORDS

Retina, photoreceptor, rod outer segment, shedding, phagosome, circadian rhythm.

INTRODUCTION

The outer segments of vertebrate rod and cone photoreceptors are renewed throughout the life of the animal (reviewed in Young, 1976). Renewal occurs through the assembly of new disc membranes at the base of the outer segment and a carefully balanced disposal mechanism in which the tips of the outer segments (phagosomes) are shed, engulfed and digested by the pigment epithelium. The shedding of rod and cone outer segments has been observed in a number of vertebrate species (reviewed in Hollyfield and Basinger, 1978a), but to date the most complete information on shedding is available for rod photoreceptors in the retinas of rats and frogs (LaVail, 1976; Basinger, Hoffman and Matthes, 1976; Hollyfield, Besharse and Rayborn, 1977; Besharse, Hollyfield and Rayborn, 1977; Basinger, 1978; Currie, Hollyfield and Rayborn, 1978; Hollyfield and Basinger, 1978a, 1978b; LaVail and Ward, 1978; Tamai *et al.*, 1978; Goldman, O'Brien and Teirstein, 1979; Hollyfield and Rayborn, 1979; Teirstein, Goldman and O'Brien, 1979).

Work by LaVail and co-workers (1976, 1978) has shown that in the rat retina, rods shed their outer segment tips in a cyclic pattern shortly after light onset and may follow a circadian rhythm which persists in constant darkness. Work in O'Brien's

laboratory (Goldman, O'Brien and Teirstein, 1979; Teirstein, Goldman and O'Brien, 1979) has confirmed the apparent circadian nature of rod shedding in the rat retina and has further suggested that one or more circadian oscillators may exist which control rod shedding, possibly via the optic nerve.

Our work with adult *Rana pipiens* has shown that rod outer segment shedding follows a diurnal cycle with shedding occuring shortly after the onset of light. However, rod shedding is virtually eliminated in frogs maintained for short periods of time (up to one week) under conditions of constant dark or constant light. This evidence, and the fact that shedding can be independently initiated in either eye, suggests that rod shedding in the frog retina may not follow a true circadian rhythm but instead be driven by the onset of light or other environmental cues (Basinger, Hoffman and Matthes, 1976; Currie, Hollyfield and Rayborn, 1978; Basinger, 1978; Hollyfield and Basinger, 1978a, 1978b). The results presented here clearly demonstrate that rod shedding in the frog retina is tightly entrained to the onset of light, is unaffected by chronic optic nerve section, and can be initiated by an increase in temperature in the absence of a light cue.

METHODS

Adult *Rana pipiens* were maintained at room temperature in large plexiglass bins with a constant supply of running tap water and were fed crickets weekly. Lighting was provided by full spectrum ceiling fluorescent lamps and was approximately 450 lux at the floor of the frog bins. Automatic timers turned the lights on at 0800 hours and off at 2200 hours, a light cycle of 14L:10D. In some experiments, frogs from the 14L:10D cycle were placed under a variety of altered lighting cycles by housing in constant temperature incubators (22°) illuminated at 450 lux by full spectrum fluorescent lights. The incubators were equipped with programmable timers to turn the lights on and off. In selected experiments, the incubator temperature, instead of the light, was controlled by the timers to cycle between 15° and 26° while the incubator was maintained dark.

Frogs were killed by decapitation at selected intervals during the various cycles, and the retinas were processed for light microscopy as previously described (Basinger, Hoffman and Matthes, 1976). Rod shedding was quantitated in 0.5-1.0 μ plastic-embedded sections, and shedding is expressed as the number of newly shed phagosomes per 100 ROS.

Optic nerves were sectioned in frogs under MS-222 anesthesia. The nerves were sectioned through the roof of the mouth approximately midway between the eye and the optic chiasm. The incision was closed with sutures, and the frogs were maintained under normal cyclic lighting for at least five weeks before they were used in the experiments described below.

Rhodopsin bleaching was determined by difference spectra in 0.04M CTAB extracts of whole retinas on a Cary 118 recording spectrophotometer.

RESULTS

In frogs maintained on a diurnal lighting cycle of 14L:10D, synchronous shedding of new phagosomes occurs between 60 and 90 minutes after the onset of light, and no further shedding takes place during the remainder of the diurnal cycle. Rod shedding is temperature dependent (reflecting the temperature dependence of disc synthesis and assembly), and is normally about 20% per day in frogs maintained at 20° - 22°.

One useful way to study the nature of various biological rhythms is to alter the environmental cycles to which the animals have been entrained. Therefore, to explore the relationship between shedding and light onset, we examined the shedding pattern of frogs maintained on a variety of altered lighting cycles in which the time of light onset was advanced or delayed. We first examined the effect of acute, short-term, alterations in the time of light onset. In this experiment, frogs previously maintained for at least three months on the 14L:10D lighting cycle were presented with light onset either eight hours earlier (14L:2D) or eight hours later (14L:18D). The results of this experiment are shown in Fig. 1, and for comparison, the shedding pattern of frogs on the normal 14L:10D cycle is shown at the top.

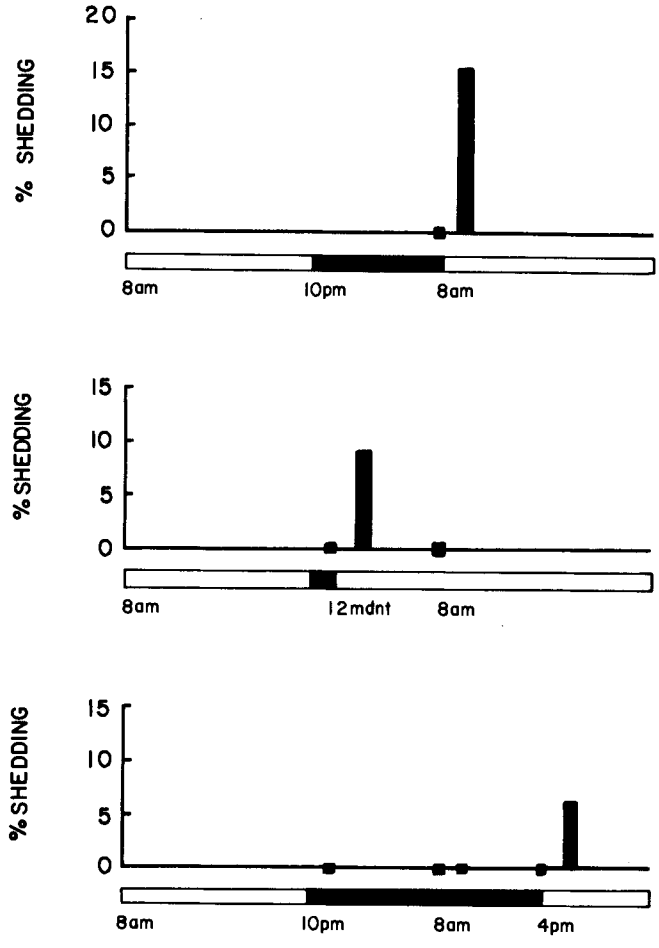

Fig. 1. Effect of short-term alterations in the time of light onset on rod shedding. The shedding pattern of frogs entrained for 2-3 months to a 14L:10D lighting cycle are shown at the top. When light onset is either shortened by 8 hours to 14L:2D (middle) or lengthened by 8 hours to 14L: 18D (bottom), rod shedding occurs only after light onset. Histograms show the extent of rod shedding at the indicated times.

As seen in Fig. 1, normal shedding occurred only after light onset when the light phase was either shortened or lengthened by eight hours. Under both conditions, there was no shedding at the time when shedding had occurred the previous day. Instead, even after months of entrainment to the 14L:10D cycle, shedding was tightly linked to the onset of light. Thus, an immediate forward or backward phase shift of eight hours had no effect on shedding. The apparent attenuation of the shedding response in the 14L:18D frogs (bottom) is within the normal range for frogs maintained at this temperature.

Figure 2 shows a light micrograph from one of the frogs examined after light onset in the 14L:2D lighting cycle. Note the presence of newly shed phagosomes immediately above the rod outer segments on the margins of this section, and the presence of older, more basally displaced phagosomes in the central portion of the micrograph. The older phagosomes were those shed after light onset the previous morning, and they have not yet been completely digested. This micrograph is also useful in demonstrating the difference between newly shed phagosomes and older ones, a characteristic which makes the frog particularly useful for these types of studies.

With no effect observed for an acute phase shift of eight hours, we then examined the shedding pattern of frogs on three significantly different lighting cycles. In this experiment, frogs previously entrained for at least three months to the 14L:10D lighting cycle were placed on three new cycles: 8L:8D, 12L:12D, and 24L:24D. After five days adaptation to the new lighting cycles, the relationship between rod shedding and light-onset was examined. As shown in Fig. 3, on each of the three new cycles, rod shedding was found to occur only at the time of light onset. This again shows that the shedding response will rapidly shift to a new time even when there are large phase shifts in the lighting cycle. Note also that the cumulative amount of shedding which occurred over the three day period was the same in all three cycles (about 55%).

Even though the frogs on the shortest cycle had twice as many shedding responses (four) as the frogs on the longest cycle (two), each shedding response of the 8L:8D frogs eliminated only half as much outer segment material as that of the 24L:24D frogs. This emphasizes how sensitive the shedding response is in controlling rod outer segment length.

Preliminary evidence from O'Brien's laboratory suggested that rod shedding in the rat retina might be mediated in part via the optic nerve (Goldman, O'Brien and Teirstein, 1979; Teirstein, Goldman and O'Brien, 1979). One of us had previously examined the immediate effect of optic nerve section on the shedding response in the frog retina and found no effect (Currie, Hollyfield and Rayborn, 1978). To further investigate the possible role of the optic nerve in controlling the shedding response, shedding was examined in frogs after chronic optic nerve section. Frogs previously entrained for at least three months on the 14L:10D lighting cycle were unilaterally (left optic nerve) or bilaterally sectioned and then maintained on the 14L:10D cycle for five weeks. All the frogs recovered from surgery and appeared normal, although the bilaterally sectioned frogs became somewhat darker, probably due to loss of inhibitory control of the hypothalamic output of melanophore-stimulating hormone.

Five weeks after unilateral or bilateral optic nerve section, the rod shedding response was examined and compared with a control group. All optic nerve sections were verified at this time.

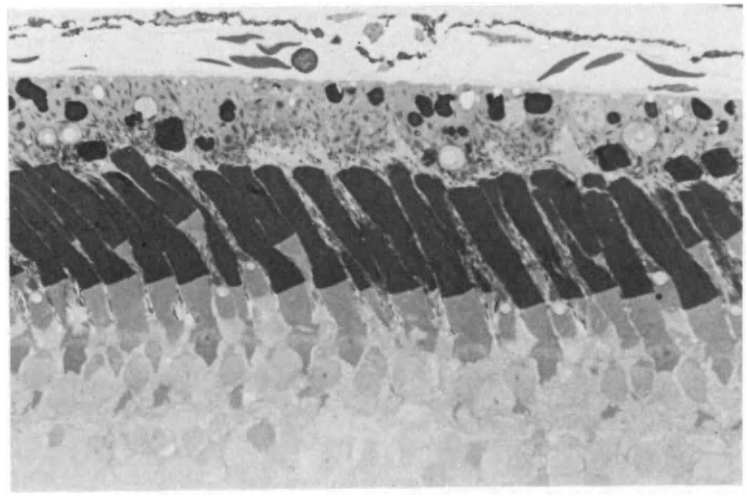

Fig. 2. Light micrograph of the photoreceptors and pigment epithelium of one of the frogs from the 14L:2D cycle. Two layers of phagosomes appear in the pigment epithelium, a layer of old phagosomes in the basal portion which were shed the previous day, and a layer of newly shed phagosomes in the apical portion adjacent to the tips of the rod outer segments. Bar = 10 microns.

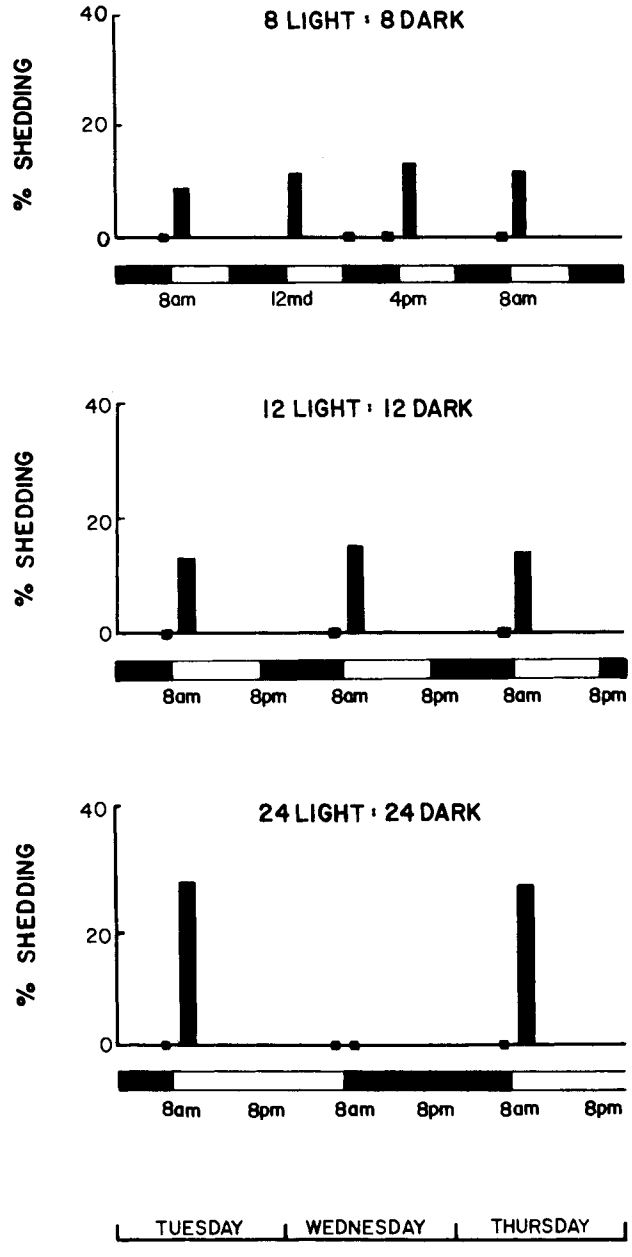

Fig. 3. Rod shedding response after five days of adaptation to three phase shifted lighting cycles. The period of each lighting cycle is indicated above the responses. The histograms show the extent of rod shedding at the indicated times. Total duration of the experiment is shown at the bottom.

TABLE 1 Effect of Chronic Optic Nerve Section on Shedding

Condition*	Percent Shedding	
	Left Eye	Right Eye
Unsectioned	18.9 ± 1.8	20.3 ± 5.4
Left Optic Nerve Sectioned	17.4 ± 3.2	14.1 ± 5.9
Both Optic Nerves Sectioned	18.5 ± 1.3	14.6 ± 2.6

*After surgery, frogs were maintained under normal diurnal lighting conditions for 5 weeks. Shedding was quantitated in frogs killed 90 minutes after light onset. Three frogs from each group were kept dark to serve as controls. No shedding occurred in any of these animals.

Table 1 shows that a normal shedding response was seen in all animals. No significant differences in shedding were seen when the unilaterally or bilaterally sectioned animals were compared to controls, nor was there any difference between the sectioned and unsectioned eyes of the unilaterally sectioned animals. Thus, in the frog retina, the presence of an intact optic nerve is not necessary for the normal expression of the shedding response, and severing the optic nerve does not alter the link between light onset and rod shedding.

In many species, particularly lower vertebrates, periodic, diurnal rhythms can be entrained to environmental cues other than light, for example temperature (Menaker, Takahashi and Eskin, 1978). To test this possibility for rod shedding we compared frogs kept in constant darkness at a constant temperature of 22^{o} to frogs kept in constant darkness with a cyclic temperature of 14 hours at 26^{o} and 10 hours at 15^{o}. The frogs were first adapted for three weeks to these respective conditions, and were then examined at regular intervals over the course of 24 hours for evidence of synchronous rod shedding.

As shown at the top of Fig. 4, the frogs maintained in constant darkness at a constant temperature showed only random shedding throughout the 24 hour period, a pattern typical of frogs maintained in constant darkness for periods up to four weeks. Low levels of random shedding are found at all time intervals examined, but there is no evidence of a burst of shedding entrained to a particular time of day. However, as shown in the bottom portion of Fig. 4, the frogs maintained in constant darkness in the presence of a 14:10 temperature cycle showed a normal shedding response coincident with the time of the temperature increase. Virtually no shedding occurred at any of the other time periods examined. Thus, in the absence of a light cue, frogs can use another environmental cue to synchronize their shedding response.

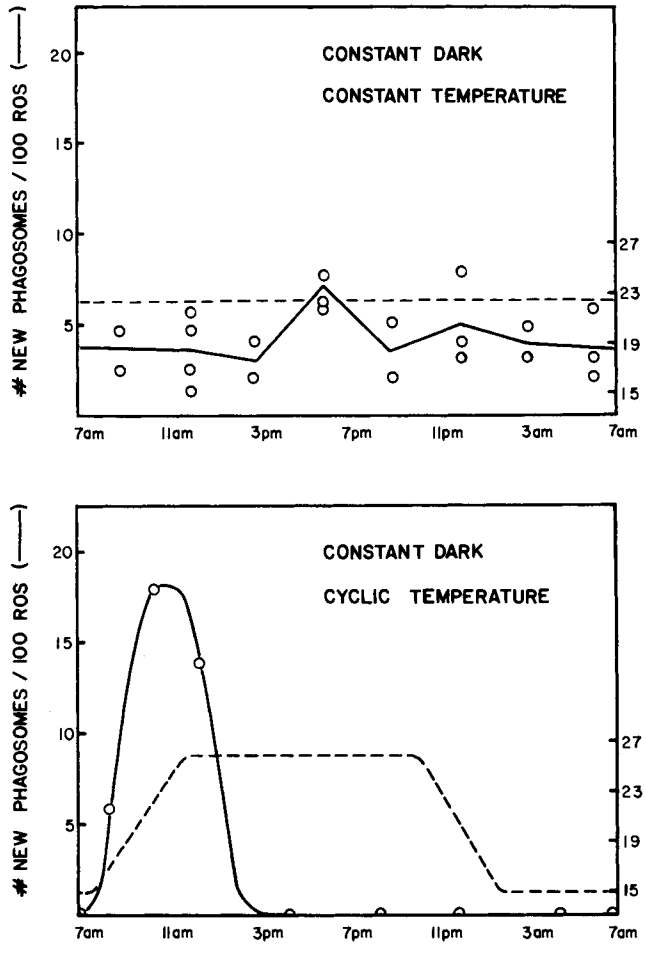

Fig. 4. A comparison of the shedding response in frogs maintained in constant darkness and kept at either a constant temperature of 22° (top) or at a cyclic temperature of 14 hrs at 26° and 10 hrs at 15° (bottom). The dotted line shows the temperature and the circles and solid line the percentage of newly shed phagosomes at the indicated times.

The strong dependence of the shedding response on light onset led us to investigate the amount of light required to initiate shedding. A useful way to do this is to compare the extent of shedding with the amount of rhodopsin bleach in the retina.

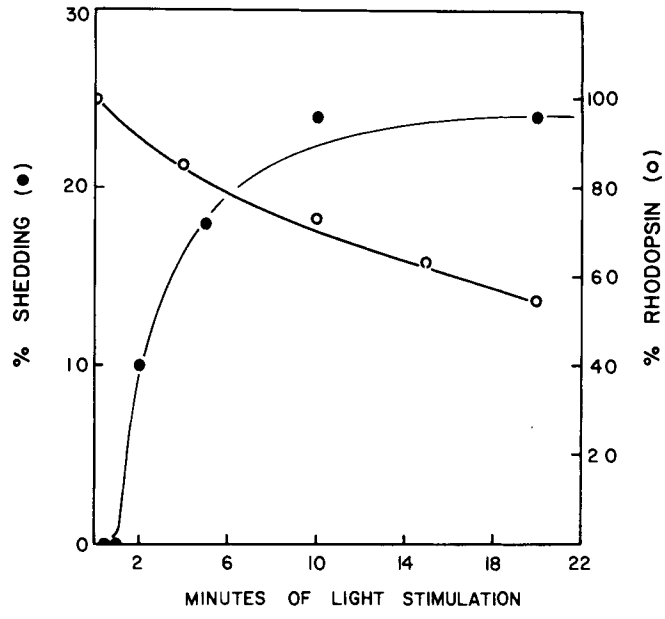

Fig. 5. Rod outer segment shedding as a function of rhodopsin bleach. Filled circles are the shedding response, and open circles are the amount of rhodopsin bleached as determined by difference spectrum.

This relationship is shown above in Fig. 5, and perhaps surprisingly, shows that a significant rhodopsin bleach was required to initiate shedding (about 5-10% bleached), and the full shedding response was not expressed until almost a 20% bleach had occurred. In physiological terms, this amount of bleaching would increase the electroretinogram threshold between one and two log units, and thus would certainly be considered a physiologically significant level of illumination.

DISCUSSION

A widely accepted definition of a circadian rhythm is one which is cued but not driven by environmental stimuli. Work in the laboratories of LaVail and O'Brien indicates that rod outer segment shedding in the rat retina may fit this definition. However, our previous work and the results presented here strongly suggest that rod shedding in the adult frog retina is tightly linked to ("driven by") the onset of light. Most well-characterized circadian rhythms have two additional characteristics: they persist in constant darkness, usually with a "free running" period of approximately 24 hours; and when presented with large phase advances or delays, the animals adapt rather slowly to the new cycle. Neither of these two additional criteria are met by rod outer segment shedding in the frog retina. Instead, rod shedding in the frog adapts quickly and completely to a variety of phase shifted lighting cycles, and shows only random, non-rhythmic shedding in constant darkness, even on the first day. Thus, rod shedding in the frog retina should not be considered a classical circadian rhythm.

The experiment in which the normal rod shedding response became entrained to an increase in temperature rather than light onset raises some significant questions about the molecular mechanisms involved in the rod shedding response, for example, the apparent requirement of light. Previous experiments in our laboratories have clearly demonstrated that light is not absolutely required to initiate shedding. Frogs placed for 2-3 weeks in constant darkness have a constant background shedding response of about 3-5%. Under conditions of constant light, virtually no shedding takes place except for occasional large bursts (20-40%) of shedding which appear to occur at random. Thus, for normal rod shedding, the requirement for a dark period appears equally important to the need for light stimulation, and of course the two together provide sufficient conditions to maintain normal shedding. However, we must tentatively conclude that the mechanism which entrains and initiates the shedding response is sensitive to a variety of external cues, including light, dark, and temperature. The localization of this mechanism to within the retina is suggested by the absence of any effect on the shedding response after optic nerve section, and our demonstration that shedding can be initiated independently in either eye (Hollyfield and Basinger, 1978b).

Finally, under normal cyclic light conditions, the amount of light required to initiate the full shedding response results in a significant rhodopsin bleach. Previous results by one of us (Basinger, 1978) suggested that light in the green part of the visible spectrum was most effective in initiating shedding, but as yet no intensity versus time relationship nor action spectrum has been determined for the rod shedding response. Relating the shedding response to the electrophysiology of the visual process remains an important, and as yet undone, experimental study.

In summary, the rod shedding process and its associated control mechanisms are essential factors in outer segment development and renewal, and the maintenance of a normal healthy retina absolutely depend upon these factors. Learning the details of the molecular events which underlie a process through which shedding is synchronously initiated in millions of outer segments each day will be an important step toward our basic understanding of membrane function and retinal disease.

ACKNOWLEDGEMENTS

This investigation was supported by grants from the Retina Research Foundation, Houston, Texas; the Retinitis Pigmentosa Foundation, Baltimore, Maryland; and grants EY 01406 and EY 02363 from N.I.H. The authors are recipients of Research Career Development Awards from the National Eye Institute. We thank Rosemary Hoffman, Luis Marroquin and Michael Matthes for expert technical assistance. As always, special thanks to Kermit.

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Neurochemistry Vol. 1, pp. 93-101.
Pergamon Press Ltd. 1980. Printed in Great Britain.

RETINAL DEVELOPMENT: TIME AND ORDER OF APPEARANCE OF SPECIFIC NEURONAL PROPERTIES

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ABSTRACT

The high affinity uptake, biosynthesis and K^+-stimulated release of certain neurotransmitter candidates was studied in adult and developing retinas of *Xenopus laevis*. In the adult retina, ^{3}H-GABA was accumulated predominantly by horizontal cells while ^{3}H-glycine and ^{3}H-dopamine were accumulated by cells located deeper in the inner nuclear layer (possibly a type of amacrine or interplexiform cells). This retina also synthesized GABA, dopamine and acetylcholine from their precursors supplied exogenously. Furthermore, adult retinas preloaded with ^{3}H-GABA, ^{3}H-glycine and ^{3}H-dopamine, released these transmitters in response to increased K^+-concentration in the medium. We have determined the time of appearance and maturation of these properties during embryonic development. With GABA, the appearance of the high affinity uptake system appeared first, preceding GABA synthesis which was followed by the development of K^+-stimulated transmitter release mechanism. Similary, ^{3}H-glycine uptake appeared several stages before its release. In the case of dopamine, however, its biosynthesis occurred first, followed by the development of the high affinity uptake system and finally the release mechanism appeared. The initiation of this sequence of events for the three transmitter systems studied occurred at different developmental stages: the GABA-ergic properties appeared first, followed shortly by the glycinergic properties, which preceded the dopaminergic properties.

KEYWORDS

Retinal development; neurotransmitter release; neurotransmitter synthesis; neurotransmitter uptake; GABA; glycine; dopamine; *Xenopus laevis*.

INTRODUCTION

Studies of neuronal differentiation using morphological techniques have largely been confined to descriptions of the time of appearance of various synaptic configurations as well as the emergence of specific neuronal shapes during neurogenesis. Biochemical studies on the other hand, have documented the time course of synthesis of putative neurotransmitters and their enzyme systems, and in a few cases the appearance of high affinity uptake of the transmitters or their precursors. Most of these studies have been performed independently with little or no attempts to correlate biochemical data with morphological changes. We have recently begun to study the appearance and maturation of specific neuronal properties in the developing retina of *Xenopus laevis*

utilizing a combination of morphological, physiological and biochemical techniques. The primary thrust of our studies has been to determine the interrelationship between the onset of neurotransmitter synthesis with the development of mechanisms for its release as well as inactivation by high affinity uptake systems. In this paper we summarize some of our recent findings on the temporal and spatial sequence in the appearance of biosynthesis, high affinity uptake and release of GABA, glycine and dopamine during retinal differentiation.

MATERIALS AND METHODS

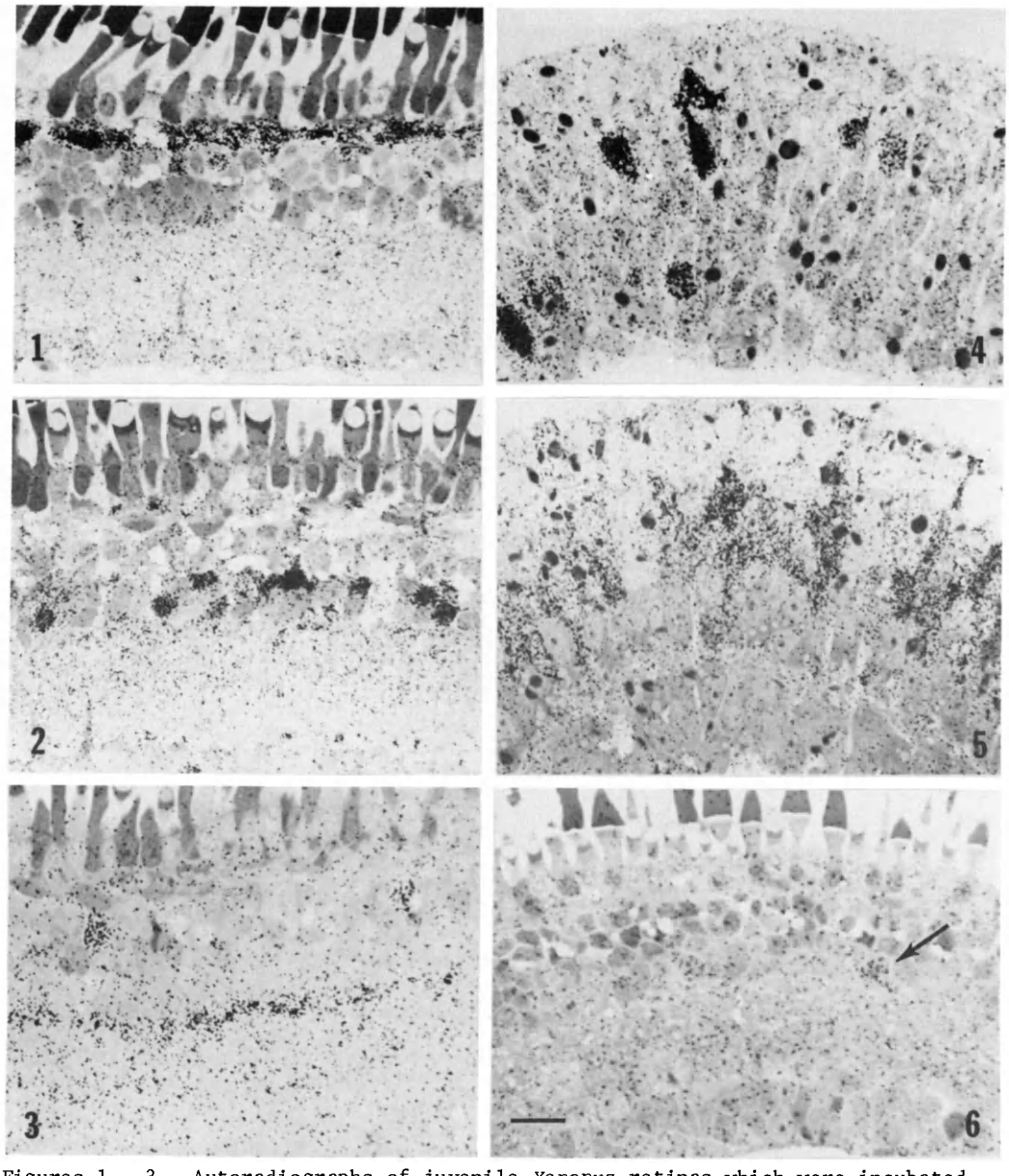
Laboratory spawned embryos, staged according to Nieuwkoop and Faber (1957) were used in these studies. Autoradiographic evaluations of the high affinity uptake system were based on the ability of glutaraldehyde to cross-link compounds containing free amino groups into cells. Eye rudiments were dissected from embryo stages 27 through 45 and were incubated for 10 to 30 minutes with either ^{3}H-GABA (100μCi/ml, 44 Ci/mMol), ^{3}H-glycine (100μCi/ml, 44 Ci/mMol) or ^{3}H-dopamine (100μCi/ml, 6.3 Ci/mMol). The retinas were washed with double strength Niu-Twitty solutions for 2 minutes and fixed on ice for 10 minutes in 2% glutaraldehyde in 50mM phosphate buffer at pH 7.2 and then post-fixed in 1% OsO_4 for an additional 20 minutes. Tissues were then routinely processed and embedded in Epon. Sections were cut at 1μm thickness and processed for light microscope autoradiography according to previously described methods (Hollyfield, Besharse and Rayborn, 1977). Transmitter synthesis was determined by incubating freshly dissected eyes in a mixture of the appropriate labeled precursor followed by transmitter separation of products using high voltage electrophoresis as previously described (Hildebrand and others, 1971).

K^{+}-stimulated release of neurotransmitters was performed using procedures modified from Sarthy and Lam (1979). Ten to twenty eyes were incubated for 15 minutes in 2X Niu-Twitty solution containing either ^{3}H-GABA, ^{3}H-glycine or ^{3}H-dopamine. After three rinses in 5 ml of medium (10 minutes per wash) two eyes were removed for determination of radioactivity and the rest were transferred to graduated conical tubes containing 10 ml of medium. After 10 minutes the medium was replaced with 2 mls of fresh media. Aliquots of 1.9 ml were withdrawn at 2 minute intervals mixed with aquesol and the radioactivity determined. Fresh medium was added to make up the 2 ml volume. At appropriate times the eyes were exposed to 2X Niu-Twitty medium containing 56mM K^{+}. Radioactivity in the eyes was counted at both the beginning and the end of the release experiments. Release data are expressed as percentage of counts per minute in the eyes before the first K^{+}-stimulation.

RESULTS

Uptake, synthesis and release of transmitter candidates in mature retinas. "Mature" retinas were obtained from tadpoles (stage 55-57) and juvenile toads which had gone through metamorphosis one to two months earlier. In autoradiographs, cells which accumulate large amounts of ^{3}H-labeled transmitter candidates were characterized by heavy deposits of silver grains over cell bodies located at characteristically different positions within the retina. ^{3}H-GABA was taken up by cells located along the sclerad border of the inner nuclear layer in a position occupied by horizontal cells (Fig. 1). In studies to be reported elsewhere we have demonstrated with EM autoradiography that these cells do indeed represent horizontal cells as evidenced by the presence of labeled processes in the outer plexiform layer which indent the synaptic base of both rod and cone photoreceptors (Hollyfield and others, 1979). The GABA-ergic horizontal cells are encountered at a frequency of 30.8±2.7 (Mean ± Standard deviation) cells per mm linear retinal expanse.

The cells which accumulate ^{3}H-glycine were located at deeper levels within the inner



Figures 1 - 3. Autoradiographs of juvenile *Xenopus* retinas which were incubated with ^{3}H-GABA (Fig. 1), ^{3}H-glycine (Fig. 2) and ^{3}H-dopamine (Fig. 3). Note distinctly different populations of cells which accumulate these putative transmitters.

Figures 4 - 6. Autoradiographs of developing *Xenopus* retinas at stages when high affinity uptake systems for putative neurotransmitters first make their appearance during retinal differentiation. ^{3}H-GABA labeled cells are first present at stage 31 (Fig. 4), ^{3}H-glycine labeled cells are first apparent at stages 33/34 (Fig. 5) and ^{3}H-dopamine labeled cells (arrow) are first present at stage 43 (Fig. 6). All micrographs at same magnification. Bar in Fig. 6 represents 20µm.

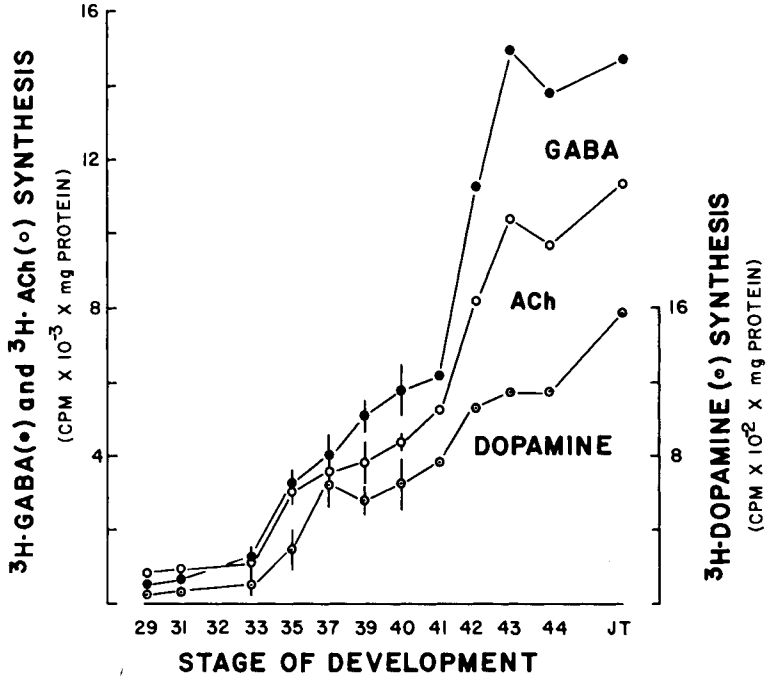


Figure 7. Synthesis and accumulation of ACh, GABA and dopamine by embryonic eyes during development. 18-20 eyes were dissected from embryos and incubated with ^{3}H-labeled choline, glutamic acid and tyrosine for 2 hrs at room temperature. The transmitters were separated by high voltage electrophoresis and counted for their radioactivity.

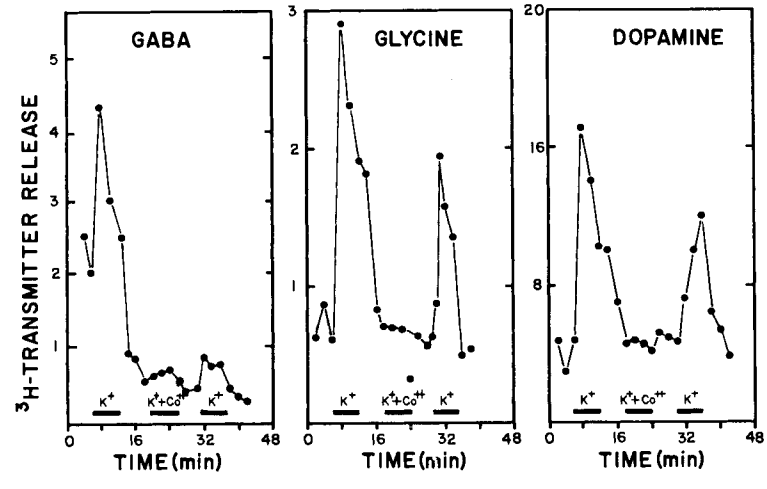


Figure 8. K^+-stimulated release of ^{3}H-GABA, -glycine and -dopamine from mature retinas. Isolated retinas were preloaded with ^{3}H-labeled transmitters, washed and exposed to three pulses of 56mM K^+. The second pulse contained 10mM Co^{2+}. Radioactivity in the eluates were monitored by scintillation counting. Ordinate values are in cpm X 10^{-3} per ml medium.

nuclear layer (Fig. 2). Though we do not know precisely the cell type which accumulates ^{3}H-glycine, they are probably neurons and not glial cells, since grain counts over cells which can clearly be identified as Müller cells (by their angular nuclear profiles and slightly darker staining affinities) have only background levels of radioactivity in tadpole and adult retinas. These glycine accumulating cells in the juvenile toad retina are present at a frequency of 99±13.2 cells per mm linear retinal expanse.

The cells which accumulate ^{3}H-dopamine were located at the vitread border of the inner nuclear layer and were relatively few in number (2.1±1.9 cells per mm linear retinal expanse). When appropriate exposure of autoradiographs was performed a continuous band of radioactivity was also observed in the inner plexiform layer in close proximity to the inner nuclear layer (Fig. 3).

Neurotransmitter synthesis was studied in isolated retinas incubated with ^{3}H-labeled choline, glutamic acid and tyrosine. The products were separated and identified as described by Hildebrand and others (1971). As shown in Fig. 7, retinas from post-metamorphic *Xenopus laevis* synthesized and accumulated significant amounts of ACh (11,300 cpm/mg protein), GABA (14,700 cpm/mg protein) and dopamine (1,580 cpm/mg protein). ACh, GABA and dopamine synthesis increased linearly during the first 3 hr of incubation. The synthesis data shown in Fig. 7 was determined following a 2 hr incubation.

An important property of a mature neuron is its capacity to release its neurotransmitter by a Ca^{++}-dependent, K^+-stimulated mechanism. As shown in Fig. 8, ^{3}H-GABA, ^{3}H-glycine and ^{3}H-dopamine accumulated by juvenile toad retinas could be released by increasing the K^+-concentration (56mM) in the medium. In each case, this release mechanism is probably Ca^{++}-dependent since this function is almost completely abolished in the presence of 10mM Co^{++}. Moreover, a subsequent pulse of K^+ in the presence of Ca^{++} and without Co^{++} stimulates release of a significant amount of the labeled transmitter.

Uptake, synthesis and release of transmitter candidates during retinal development. Heavy labeling of developing retinal cells occurred earliest following incubations with ^{3}H-GABA. Prior to stage 31, no specific labeling of retinal cells was noted, but at stage 31 or 32 some cells in the retinal neuroepithelium showed specific accumulation of ^{3}H-GABA (Fig. 4). In contrast, the first stage at which specific localization of ^{3}H-glycine was observed occurred later at stage 33/34 embryos (Fig. 5). Although some stratification of retinal cells into the three nuclear lamina was seen at this stage, very little elaboration of the neuropile layer was evident. ^{3}H-dopamine uptake was not evident until much later at stage 43 when silver grain densities were elevated above background levels over a few cell bodies located in the inner nuclear layer. However, it was not until stage 44 to 45 (Fig. 6) that relatively high amounts of ^{3}H-dopamine label was evident on cell bodies that were located along the vitread border of the inner nuclear layer. At this stage the frequency of occurrence of these cells and their location was identical to the distribution and number of cells which take up ^{3}H-dopamine in the tadpole and adult retinas.

The time course of biosynthesis and accumulation of the putative transmitter candidates ACh, GABA and dopamine in developing retinas is presented in Fig. 7. Of the three putative transmitters examined in the uptake studies, the synthesis of GABA and dopamine alone were studied during development. In addition, we also followed the synthesis of ACh during development. We found that the onset of synthesis of the three putative transmitters occurred contemporaneously sometime between stages 33/34 and 35/36.

The release of GABA, glycine and dopamine was studied by monitoring the radioactivity in washes from embryonic retinas preloaded with radioactive transmitters (Fig. 9). Although the uptake of GABA was found to occur at stage 31, glycine at stage 33/34

and dopamine at stage 43, release of these transmitters did not occur until stage 37 for GABA, stage 43 for glycine and stage 46 for dopamine, respectively. In addition, release of these transmitters from preloaded tadpole and juvenile toad retinas is Ca^{++}-dependent.

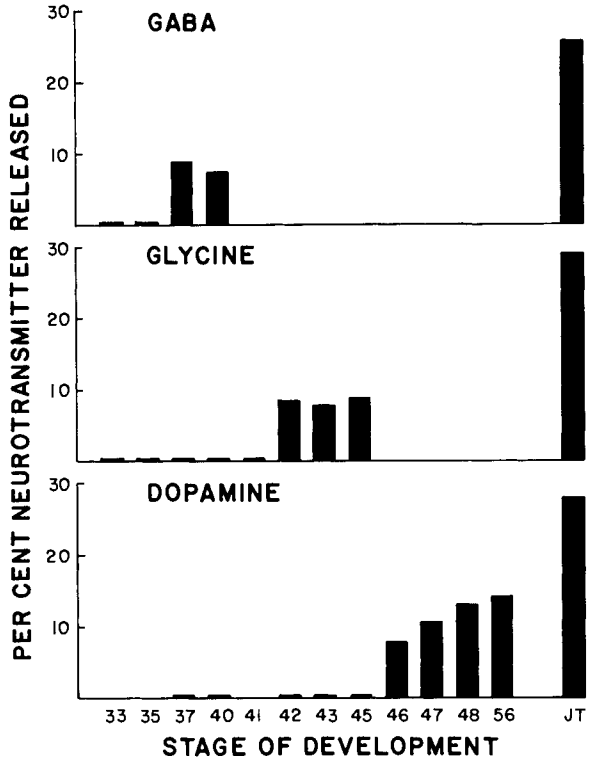


Figure 9. Release of preloaded transmitters from embryonic eyes by 56mM K^+. Freshly dissected eyes were incubated with ^3H-labeled transmitters and the radioactivity released by the first K^+-pulse was divided by the total radioactivity in the eye before stimulation to obtain the percentage of K^+-induced transmitter release.

DISCUSSION

The biosynthesis and release of neurotransmitters are unique characteristics of neurons. In many cases, neurons using a specific neurotransmitter also possess a selective, high affinity uptake mechanism for the accumulation of this transmitter or its immediate precursor. In the present study, we have shown that retinas from post-metamorphic *Xenopus laevis* synthesize GABA, dopamine and acetylcholine and release GABA, dopamine and glycine in a Ca^{++}-dependent, K^+-stimulated manner. In addition, our autoradiographic studies show that there are at least three distinct cell populations in the inner nuclear layer of the mature *Xenopus laevis* retina which can be distinguished by their ability to accumulate exogenous transmitter candidates using high affinity uptake mechanisms. ^3H-GABA is taken up predominately by horizontal cells located at the outer border of the inner nuclear layer (Hollyfield and others, 1979); ^3H-glycine

is taken up by a large population of cells located in the middle of the inner nuclear layer (Lam and others, 1980); and ^{3}H-dopamine is taken up by a sparse population of possibly amacrine cells located at the vitread side of the inner nuclear layer (Sarthy and others, 1980).

In this study, we have used the uptake, synthesis and release of these transmitters to follow the emergence of neuronal specific characteristics during retinal development. Our autoradiographic result on GABA uptake suggests that at least some retinal neurons, presumably horizontal cells, are committed to be GABA-ergic as early as stage 31, a stage shortly after the first retinal cells become post-mitotic and when the developing retina still retains characteristics of a neuroepithelium with no clear evidence of stratification of the various neuronal laminae or the presence of synapses. In addition, the finding that high affinity GABA uptake into certain retinal neurons appear prior to synapse formation indicates that uptake sites for GABA are present on the somas of probable GABA-ergic neurons and these sites may be distinct from the Ca^{++}-dependent, K^{+}-stimulated sites for GABA release which are presumably present only at the synaptic regions. Similarly, the autoradiographic demonstration of high affinity glycine uptake into specific neurons at stage 33/34 indicate that these neurons are probably committed to be glycinergic and that the uptake sites are probably present on the somas of these cells as well. Thus, for both amino acid neurotransmitters examined in this study, the neurons destined to be GABA-ergic and glycinergic are determined fairly early during retinal differentiation. Furthermore, there exist somatic uptake sites distinct from the terminal release sites for these neurotransmitters.

In contrast to both GABA and glycine, the high affinity uptake mechanism for dopamine first appears at stage 43, when the retina shows distinct tri-laminated nuclear arrays, and when morphologically distinct synapses are already present. In an earlier study, Sarthy and Lam (1979) showed that when goldfish retinas were incubated with ^{3}H-dopamine for various time intervals, the presynaptic terminals of dopaminergic cells are labeled much earlier than the corresponding somas. This result indicates that the uptake sites for dopamine are distributed predominantly on the presynaptic terminals rather than on the somas. The present study which shows that synapse formation occurs concurrently with the appearance of dopamine uptake is therefore consistent with our findings on dopamine uptake in adult goldfish retinas.

Unlike the emergence of the high affinity uptake mechanism which was spread over many developmental stages, the first detectable synthesis and accumulation of GABA, dopamine and ACh occurred almost concurrently between stages 33/34 and 35/36. The onset of ACh and GABA synthesis also coincided with the first stages at which choline acetyltransferase and glutamic acid decarboxylase activities could be detected. The appearance of dopamine synthesis at stage 35/36 suggests that dopaminergic neurons in the retina are "determined" by this stage even though the uptake mechanism for dopamine has not yet developed.

Although the appearance of transmitter uptake and synthesis does not follow any apparent temporal order for the neurotransmitter systems examined, of the three neuronal specific properties followed in this study, the last to develop is invariably the mechanism for Ca^{++}-dependent, K^{+}-stimulated release of transmitters. In addition, as to be expected from numerous other studies, transmitter release always appears concurrently with or following morphological demonstration of synapse formation. For instance, the stage at which we first detected ^{3}H-GABA release from horizontal cells is also the stage when synaptic contacts between horizontal cells and photoreceptors first appear in the outer plexiform layer (stage 37/38, Chen and Witkovsky, 1979).

The coupling of high affinity uptake with autoradiographic studies provides a new dimension in studies of neuronal differentiation. With morphological techniques alone specific neuronal types can only be vaguely identified at the time when

specific synaptic configurations first make their appearance. Our studies indicate that the high affinity uptake system for GABA and glycine in specific neuronal types appears before synaptic specialization. This characteristic not only provides a means for the identification of specific neuronal types much earlier than previously possible but also allows us to distinguish between different neurons which share similar synaptic characteristics on the basis of the uptake of different neurotransmitters. We envision continued exploitation of the high affinity uptake system with autoradiography as an important tool in further studies on the patterns and timing of neuronal differentiation.

The main conclusion from the present study is that the appearance of certain neuronal specific properties follows a precise temporal pattern for each of the transmitter systems studied: In the case of GABA, high affinity uptake precedes transmitter synthesis which is followed by the K^+-stimulated release mechanism. Similarly, the high affinity uptake system for glycine appears several stages earlier than its release. In contrast to GABA, dopamine synthesis precedes the development of the high affinity uptake system which is followed several stages later by the appearance of the dopamine release mechanism. Moreover, the initiation of this sequence of events in each of these specific neuronal populations begins at distinctly different stages during retinal differentiation.

ACKNOWLEDGEMENT

We thank Ms. Gill Verner for her help with the retinal dissections and incubations, Ms. Susan Robbins and Ms. Donna Medford for preparation of the autoradiographs, Ms. Laun Pearson for typing the manuscript and Mr. Luis Marroquin for the photographic work. This study was supported by grants from the Retina Research Foundation to JGH and DMKL and grants from the National Institutes of Health. JGH and DMKL are recipients of Research Career Development Awards from the National Institutes of Health (USA).

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Neurochemistry Vol. 1, pp. 103-112.
Pergamon Press Ltd. 1980. Printed in Great Britain.

CYCLIC METABOLISM OF PHOTORECEPTORS AND RETINAL PIGMENT EPITHELIUM IN THE FROG

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ABSTRACT

The diurnal patterns of ^{3}H-uridine, ^{3}H-leucine and ^{3}H-mannose incorporation by red rods, single cones and pigment epithelial cells were evaluated utilizing quantitative autoradiography. Adult *Rana pipiens*, previously maintained under a 14L:10D lighting cycle for 3-4 months, were injected with ^{3}H-labeled precursors 3 hrs prior to killing and were sampled throughout a diurnal cycle. In rods, the rate of ^{3}H-uridine incorporation was highest during the middle of the light cycle and was lowest in the middle of the dark cycle, whereas ^{3}H-leucine and ^{3}H-mannose incorporation was more rapid during the latter hours of the dark cycle and lowest during the light cycle, out of phase by approximately 12 hrs from the pattern of ^{3}H-uridine incorporation. In cones, ^{3}H-uridine incorporation was highest during the middle of the light cycle and was lowest during the dark cycle, similar in pattern but reduced in amplitude from the changes observed in ^{3}H-uridine incorporation in rods. ^{3}H-leucine incorporation was high a few hours into the light cycle and just prior to the onset of the dark cycle. Relatively few grains were present over the cone myoids following ^{3}H-mannose injection with no cyclic changes evident. In the pigment epithelium, ^{3}H-uridine incorporation showed two peaks of rapid incorporation, both during the light portion of the diurnal cycle, whereas incorporation dropped to a low level in the middle of the dark cycle. ^{3}H-leucine incorporation was most rapid a few hours into the light cycle, decreasing through the middle portion of the light cycle and then increasing prior to the onset of the dark cycle with this rate sustained throughout the remainder of the dark cycle. ^{3}H-mannose incorporation showed little change throughout the diurnal cycle. In each of these three cell types, ^{3}H-uridine (RNA) incorporation occurred most rapidly during the early part of the light cycle followed by rapid rates of ^{3}H-leucine (protein) incorporation at later times in the day. These cyclic changes in the incorporation of precursors of RNA, protein and glycoprotein in the photoreceptor pigment epithelium complex are discussed in relationship to the times of membrane addition and loss from photoreceptors and times of phagocytosis by the pigment epithelium.

KEYWORDS

Cyclic metabolism; retina; photoreceptors; rods; cones; pigment epithelium; RNA; protein; glycoprotein; ^{3}H-uridine; ^{3}H-leucine; ^{3}H-mannose.

INTRODUCTION

The early autoradiographic studies of Young, (1967, 1968) clearly established that the outer segment membranes of rod photoreceptors were renewed throughout life. Within the past few years it has become apparent that the daily rates of membrane replacement are not constant but undergo cyclic changes. Studies of the diurnal variation in the time of membrane loss through shedding indicate that this process in rod photoreceptors occurs shortly after the onset of light in animals entrained to controlled lighting cycles (LaVail, 1976; Basinger, Hoffman and Matthes, 1976; Hollyfield, Besharse and Rayborn, 1976), whereas cone photoreceptors lose their apical membranes following the onset of the dark cycle (Young 1977, 1978). The rates of membrane addition in rods also show cyclic changes with approximately 80% of the daily allotment of new membrane added to the outer segment during the first 8 hrs of the lighting cycle (Besharse, Hollyfield and Rayborn, 1977).

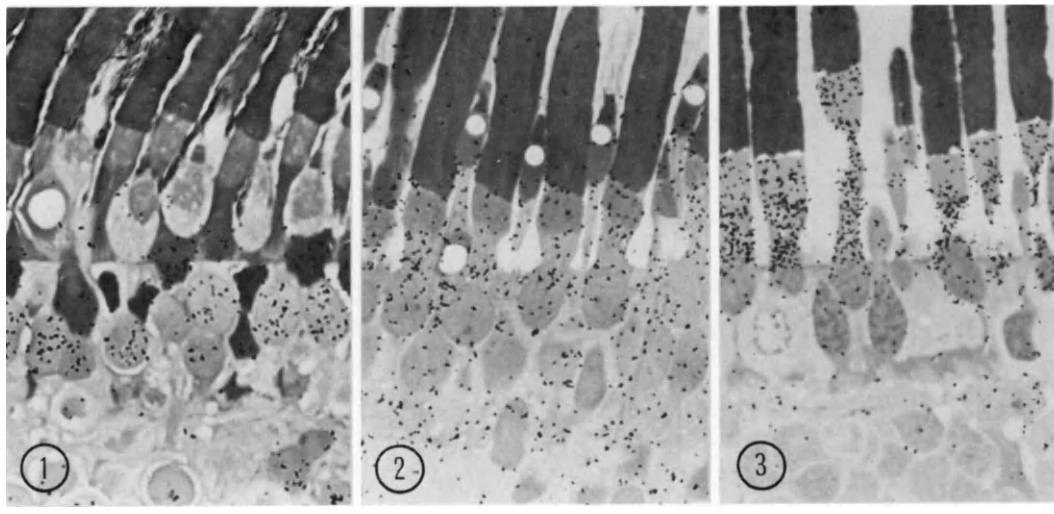
With this evidence of daily variations in the rates of membrane addition and loss in photoreceptors as background, we have addressed the following question: Do the more primary aspects of metabolism in photoreceptors and pigment epithelium involved in membrane biogenesis and degradation undergo cyclic changes during the day? To these ends we have utilized quantitative autoradiography to follow the incorporation of ^{3}H-labeled precursors of RNA, protein and glycoprotein in the retinas of frogs entrained for at least 3 months to a diurnal cycle of 14 hrs light: 10 hrs darkness. Our results suggest that RNA synthesis is highest during the early light phase of the cycle, whereas protein and glycoprotein synthesis are highest during the dark phase of the cycle.

METHODS

Adult *Rana pipiens* (20 to 25 grams) were used in these studies. Prior to use in these experiments, frogs were kept in leucite chambers with a continuous flow of tap water under ceiling fluorescent lighting on a cycle of 14 hrs light:10 hrs darkness. During the 3 to 4 months of acclimation to this lighting regimen, the animals were fed weekly with live crickets.

With each radioactive precursor, we first determined the rate of incorporation into either retinal RNA or retinal protein as a function of post-injection time. During the first four hours following injection, incorporation was linear. We chose a 3 hr post-injection interval for study because this period provides sufficient time for incorporation of the precursors, yet, since incorporation is still increasing, this interval will be sensitive to any changes during the day in synthetic rates.

Frogs were injected in the dorsal lymph sac with 100μCi of either (5,6 ^{3}H)-uridine (41.3 Ci/mMol), L-(4-5 ^{3}H)-leucine (62 Ci/mMol), or D-(2 ^{3}H)-mannose (18.5 Ci/mMol) and were sacrificed 3 hrs later. Using this protocol, frogs were studied at successive 3 hr intervals during a complete diurnal lighting cycle with an overlapping sample at the end of each 24 hour cycle. Eyes were removed, slit at the limbus and fixed either in 3% formaldehyde, 2.5% glutaraldehyde in 0.087M phosphate buffer, pH 7.4 (for ^{3}H-uridine and ^{3}H-mannose injected animals) or in the same mixture without glutaraldehyde (for ^{3}H-leucine injected frogs). After several hours, small rectangles around the optic nerve were cut from each eye, rinsed in buffer and postfixed in 1% OsO_4 for an hour and processed for plastic embedment. One micron sections were cut and placed on precleaned glass microscope slides, then dipped in Kodak Nuclear Track emulsion according to methods described previously (Hollyfield, Besharse and Rayborn, 1977). After an appropriate exposure interval (1 to 3 weeks) grain counts were made from the appropriate cellular areas using a 100X oil immersion lens. The areas from which counts were taken were then determined using either a calibrated ocular micrometer or with the aid of a Zeiss MOP-3 image analysis system. The relative grain counts were adjusted using a correction factor based on the serum levels of radioactivity present in the individual animal at the time of sacrifice.



Figures 1 - 3. Autoradiographs of retinas from frogs injected with ^{3}H-uridine (Fig.1), ^{3}H-leucine (Fig. 2) or ^{3}H-mannose (Fig. 3) three hours before the eyes were recovered for autoradiography. Silver grains in the ^{3}H-uridine experiment are restricted primarily to the nuclei whereas ^{3}H-leucine is found throughout the retina but most notably over the myoid region of the photoreceptors. ^{3}H-mannose radioactivity is localized primarily over the rod myoid regions.

The serum level was obtained by counting 10µl of plasma taken immediately after the animal was killed. This procedure normalizes the differences in available precursor which result from variations of the amount of isotope in the circulation.

RESULTS

Distribution of radioactivity. 3 hrs following injection of ^{3}H-uridine, the incorporated label was restricted to nuclei of retinal cells as evidenced by the presence of large numbers of silver grains over nuclear profiles (Fig. 1). Very little radioactivity was found in any other cellular compartment, ^{3}H-leucine, in contrast, shows a much more generalized distribution throughout the photoreceptor layer. It is present to some extent over nuclei, myoid, elipsoid, the outer plexiform layer and throughout the cytoplasm of the pigment epithelium, with the largest concentrations of silver grains found over the myoid regions of the photoreceptors (Fig. 2). ^{3}H-mannose labeled the myoid region of rods almost exclusively, with little labeling in cone or pigment epithelium cells (Fig. 3). Final quantitation was based on nuclear grain counts for ^{3}H-uridine, whereas grain counts in the ^{3}H-leucine and ^{3}H-mannose experiments were taken from the myoid region of photoreceptors and cytoplasmic regions in the pigment epithelium.

Patterns of incorporation

Red rods. The patterns of incorporation of ^{3}H-uridine in red rods are presented in Fig. 4. At the time of light onset, the grain counts over rod nuclei were relatively low but quickly increased by 2-3 fold, reaching their highest density in animals killed in the middle of the light cycle. Thereafter the grain density decreased to lower levels, finally falling precipitously after the onset of the dark phase of the diurnal cycle. Grain counts were lowest during the middle of the dark cycle, slowly increased throughout the remainder of the night, and abruptly increased following the onset of the light. At the beginning of the day, ^{3}H-leucine incorporation in rod myoids was relatively high, and it fell to its lowest level in the middle of the light cycle. Grain counts increased thereafter and were highest shortly before the onset of the dark phase of the diurnal cycle. The grain density remained at relatively high levels throughout the remainder of the night. ^{3}H-mannose incorporation was lowest throughout the light phase of the cycle but increased rapidly during the dark portion of the diurnal cycle, becomming approximately 3-4 folds higher than at any time earlier in the day.

Cones. The patterns of incorporation in cone photoreceptors are presented in Fig. 5. at the beginning of the diurnal cycle the rates of ^{3}H-uridine incorporation were increasing and were sustained at a high level throughout the light cycle. At the onset of the dark cycle, the rates fell precipitously and remained at a low level throughout the night. ^{3}H-leucine incorporation was highest for a brief period during the early part of the light cycle, falling off thereafter and finally gradually increasing during the remainder of the light cycle, reaching a second plateau prior to the onset of the dark cycle. Throughout the remainder of the dark cycle the rates of incorporation continued to decline. For ^{3}H-mannose, relatively few grains were found over the myoid region of cones, and those present did not vary significantly during the diurnal cycle.

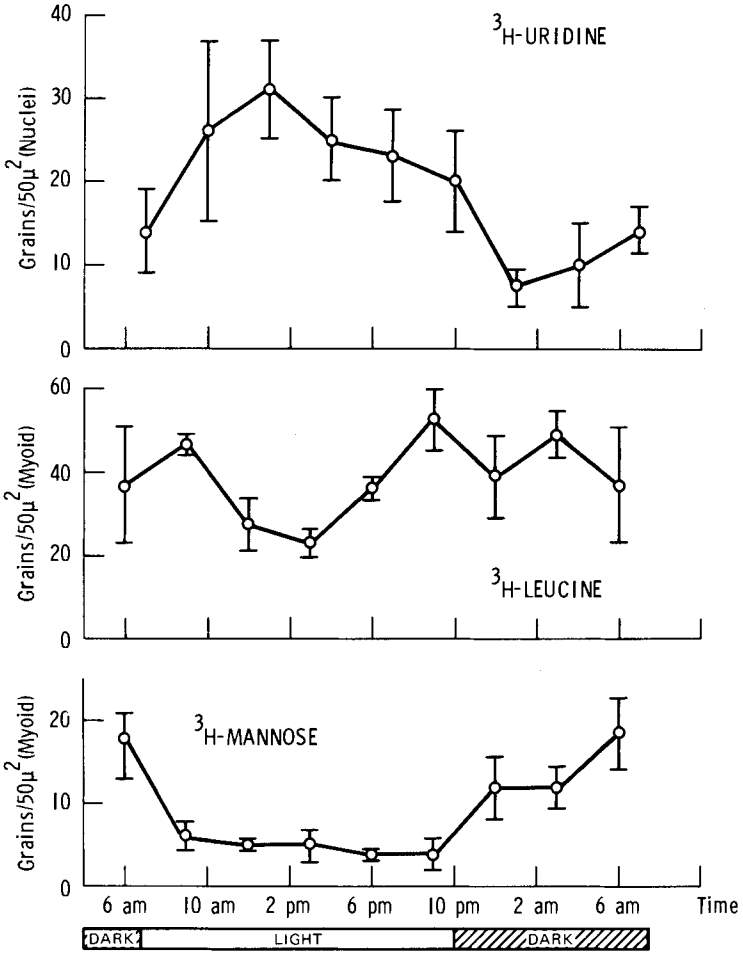


Figure 4. Diurnal patterns of incorporation of the indicated ^{3}H-precursor in rod photoreceptors during the day. Bar around individual sample means represent one standard deviation.

Pigment epithelium. The patterns of incorporation in the pigment epithelium for the three precursors are shown in Fig. 6. The rate of incorporation of ^{3}H-uridine is highest during the light portion of the diurnal cycle where it shows two peaks. Following the onset of the dark cycle the rates of incorporation decreased in a manner similar to that observed for ^{3}H-leucine in rod and cone photoreceptors described above. The rates of incorporation of ^{3}H-leucine were lowest during the middle of

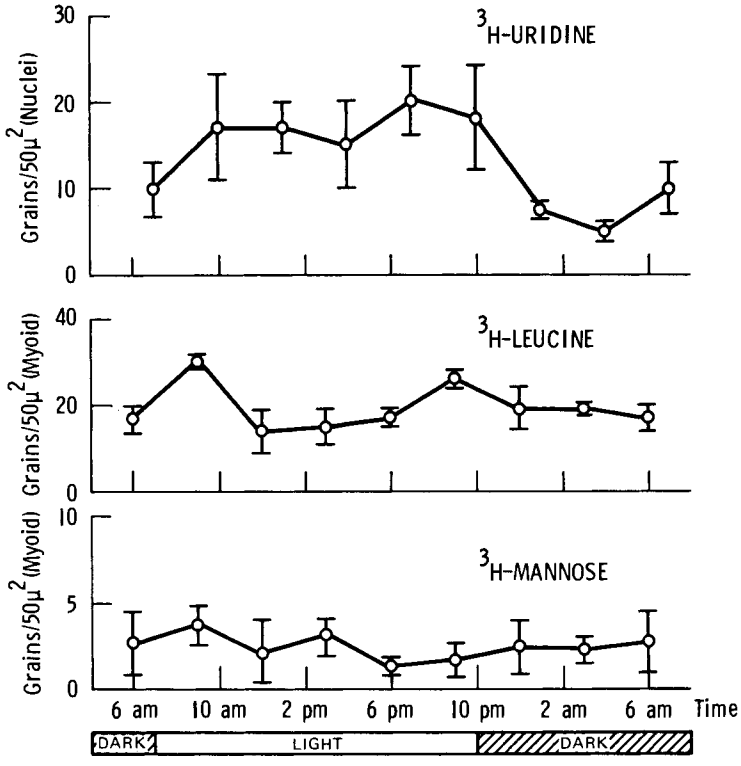


Figure 5. Patterns of incorporation of the indicated ^{3}H-precursors in cone photoreceptors during the day. Bar around individual sample means represent one standard deviation.

the light cycle and increased to higher levels prior to the onset of the dark cycle, where they remained throughout the remainder of the dark phase and into the early morning. ^{3}H-mannose was not incorporated by the pigment epithelium to any appreciable extent, though grain counts were elevated slightly during the middle of the light cycle as compared to other times of the day.

DISCUSSION

These experiments reveal significant differences in the rates of incorporation of ^{3}H-uridine, ^{3}H-leucine and ^{3}H-mannose throughout the day. The pattern of ^{3}H-uridine incorporation suggests that a period of rapid RNA synthesis takes place during the early part of the day, while the patterns of ^{3}H-leucine and ^{3}H-mannose incorporation

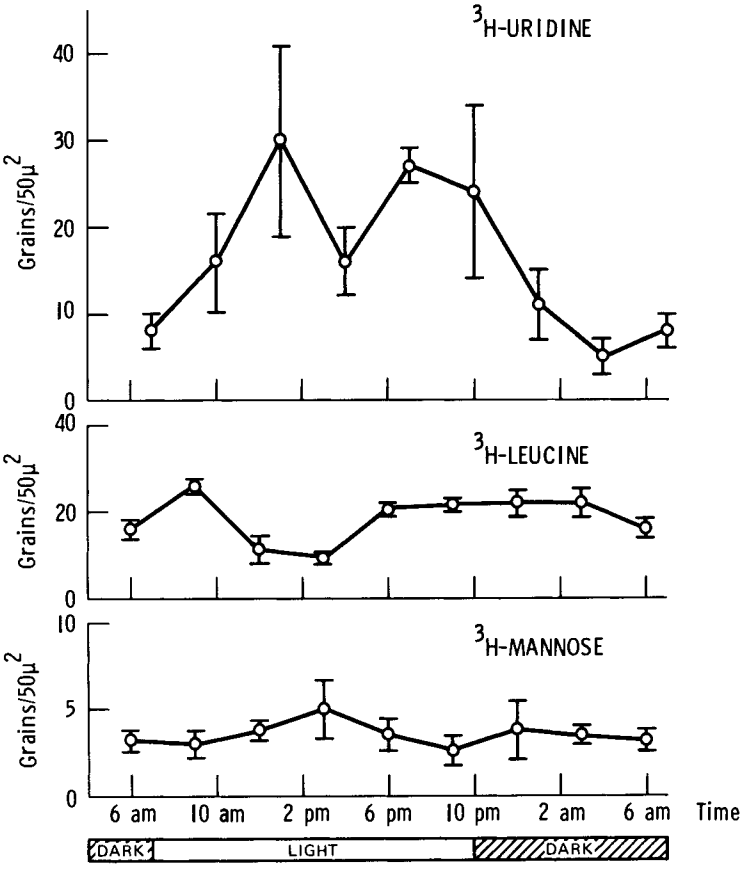


Figure 6. Patterns of incorporation of the indicated ^{3}H-precursors in the pigment epithelium during the day. Bar around individual sample means represents one standard deviation.

seem to indicate that protein synthesis and glycosylation are more active in the latter portion of the night. Taken as a whole, this sequence of metabolic events suggests that in rods, the synthesis of membrane precursors occurs in the latter portion of the night, prior to both the time of rod outer segment shedding and the time when new membrane precursors will be most rapidly assembled at the outer segment base. Though the diurnal pattern of membrane addition to cone photoreceptors is not known, in most animals membrane loss from cones has been observed to occur at a time approximately 12 hrs out of phase from the time of rod shedding (i.e. at the beginning of the dark phase of the diurnal cycle). One might predict that if the changes in metabolic patterns which underlie membrane biogenesis were linked to the time of membrane loss through shedding, then the diurnal metabolic patterns in cones should be out of phase by approximately 12 hrs from the metabolic patterns observed in rods. This, however, was not what we observed. ^{3}H-uridine incorporation

follows a pattern in cones similar to rods with the most rapid period of incorporation occurring in the light phase of the diurnal cycle and the slowest period during the dark phase. Similarly, ^{3}H-leucine incorporation also shows a similar pattern in both rods and cones, indicating that the patterns of protein synthesis in both were occurring at similar rates during the same time of day. We interpret these observations as indicating that the control mechanism(s) that regulate the time of membrane loss in these two photoreceptor types are distinctly different from the mechanisms regulating the cyclic metabolic changes presented in this study.

The pigment epithelium shows two peaks of ^{3}H-uridine incorporation during the light phase of the diurnal cycle. In experiments to be reported elsewhere, we have found that the second peak of incorporation may be related to the entry of phagocytized debris into the pigment epithelium (Hollyfield and Rayborn, 1979). When animals have been manipulated so that 3 to 4 fold more outer segment material is phagocytized by the pigment epithelium, ^{3}H-uridine incorporation in the pigment epithelium is increased in direct response to the increase in the phagocytic load. This may be similar to the increases in ^{3}H-uridine incorporation seen in alveolar macrophages following a phagocytic event (Low, 1977).

The pigment epithelium and cone photoreceptors show very little incorporation of ^{3}H-mannose, whereas rod photoreceptors were heavily labeled. It is known that mannose comprises part of the carbohydrate moiety of opsin synthesized by the rods. If the increased number of silver grains over rod myoids near the latter portion of the dark cycle is due to the glycosylation of newly synthesized opsin, this pattern of ^{3}H-mannose incorporation suggests that opsin biosynthesis occurs most rapidly during the latter portion of the dark cycle. Thus, the synthesis of the principal outer segment membrane protein is enhanced at a time prior to its rapid assembly into membranes following the onset of the light (Besharse, Hollyfield and Rayborn, 1977).

Although these observations present new information on changes in the metabolism of the photoreceptors and pigment epithelium during the day which correlate with changes in the diurnal lighting cycle, they do not provide information as to how these changes in rates are mediated. Additional studies are underway to determine whether the level of control of these metabolic events is within the retina or through central regulatory mechanisms.

ACKNOWLEDGMENTS

This investigation was supported by grants from the Retina Research Foundation, Houston, Texas; The Retinitis Pigmentosa Foundation, Baltimore, Maryland; and grants EY01406, EY02362 and EY02363 from the National Institutes of Health, Bethesda, Maryland. The authors are recipients of Research Career Development Awards from the National Eye Institute. We thank Ms. Donna Medford and Ms. Gill Verner for their help in quantitating the autoradiographic data and Ms. Laun Pearson for typing the manuscript.

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Neurochemistry Vol. 1, pp. 113-122.
Pergamon Press Ltd. 1980. Printed in Great Britain.

AN HYPOTHESIS FOR A VITAMIN A CYCLE IN THE PIGMENT EPITHELIUM OF BOVINE RETINA

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ABSTRACT

Several metabolic transformations of vitamin A have now been found in the pigment epithelium of cattle retina, leading to the hypothesis that there may be a vitamin A cycle in these cells. A retinol binding protein of MW ≃ 17,000 in pigment epithelial cytosol has been identified independently in several laboratories. In addition, a very active esterifying system, capable of utilizing either ^{3}H-retinol or ^{3}H-retinol bound to cellular retinol binding protein as substrates, has been demonstrated in the microsomal fraction of the cell. Finally, a retinyl ester hydrolase, with optimum activity between pH 4.0-4.5, utilizing physiological substrate prepared from pigment epithelial microsomes, has been localized in the lysosomal fraction of pigment epithelial cells. When considered together, these transformations could provide a unique cyclic mechanism for storage and mobilization of vitamin A in the pigment epithelium.

KEYWORDS

Retinal pigment epithelium; vitamin A cycle; retinol esterification; retinyl ester hydrolase; retinol binding protein.

INTRODUCTION

The retinal pigment epithelium (RPE) is considered to be the principal storage depot for vitamin A in the eye. The vitamin reaches this cell layer from two principal sources: (a) it flows in from the retina during light adaptation (Dowling, 1960), and (b) it is taken up from the blood (Young and Bok, 1970; Hall and Bok, 1974), where it circulates as a 1:1 molar complex with retinol binding protein pre-albumin. The circulating vitamin that enters the pigment epithelial cell from the blood is in the form of the alcohol; yet, inside the cell, about 92% of it is in the ester form. Cell fractionation studies have shown that the principal storage site of retinyl ester in cattle RPE is in the microsomes (Berman, Segal and Feeney, 1979). A second, smaller, storage pool for the ester in mammalian RPE appears to be lipid droplets in the cytoplasm (Hirosawa and Yamada, 1976; Robison and Kuwabara, 1977; Berman and co-workers, 1979). On the other hand, unesterified retinol (vitamin A alcohol) of the RPE is localized exclusively in the soluble portion of the cell, all of it bound

to cytosol retinol binding protein (MW ≃ 17,000). Metabolic transformations of these different forms of vitamin A within the RPE are poorly understood. We have recently confirmed the early observation of Krinsky (1958) showing a very active esterifying enzyme for retinol; it is concentrated mainly in the microsomes of the RPE cell (Berman and co-workers, 1980). The present report describes three different aspects of retinol metabolism in bovine RPE which, taken together, suggest the possible existence of a vitamin A cycle in cattle RPE.

MATERIALS AND METHODS

All studies were carried out on cattle eyes brought to the laboratory in cooled containers within three hours after death of the animals. Preparation of pigment epithelial cells and fractionation of their subcellular organelles have been described previously (Berman, Schwell and Feeney, 1974; Rothman, Feeney and Berman, 1976; Berman and Feeney, 1976; Berman and co-workers, 1979). Three morphologically and biochemically defined fractions were isolated and used in the present study. Further descriptions are given in the text.

The ^{3}H-all-trans-retinol (1.21 Ci/mmole) was purchased from New England Nuclear Corp., Boston, MA. Sephadex G-100 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden, and alumina from Riedel-de Haen, AG, Hanover, Germany.

The chromatographic procedures used for isolating retinol-retinol binding protein complex from RPE cytosol, either labeled (Saari and co-workers, 1977) or unlabeled (Berman and co-workers, 1979), have been described previously. Vitamin A alcohol (retinol) and retinyl ester were separated on columns of water-weakened alumina. Protein was measured according to Lowry and co-workers (1951) using bovine albumin as standard.

The methods for studying retinol esterification have been described in detail elsewhere (Berman and co-workers, 1980). Briefly this consists of incubating either ^{3}H-retinol or ^{3}H-retinol bound to cellular retinol binding protein with appropriate subcellular fractions at 30° for 15 min under subdued light. The reaction is terminated by addition of cold ethanol; vitamin A compounds are extracted with petroleum ether, separated on columns of alumina and counted in a Packard Tri-Carb Liquid Scintillation Counter Model 3380.

Retinyl ester hydrolase was studied using as substrate labeled ester extracted from RPE microsomes after incubation with ^{3}H-retinol. In a typical preparation, 100 µg of microsomal protein from cattle RPE was incubated at pH 7.5 with 10 µCi of ^{3}H-retinol for one hr at 30°. The retinyl ester and the unreacted ^{3}H-retinol substrate were extracted by successive addition of ethanol and petroleum ether. Afterward ^{3}H-retinyl ester was isolated free of ^{3}H-retinol by alumina chromatography. Retinyl ester prepared in this manner had a specific activity of about 0.4 to 0.6 Ci/mmole. Details of the assay for hydrolase activity are given in the text.

RESULTS

Binding of Retinol to Cellular Retinol Binding Protein

A retinol binding protein of MW ≃ 17,000 has been identified in RPE cytosol in a variety of animal species (Wiggert and Chader, 1975; Wiggert and co-workers, 1977a; Wiggert and co-workers, 1977b; Saari and co-workers, 1977). Although its exact "concentration" in RPE cytosol has not been determined, Saari and co-workers

(1977) calculated that in cattle cytosol, approximately 190 pmoles of ^{3}H-retinol are bound per mg of protein, which corresponds to 115 pmoles per eye. Measurement of the endogenous retinol binding protein in cattle RPE cytosol by fluorescence techniques gave an approximate value of 1,000 pmoles of retinol-retinol binding protein complex per eye (Berman and co-workers, 1979). These findings suggest that about 10% of the sites may be available for retinol binding by this specific protein in RPE cytosol. Since no free retinol could be detected in the RPE (Berman and co-workers, 1979), binding could be the first step in the handling of retinol entering the pigment epithelium.

Esterification of Vitamin A in RPE and Retina

A brief summary of our present knowledge concerning esterification of retinol in the pigment epithelium is given in Table 1. This enzyme is more active in the

TABLE 1 Retinol Esterifying Enzyme in RPE*

Optimum pH	7.0 - 7.5
Cellular localization	Microsomes
Cofactor requirements	None
Apparent Km	
^{3}H-retinol	16.6×10^{-6} M
^{3}H-retinol bound to cellular retinol binding protein	5.5×10^{-6} M
Apparent Vmax	
^{3}H-retinol	500**
^{3}H-retinol bound to cellular retinol binding protein	180**

* Adapted from Berman and co-workers (1980)
** nmoles ester formed/hr/mg protein

RPE than any other tissue examined. The activity can be detected using as little as 20-30 µg of RPE microsomal protein, but under the same conditions, the only other tissues showing any measurable activity is intestine. It is however possible to demonstrate retinol esterifying enzyme in the retina as well, but this requires higher concentrations of enzyme and longer incubation periods than for RPE. Approximately 1 mg of microsomal protein from cattle retina incubated for one-half hr with 0.3 nmoles of ^{3}H-retinol results in about 1 to 2% esterification. Although this activity seems relatively low when compared to the RPE microsomal activity, it is in fact close to the value reported by Andrews and Futterman (1964) for microsomes isolated from cattle retina.

The question of whether rod outer segments are able to esterify retinol has been controversial. Krinsky (1958) found active esterification in lyophilized bleached rod outer segments (prepared from dark-adapted cattle retinas), provided that NADH and alcohol dehydrogenase were present during the bleaching process. Andrews and

Futterman (1964) were unable to confirm this activity in freshly isolated dark-adapted rod outer segments after exposure to light. Recent findings in our laboratory support the view that rod outer segments are in fact able to esterify retinol if proper conditions are chosen and if the assay is sensitive enough. Using about 1 mg of rod outer segment protein, and incubating for one-half hr with 0.3 nmoles of ^{3}H-retinol results in approximately 12% ester being formed when using dark-adapted preparations (Table 2). It is approximately 50% higher in light-adapted rod outer segments. That there is far less activity in rod outer segments (either light- or dark-adapted) than in microsomes of RPE is apparent from the data shown in Table 2, where similar concentrations of protein were used.

TABLE 2 Effect of Light* and Dark** on Retinol Esterification†

Tissue	Number of experiments	% esterification
Retinal pigment epithelium		
Dark	3	83
Light	3	79
Rod outer segments		
Dark	7	12.3 ± 1.1††
Light	7	18.5 ± 2.0††

* Light refers to RPE and rod outer segments isolated from dark-adapted tissue and then exposured to normal indoor room illumination for 1½ hr.

** Dark refers to RPE and rod outer segments isolated from cattle eyes kept in a cold, light-tight box for 3 hrs. Dissections and enzyme assay were carried out under dim red light.

† Adapted from Berman and co-workers (1980)

†† Mean ± S.E. of the mean

Retinol esterification by both rod outer segments and RPE microsomes can also be demonstrated using ^{3}H-retinol bound to cellular retinol binding protein as substrate. When prepared as described previously (Saari and co-workers, 1977) and incubated under optimum conditions with either RPE microsomes or with rod outer segments, approximately the same percentage of esterification is found as that using ^{3}H-retinol (Berman and co-workers, 1980). This is the first time that a metabolic transformation of ^{3}H-retinol bound to cellular retinol binding protein has been demonstrated.

Hydrolysis of Retinyl Ester

Previous studies (Krinsky, 1958) were inconclusive in detecting hydrolytic activity toward either natural or synthetic esters of retinol in RPE homogenates, even though under the same experimental conditions, such activity was clearly demons-

trable in preparations of lyophilized retina. It seems possible that retinyl ester hydrolase in the RPE may have been missed in these early experiments due to lack of a methodology sensitive enough to detect what may be relatively low levels of activity in these cells. Krinsky (1958) used unlabeled retinyl ester as substrate and even today, long chain fatty acid esters of retinol suitably labeled for metabolic experiments are not commercially available. However, from the experiments described above on esterifying enzyme in the pigment epithelium, it was clear that enzymatically synthesized labeled retinyl ester could be obtained in good yield from RPE microsomes. We have synthesized ^{3}H-retinyl ester by this method and purified it, as described in MATERIALS AND METHODS. Preliminary experiments using RPE cell homogenates, together with appropriate controls consisting of substrate without enzyme source, showed that about 10-20% of the labeled retinyl ester was hydrolysed after incubation at 30° for one hr.

Further studies to determine the intracellular localization of this hydrolytic activity utilized three clearly defined cell fractions: lysosomal-mitochondrial, microsomal and cytosol. These experiments showed that the retinyl ester hydrolase of pigment epithelium is localized principally in the lysosomal-mitochondrial fraction of the cell. Very little activity could be detected in either microsomes or cytosol. The optimum pH for retinyl ester hydrolase activity in the lysosomal-mitochondrial fraction was found to be at pH 4.0-4.5. The retinyl ester hydrolase activity is proportional to enzyme concentration up to about 200 µg of lysosomal-mitochondrial protein (Fig. 1) and the rate of hydrolysis

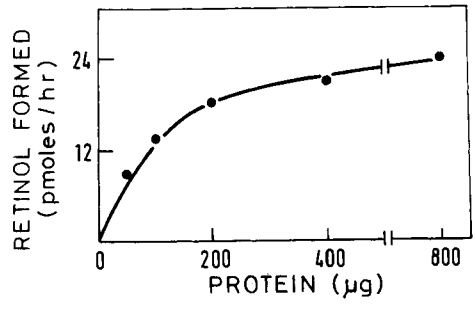

Fig. 1. Effect of protein concentration on retinyl ester hydrolase activity in the lysosomal-mitochondrial fraction of RPE. The reaction mixture contained 50 µmoles of citrate-phosphate buffer, pH 4.5, 40 pmoles of ^{3}H-retinyl ester (2.5 x 10^{4} cpm) and varying amounts of enzyme protein. Incubation was at 30° for one-half hr.

using 200 µg of lysosomal-mitochondrial protein is linear with time for approximately 30 min (Fig. 2).

The enzyme is readily extractable from the lysosomal-mitochondrial pellet either by glass-Teflon homogenization or by mild sonication. It is stable when frozen for periods up to approximately one month, and is inactivated after 5 min at temperatures of 56° or higher. Some of the properties of the retinyl ester hydrolase

have been compared to the previously described acid lipase of RPE which was investigated using 4-methylumbelliferyl palmitate as substrate (Rothman and co-workers, 1976). These findings will be reported separately, but preliminary data suggest that we are dealing with two separate enzymes.

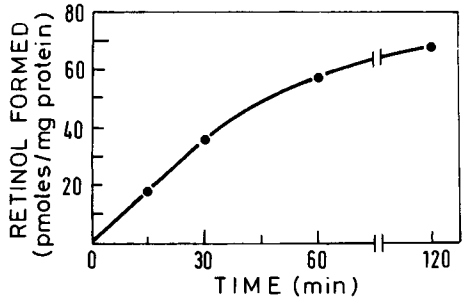

Fig. 2. Time course of ^{3}H-retinyl ester hydrolysis by lysosomal-mitochondrial fraction of RPE cells. The reaction conditions were the same as those described in Fig. 1.

In these studies on retinyl ester hydrolase of the RPE, the enzyme activity was calculated from the amount of radioactive retinol recovered from alumina columns. This technique requires direct extraction of retinol from the incubation mixture with ethanol and petroleum ether as described in MATERIALS AND METHODS. However, organic solvent extraction, although both accurate and reproducible for studying retinyl ester hydrolase, does not give any information as to the form or physical state in which the liberated retinol is present after the hydrolysis. Considering possible in vivo situations, the retinol could be present either as a free lipid, or - as the hydrolytic reaction proceeds - it could become bound to the cellular retinol binding protein of RPE. To examine the latter possibility, unlabeled retinol binding protein of RPE was prepared from the cytosol fraction of the cell as described previously (Berman and co-workers, 1979) and added to the incubation mixture. At the end of 30 min, the mixture was centrifuged at 110,000 x g for one hr and the supernatant applied to a column of Sephadex G-100. The results of a typical experiment are shown in Fig. 3. One major and two minor radioactive peaks were observed. The first, in the void volume (fractions 22-27), probably represents unreacted substrate, possibly micelles of retinyl ester, judging from the elution patterns in gel chromatography noted previously (Berman and co-workers, 1979). The third radioactive peak (fractions 56-65) would be consistent with unbound monomeric forms of retinol or retinyl ester since low molecular weight substances would be eluted in this position.

Of major interest was the largest (middle) peak eluting in fractions 38-52. The Ve/Vo of this radioactive peak was 1.88, which corresponds to the eluting position of retinol bound to retinol binding protein in cytosol of cattle RPE (Saari and co-workers, 1977; Berman and co-workers, 1979). Thus it is reasonable to assume that most of the retinol released by the action of RPE retinyl ester hydrolase can, in the presence of cytosol retinol binding protein, form a stable complex with this component, a process that could readily be occurring in vivo as well. Quantitative aspects of this reaction are now being examined.

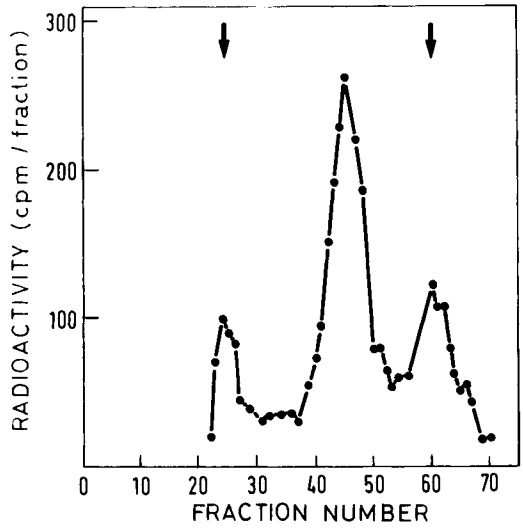

Fig. 3. Gel filtration of the incubation mixture of lysosomal-mitochondrial fraction of RPE. The reaction, in 0.4 ml, contained 200 µg of enzyme protein, 50 µmoles of citrate-phosphate buffer, pH 4.5, 28 µg of cytosol retinol binding protein from RPE and 40 pmoles (2.5×10^4 cpm) of ^{3}H-retinyl ester. After incubation at 30° for one-half hr, the reaction mixture was centrifuged at 110,000 x g for 1 hr and the supernatant applied to a column (1.2 x 55 cm) of Sephadex G-100 equilibrated with 0.2 M NaCl-50 mM Tris, pH 7.5. Other details have been described previously (Saari and co-workers, 1977; Berman and co-workers, 1979). The arrows indicate the void volume (Vo) and the total volume (Vt) of the column.

DISCUSSION

Injected vitamin A is taken up from the circulation and becomes localized within the pigment epithelial cell in a matter of minutes (Young and Bok, 1970) or hours (Hall and Bok, 1974). Radioautographic studies have shown that after injection of vitamin A in the frog, most of it is concentrated in oil droplets (Young and Bok, 1970). In mice however, it is found in both the smooth endoplasmic reticulum and in lipid droplets (Hirosawa and Yamada, 1976; Robison and Kuwabara, 1977). The size and number of vitamin A-storing lipid droplets is related to the amount of vitamin administered as well as to the nutritional status of the animal. Once inside the cell, it undergoes metabolic transformations, at least three of which are now known. These reactions, considered together, could serve as an efficient mechanism for the storage and mobilization of vitamin A by RPE cells. Their presence in three different intracellular compartments suggests a unique structural and functional interdependency of vitamin A metabolism within the pigment epithelium. This is depicted schematically in Fig. 4.

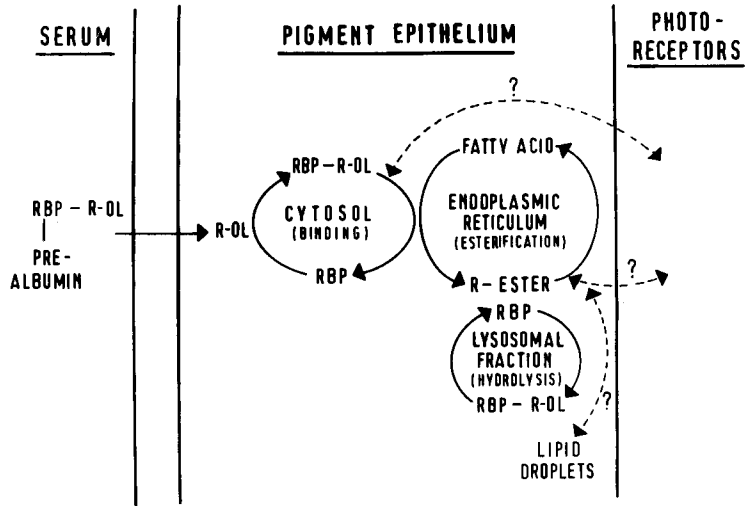

Fig. 4. Possible metabolic interrelationships of various vitamin A compounds in the pigment epithelium of cattle retina.

Although not yet proven directly, it seems reasonable to consider that the first step occurring after vitamin A enters the RPE cell is binding to the endogenous retinol binding protein (Wiggert and Chader, 1975; Wiggert and co-workers, 1977; Saari and co-workers, 1977).

Not only free retinol, but also retinol bound to cellular retinol binding protein, can serve as substrates for esterification, a reaction taking place mainly in the microsomal fraction of RPE. The esterifying enzyme of RPE microsomes is more active than that found in microsomes of any other tissue, or in rod outer segments (Table 2). In the pigment epithelium it is not influenced by the degree of light- or dark-adaptation, as far as we are able to determine, and cofactors are not required to obtain maximum activity. Approximately 180-500 nmoles of ester are formed from either ^{3}H-retinol or ^{3}H-retinol bound to cellular retinol binding protein per hour per mg protein (Table 1). Microsomes are the principal storage site of the ester in cattle RPE, although both morphological (Hirosawa and Yamada, 1976; Robison and Kuwabara, 1977) and biochemical (Berman and co-workers, 1979) evidence point to a second pool of ester in the form of lipid droplets.

Although retinol is esterified rapidly, hydrolysis appears to be much slower. As shown in Figs. 1 and 2, under optimum conditions of pH, time and enzyme concentration, approximately 100 pmoles of labeled retinyl ester are hydrolysed per mg of lysosomal protein per hr. Thus the equilibrium between esterification and hydrolysis is strongly in favor of esterification by a factor of approximately 10^{3}, suggesting that RPE cells have an enormous capacity to store vitamin A ester. This relatively slow rate of hydrolysis may explain why the retinyl ester hydrolase was not detected previously (Krinsky, 1958). We have only been able to demonstrate it using radioactive substrate, which provides a sensitivity and accuracy not possible with unlabeled substrates. Moreover the substrate used, which represents enzymatically synthesized retinyl ester, is as close to the physiological substrate as can be found for in vitro biochemical experiments. Most of the hydro-

lytic activity is present in the fraction isolated from RPE containing both lysosomes and mitochondria (Berman and Feeney, 1976; Rothman and co-workers, 1976; Berman and co-workers, 1980). The acidic pH optimum of the hydrolysis as well as the localization of nearly all other known hydrolytic enzymes in lysosomes favors localization of retinyl ester hydrolase of RPE in lysosomes as well. The presence of a well developed autophagic system in the pigment epithelium (Feeney, 1973) lends further support to this view. Nevertheless a possible localization of the activity in mitochondria, analogous to the pH 4.2 cholesteryl ester hydrolase in rat brain, cannot be completely excluded (Eto and Suzuki, 1971).

With the finding (Fig. 3) that retinol released by the action of retinyl ester hydrolase can be complexed to cytosol retinol binding protein, we have come a full circle. The dynamic process of storage and mobilization of vitamin A in the RPE, occurring in the microsomes and lysosomes respectively, could be controlled by subtle changes in either pH, availability of retinol, or other yet unknown factors. Both the esterification and hydrolysis are intimately associated with the pool of soluble retinol binding protein in the cell cytoplasm. The activities of the pigment epithelial enzymes do not appear to be light-dependent. However, in the photoreceptors, both binding (Wiggert and co-workers, 1979) and esterification (Berman and co-workers, 1980) of retinol are enhanced in light-adapted rod outer segments.

The present investigations, while supporting the possibility of a vitamin A cycle in the RPE, still leave many questions unanswered. Some of these are indicated in Fig. 4. We do not know if there is any metabolic interrelationship between the pools of retinyl ester in the microsomal membranes and the lipid droplets. Judging from the more rapid association of injected vitamin A with the lipid droplets than with the endoplasmic reticulum (Hirosawa and Yamada, 1976), the former may be the more rapidly turning over pool. In what form is retinol transported between the RPE and the photoreceptors during light- and dark-adaptation? Is it as the ester, perhaps lipid droplets, or is it as retinol bound to a retinol binding protein? Finally, oxidative as well as stereoisomeric conversions of retinol are probably occurring in the RPE. Circulating retinol is the all-trans isomer, yet most of the retinol in RPE is the 11-cis form (Krinsky, 1958). Experiments designed to answer some of these questions are now under way.

ACKNOWLEDGMENT

This work was supported in part by the U.S.-Israel Binational Science Foundation, Jerusalem, and National Institutes of Health Grants EY00715 and EY01131.

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Neurochemistry Vol. 1, pp. 123-142.
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THE CHEMISTRY OF THE RETINA: FUNCTION, RENEWAL, RHYTHMS, AND THE NUCLEUS

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ABSTRACT

Investigation of the chemical activities of retinal cells began with *functional* chemistry, an origin which can be traced to the pioneering studies of Kühne and Wald. Next to develop was *renewal* chemistry, which is concerned with the continued replacement of retinal cell structure. A third field of retinal chemistry, more recently evolved, is the analysis of chemical change associated with daily *rhythms*. In the living cell, all such groups of chemical reactions are merged into a single, integrated metabolism which ultimately is regulated by molecular systems sequestered within the nucleus. Although *nuclear* chemistry has so far been neglected in vision research, a theory of nuclear metabolism has been maturing in other fields of biological chemistry. One salient aspect of the theory is the temporal stability of the fundamental constituents of the chromosomes: DNA and histone. Alone among all cellular molecules, they may persist indefinitely. Nuclear theory has proved to have important predictive value in preliminary applications to analysis of the mammalian retina. Evidence is presented in the following report which indicates that retinal cells can *repair* DNA lesions provoked by UV radiation. Other experiments suggest that in retinal nuclei there is a reversible acetylation of histones and non-histone chromosomal proteins--a process which is implicated in gene regulation.

KEYWORDS

Retina; renewal; repair; nucleus; histones; DNA; genetic regulation

INTRODUCTION

The study of the chemical reactions which take place in the retina was initiated a century ago by Kühne (1878), who took up the study of rhodopsin shortly after Boll's discovery of it. According to Wald, in his Nobel Prize lecture (1968), "in 2 extraordinary years he and his coworker Ewald learned almost everything known about it for another half century." Clearly, the pace of research was more leisurely in those days. In the last decade or two, more has been learned about the chemical activities of the retina than in all previous recorded history.

In its modern form, the field of retinal chemistry had its origin when Wald discovered vitamin A in the retina (1933, 1934-35). He showed that opsin is a protein

which under the influence of light engages in a cycle of molecular interactions with retinol and retinal. Working with Hubbard and others, he discovered the oxidation of retinol by alcohol dehydrogenase, and the cycle of cis-trans isomerization which proved to be a part of every visual system known. Wald provided a clear and accurate insight into the profoundly significant question of how the eye responds to radiant energy and transmits its excitation to the brain through a sequence of physico-chemical changes. In short, he revealed the essence of *how we see*. These magnificent discoveries generated new ways of thinking about the chemistry of vision which still have not lost their explanatory power. Even today, opsin and its chromophore stand out as the key concept. The shifting waves of ions, the rise and fall of cyclic nucleotides, and the transient phosphorylation of membrane proteins--all are recognized as part of the complex series of chemical changes which follow the crucial initial events revealed by Wald.

FUNCTIONAL CHEMISTRY

In opening up the field of retinal chemistry, Wald focussed attention on questions of *function*. I use the term in the sense intended when we say that the function of visual cells is to detect and signal the presence of light; or, when we acknowledge that the eye is concerned with "visual function." Functional activities are those which are construed to be of benefit for some level of organization of which the functioning entity is a part. In the cellular context, function refers to the chemical activities of particular cells which are judged to be beneficial for other cells, or for the organism as a whole. The field of study primarily concerned with the chemical changes that occur when the retina is stimulated by light is *functional* chemistry.

RENEWAL CHEMISTRY

About 15 years ago, investigation of a second aspect of retinal chemistry was initiated. Discovery of the continued replacement of the rhodopsin-containing membranes of the rod outer segments, and the phagocytic properties of the pigment epithelium (Young, 1967; Young and Bok, 1969) led to the development of a body of information concerned with the chemistry of *renewal*. This field is primarily devoted to analysis of the continued synthesis of new molecules and the destruction of old ones. Whereas the chemical reactions of visual *function* take place only when the retina is stimulated by light, *renewal* processes go on incessantly. Functional chemistry involves relationships which go beyond the chemical requirements of individual cells, and involve more complex levels of hierarchical organization. Renewal chemistry concerns the self-rebuilding of cells.

In visual cells, as in other cells, renewal seems to represent the predominant aspect of the overall chemical activity (Young, 1976, 1979). In other words, most of the chemical reactions seem to be concerned with reconstruction of the cell, rather than with providing benefits for other cells. In mammals, for example, a new double-membrane disc is formed in each rod outer segment every 15 minutes. The cell replaces about 10 per cent of its light-detecting apparatus daily (Young, 1967, 1971). If we assume that each disc contains 10,000 rhodopsin molecules, and that each rod produces 100 discs daily, this means one million rhodopsin molecules are synthesized each day by every rod visual cell. With 250 million rods in both eyes, there is a production of 250 trillion (2.5×10^{14}) rhodopsin molecules per day, throughout life. This would require an enormous energy expenditure for the formation of peptide bonds alone, not to mention the synthesis of 30 or more phospholipids which are associated with each rhodopsin molecule. The major chemical activity in visual cells may well be the production of membranes.

In the years following the discovery of outer-segment renewal, it was gradually documented that practically every species of molecule in visual cells is continually replaced. Molecular constituents are destroyed or discarded as rapidly as they are produced. This results in a steady-state in which the appearance of structural stability is maintained despite continuous chemical renovation (Young, 1976). The lifetime of most of the cells' constituents is a matter of a few days or less.

RHYTHMIC CHEMISTRY

It was analysis of renewal processes in visual cells which uncovered a third aspect of retinal chemistry--its rhythmicity. The discovery that rods preferentially shed membranes from the tips of the outer segments shortly after the beginning of the day (LaVail, 1976) led to the observation that cones preferentially shed membranes soon after the beginning of the night (O'Day and Young, 1978; Young, 1977, 1978a). No exceptions to this cyclic disposal of membranes by rods and cones have been found, indicating that it is a general rule. That the generalization may have even wider scope is suggested by a report that invertebrate photoreceptors may also renew their light-sensitive membranes according to a daily rhythm (Blest, 1978). Additional symptoms of the chemical rhythmicity of renewal processes have been observed in studies of autophagy, membrane assembly, and the synthesis of RNA and glycoprotein (Besharse and coworkers, 1977; Hollyfield and coworkers, 1979; Reme, 1978; Reme and Sulser, 1977). It seems certain that many further examples will soon be revealed. This work has introduced a new variable into the study of retinal chemistry: *time of day*.

INTEGRATION OF CELL CHEMISTRY

That the *functional* chemistry of visual cells also proceeds with a daily rhythm is implicit in Schultze's generalization, that rods mediate visual function in the dim light of the night, whereas cones are active as light receptors during the day (Schultze, 1866). It is apparent that the intermittent shedding of membranes--a feature of *renewal* chemistry--is coordinated with the functional chemistry of rods and cones: Each class of photoreceptors discards membranes at the termination of its period of functional activity. Furthermore, these *rhythmic* aspects of cell chemistry are synchronized by the environmental light cycle (Basinger, 1977) acting through *functional* chemical pathways. By relaying to the brain information concerning the rhythmic daily fluctuations in visible radiation, the retina also serves the function of synchronizing the chemical activities of the other rhythmic cells in the vertebrate body (Young, 1978b).

These interrelationships bring out the point that within the cell all chemical reactions are interdependent. Fundamentally, there is only one kind of chemistry: cell chemistry. How the functional, renewal, rhythmical and other aspects of the overall metabolic system are coordinated and unified we can at present but dimly perceive. In the cytoplasm, the organization of unceasing molecular rearrangement may depend upon such features as compartmentalization of groups of related chemical pathways, integrated by molecular feedback circuits involving conformational changes in proteins. However, the primary source of control of these intricate chemical activities resides not in the cytoplasm, but in the limited classes of molecules and associated chemical reactions of the nucleus. Ultimately, it resides in the chemical properties of a single molecule: deoxyribonucleic acid.

THE NUCLEUS

The chemical activities of retinal nuclei have yet to be explored. The only path-

way of nuclear metabolism demonstrated in the retina is the production of RNA in visual cells. This lack of attention to the nucleus may be due to the belief that the truly distinctive chemical activities which characterize the different varieties of retinal cells are situated in the cytoplasm. There is some justification for this view, for the nucleus is noted for an astonishing evolutionary conservatism. In organisms as varied as peas and men, the nuclei of cells are remarkably similar in their chemical composition and metabolic behavior. Nuclear chemists freely amalgamate into their schemes information derived from analysis of slime molds, cancer cells, sea urchin eggs, and thymus glands in remarkable tribute to the biochemical unity of nature.

However, the hazard involved in ignoring the metabolism of the nucleus (even while admitting its central role in chemical integration) is also clear. Unless we test in the retina the concepts of nuclear chemistry which have been derived from investigations of other kinds of cells, we cannot be sure that they are actually pertinent. Furthermore, most of the available information concerning the chemical activities of the nucleus has been derived from study of dividing cells--and the cells of the retina are manifestly post-mitotic. The chemical activities of nuclei in dividing cells undergo continuous cyclic changes, with some sets of reactions (such as DNA synthesis and mitosis) being strictly intermittent, due to the alternating activation and repression of different genes (Mitchison, 1971; Fig. 1). In contrast, when mitosis ceases and differentiation occurs, it is assumed that one discrete cluster of genes is activated permanently, locking the cell into that particular set of continuing chemical reactions. This might involve quite a different type of nuclear chemistry from that observed in dividing cells.

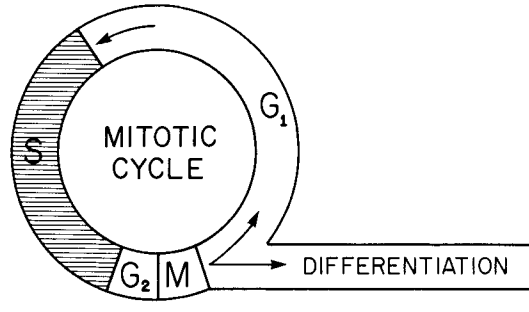

Fig. 1. Diagram of the mitotic cycle. DNA is synthesized only during the *S* phase, prior to mitosis (*M*). After retinal cells have differentiated, they do not synthesize DNA.

We must also be alert for possibly unique aspects of nuclear metabolism in the retina. There seems to be nothing in current nuclear theory, for example, to clarify reports and observations of taurine, melatonin, glucosamine, choline, and ethanolamine in visual cell nuclei, or to explain why the nuclei of mammalian rods are so much more condensed than those of ganglion cells. Nevertheless, with these cautions in mind, it is reasonable to begin to explore the patterns of nuclear chemistry in retinal cells by testing the predictive value of current nuclear theory to learn what limitations there may be to its application in the retina.

CHEMICAL COMPOSITION OF THE NUCLEUS

The major molecular constituents of the nucleus are: DNA, RNA, and two classes of

protein--histones and non-histone chromosomal proteins. Carbohydrates have not been described as nuclear components, and lipid is confined to the nuclear membrane. In the retina, the absence of carbohydrates and lipids in nuclei has received some documentation (Bibb and Young, 1974a, 1974b; Wislocki and Sidman, 1954; Young, 1968, 1976; Young and Bok, 1979).

DNA

Deoxyribonucleic acid has been demonstrated in retinal nuclei by several methods (Eichner, 1957; Sidman, 1961; Yoshida, 1958). Identification of DNA is facilitated by a specific staining procedure, the Feulgen reaction (Ris and Mirsky, 1949), and a specific enzyme, deoxyribonuclease, which selectively digests this nucleic acid.

RNA

Ribonucleic acid has also been identified in the nuclei of retinal cells by several different techniques (Bok, 1970; Byzov, 1965; Koenig, 1967, 1971). One such demonstration is given in Fig. 3, which illustrates that when nuclear RNA is labeled by providing retinal cells with a radioactive precursor of RNA, the autoradiographic reaction can be abolished by incubation of the tissue sections in a solution which contains the specific hydrolytic enzyme, ribonuclease.

Histones

Histones have not been documented in the nuclei of retinal cells. The members of this small group of unusual proteins are characterized by their unique capacity for specific binding to the double helix of DNA (Allfrey, 1971, 1977; Bradbury, 1975; Elgin and Weintraub, 1975; Johnson and coworkers, 1974; MacGillivray and Rickwood, 1974). Regions of *positive* electrical charge, due to lysine and arginine residues, are concentrated on the surface of the histone molecules. These two amino acids bear positively charged nitrogen atoms on their side chains. The recurring phosphate groups located on the outer aspect of the DNA double helix are also fully ionized. However, having lost a proton instead of gaining one, the phosphates bear a net *negative* charge. The histones and DNA are linked together by multiple ionic bonds between these electrostatically charged groups of opposite polarity.

These features provide the basis for a histochemical procedure which selectively stains histones (Alfert and Geschwind, 1953). When tissue sections are incubated at a low pH in a solution of the acid dye, fast green, the colorant is bound to practically all tissue components. However, when the pH is raised to neutrality and beyond, increasingly fewer compounds are capable of binding the dye. At pH 8, the only intracellular constituents with a residual positive charge capable of binding the negatively charged dye molecules are the histone proteins of the nucleus (Fig. 4). However, the dye will not stain the nuclei at this pH unless the DNA is first removed (by digestion with DNAse or extraction with hot trichloracetic acid). This indicates that the DNA interacts with most or all of the positively charged groups on the histone surfaces. Furthermore, if the DNA is removed, but the positively charged groups on the histones are neutralized by acetylation, the nuclei also fail to bind the dye. Additional evidence that it is the histone proteins which are stained is provided by selective extraction of tissue sections with 0.25 *N* HCl--the classical biochemical method for solubilizing this class of proteins (Brasch, 1976; Hancock, 1969; Mirsky and Pollister, 1946). Extraction removes the material stainable at pH 8 with fast green (Fig. 4), but it does not diminish the Feulgen reaction for DNA.

These experiments demonstrate that retinal nuclei contain DNA, RNA, and histone, and that each of these nuclear components can be selectively eliminated from slices of retinal tissue.

Non-Histone Chromosomal Proteins (NHCP)

Unlike the small group of histones, whose components have similar and related properties, the non-histone proteins of the nucleus comprise a large and heterogeneous collection. Many are enzymes, which mediate the varied metabolic activities of the nucleus. No specific stain or selective method for extraction or digestion is available to document their presence. However, because the histones lack the amino acid tryptophan, the presence of tryptophan-containing compounds in the nucleus, demonstrated by a histochemical procedure (Adams, 1957; Fig. 5) testifies to the presence there of non-histone proteins (MacGillivray and Rickwood, 1974; Vidali and coworkers, 1972). Extraction of the histones or nucleic acids does not abolish the staining of tryptophan-containing protein in retinal nuclei.

These preliminary results are consistent with the conclusion that retinal cells, like other nucleated cells, contain four major macromolecular nuclear constituents: DNA, RNA, histones, and non-histone proteins.

SYNTHESIS AND RENEWAL OF NUCLEAR CONSTITUENTS

RNA

All three classes of RNA--ribosomal, transfer, and messenger--are synthesized in the nucleus, although they are destined to play their *functional* roles in the cytoplasm. With one exception (histone mRNA), all are produced in the nucleus from larger RNA molecules which are subsequently modified by a series of cleavage steps that snip away excess nucleotides (Darnell, 1968; Georgiev, 1972; Sirlin, 1972). Thus, much of the newly synthesized RNA never leaves the nucleus. Instead, it is broken down and recycled. Messenger RNA, in addition to being cleaved into a smaller polynucleotide, is further modified by the addition of a long chain of repetitive adenylic acid groups (Greenberg, 1975).

Renewal of ribonucleic acid in the nuclei of retinal cells has been recorded in several species (Bok, 1970; Bok and Young, 1970; Koenig, 1971; Maraini and Franguelli, 1962; Young, 1973, 1976; see Fig. 3).

NHCP

The non-histone chromosomal proteins are also continually replaced. A wide range of variability in patterns of renewal has been recorded (Dice and Schimke, 1973; Holoubek and Crocker, 1968; MacGillivray and Rickwood, 1974). Synthesis of new molecules occurs in the cytoplasm (this now seems to be the birthplace for all proteins), and it goes on continuously, even during mitosis. Subsequently, the molecules migrate into the nucleus. This has not yet been studied in the visual system.

DNA and Histone

In contrast to the limited lifetimes of RNA and NHCP, the DNA and histone molecules appear to be stable. Once synthesized, they are not broken down. In non-dividing cells, it seems that they are not synthesized at all. In this respect *they are unique.*

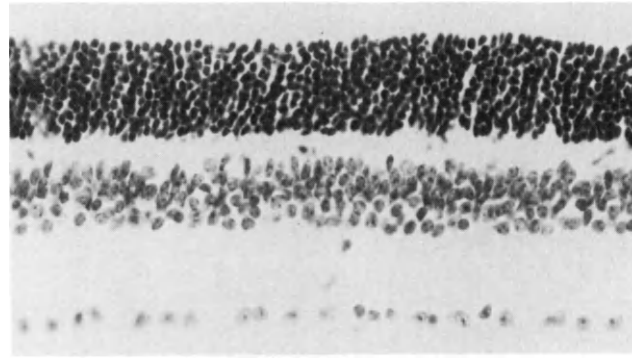

Fig. 2. Demonstration of DNA in retinal nuclei with the Feulgen reaction. Staining is abolished by prior incubation in DNAse. Mouse retina, X 350.

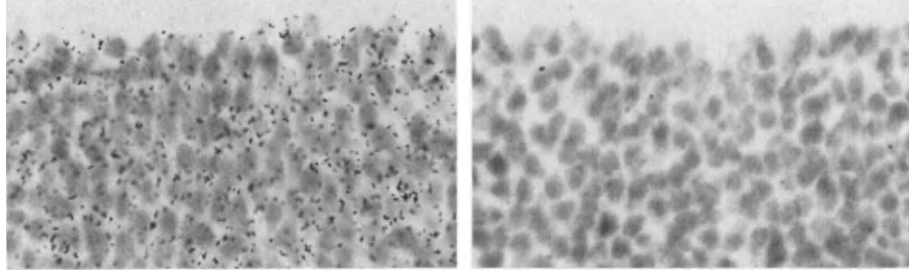

Fig. 3. Demonstration of RNA in retinal nuclei by autoradiography. 30 min after injection of H^3-uridine, visual cell nuclei are labeled (left). The radioactive material is removed by incubation in a ribonuclease solution (right). Mouse retina, X 1,000.

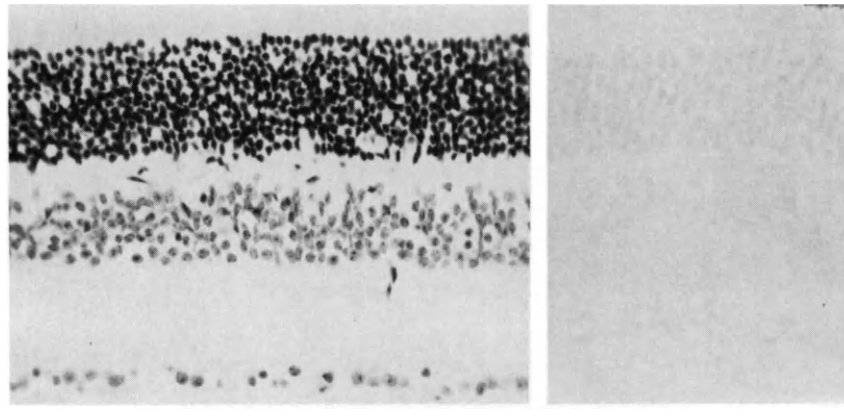

Fig. 4. Demonstration of histone proteins in retinal nuclei by staining with fast green at pH 8.0 after removal of DNA (left). Extraction of histones from tissue sections (with 0.25 *N* HCl) practically abolishes the staining reaction (right). Mouse retina, X 350.

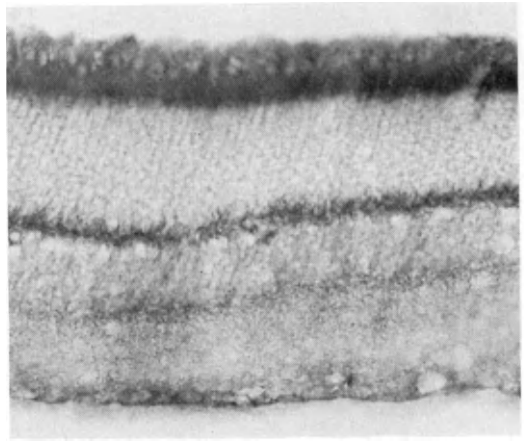

Fig. 5. Tryptophan-containing compounds are demonstrated in all retinal layers by this histochemical procedure (Adams, 1957). Because histones lack tryptophan, the staining of nuclei indicates that they contain non-histone proteins. Mouse retina, X 400.

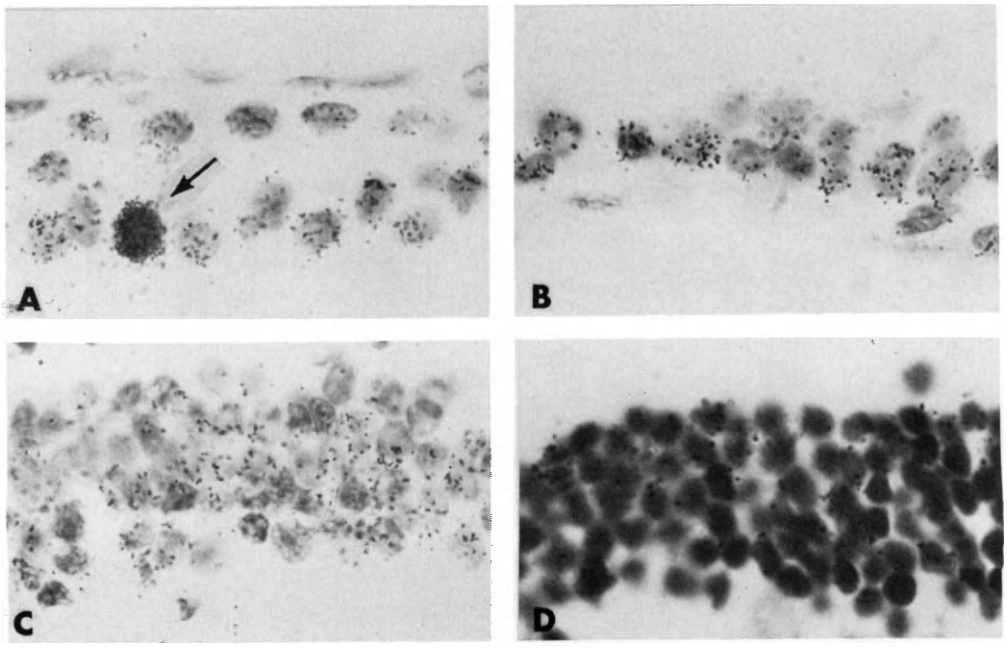

Fig. 7. Evidence of DNA repair in mouse corneal epithelium and retinal cells irradiated with 10,000 erg/mm^2 UV (254 nm), incubated in a nutrient medium containing 5 µCi/ml H^3-thymidine (37° C, 2 hr), then examined by autoradiography. In the corneal epithelium (A) a cell undergoing DNA synthesis prior to mitosis is heavily labeled (arrow). Several other cells show a weak labeling, indicative of excision repair of DNA. Evidence of DNA repair was also obtained in ganglion cells (B), cells of the inner nuclear layer (C), and in visual cells (D). X 950.

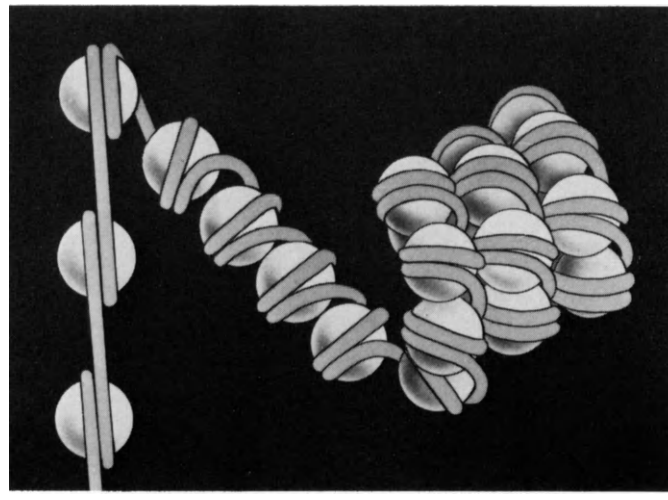

Fig. 8. Diagram of the relationship of DNA and histone in chromosomes according to the nucleosome model. Adapted from Worcel (1977).

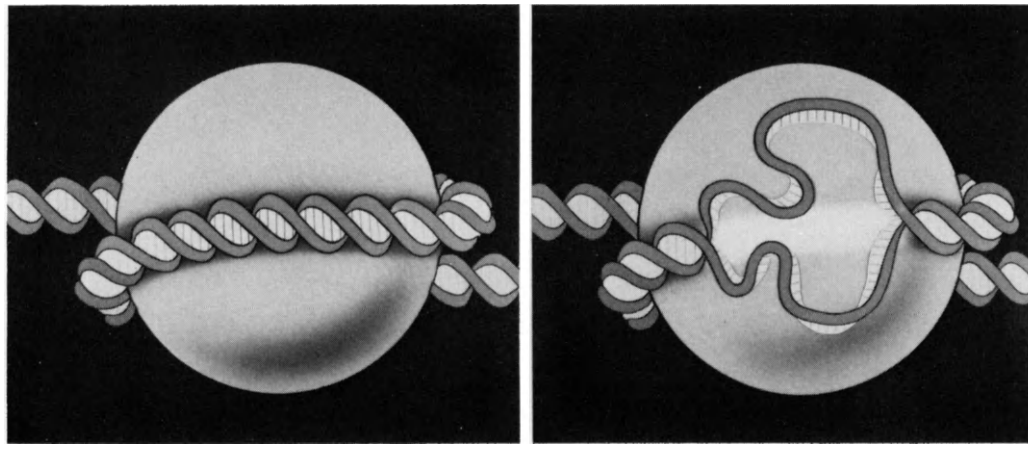

Fig. 9. Model of a molecular mechanism by which DNA can be released from its ionic bonding to the surface of the nucleosome (left) as a prelude to RNA transcription. The white horizontal streak across the center of the nucleosome on the right indicates the zone where acetate groups have been added to ε-amino groups on lysine residues, eliminating positive charges. This breaks the ionic bonds, releasing the double helix so that it can be partially unwound and separated. RNA polymerase can then transcribe the base sequence of one of the strands.

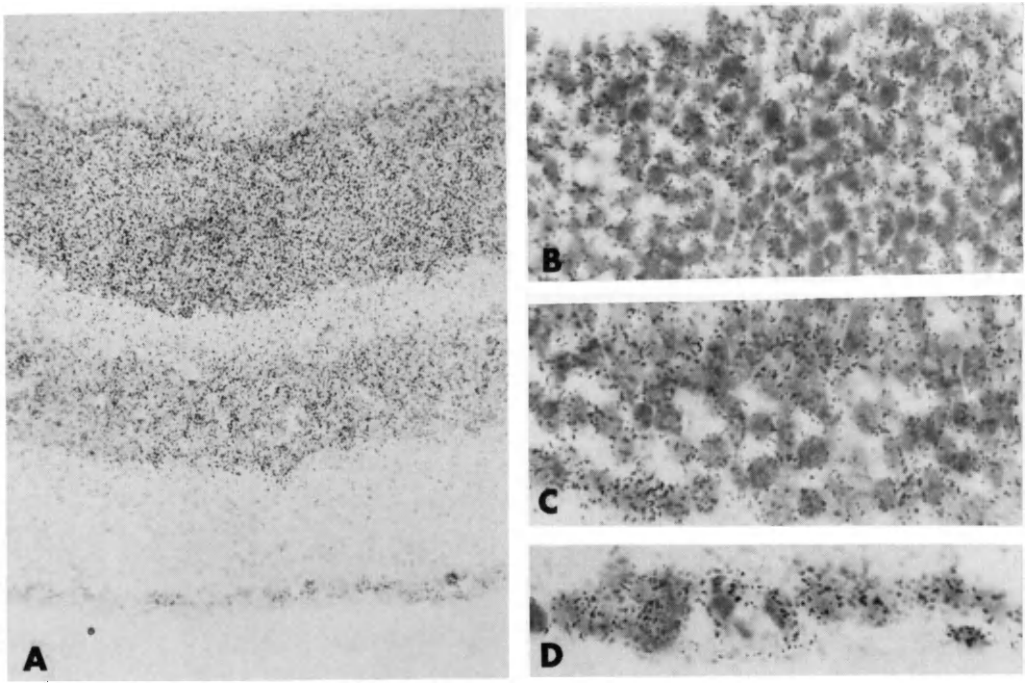

Fig. 10. Heavy labeling of retinal nuclei is revealed by autoradiography 30 min after intravenous injection of H^3-acetate in adult mice. In A, the full thickness of the retina is shown (X 400). On the right, nuclei are depicted at higher magnification (X 900). B, outer nuclear layer; C, inner nuclear layer; D, ganglion cells.

The bulk of the evidence strongly indicates that DNA and histone are synthesized simultaneously during the DNA synthesis period of the cell division cycle (*S*-phase, Fig. 1) and that this is the only time they are synthesized (Bloch and Godman, 1955; Bloch and coworkers, 1967; Gallwitz and Mueller, 1969; Gurley and Hardin, 1968; Prescott, 1966; Rasch and Woodard, 1959; Robbins and Borun, 1967; Stein and coworkers, 1977). At the time of onset of DNA synthesis (or perhaps slightly before it) the genes coding for histones are derepressed, and transcription of histone mRNA begins. Histone genes occur in multiple copies, and their mRNA molecules seem to be the only ones which pass directly to the cytoplasm without undergoing any "processing" whatsoever in the nucleus (Adesnik and coworkers, 1972; Greenberg, 1975; Jacob, 1976; Kedes and Birnstiel, 1971; Pardue, 1975). In the cytoplasm these mRNA molecules mediate the rapid synthesis of histone proteins, which migrate into the nucleus as soon as they are formed (Oliver and coworkers, 1974; Perry and Kelley, 1973; Robbins and Borun, 1967). In the nucleus, they become electrostatically bound to DNA. (Whether new histone is linked solely to new DNA is not yet known). At the end of the DNA synthesis period, when the genetic material has been completely replicated and attached to histone, the histone mRNA molecules are destroyed and histone synthesis stops (Borun and coworkers, 1967; Perry and Kelley, 1973). In the ensuing biochemical events, the cell splits in two.

Restriction of DNA and histone synthesis to the *S*-phase of the mitotic cycle has important implications for non-dividing cells such as those of the retina and brain. It means that these two molecules, the fundamental elements of the chromosomes, differ from the other molecular constituents of the cell in that they are the *only* permanent members of the entire assemblage. DNA and histone, synthesized simultaneously, then immediately bonded into a *nucleohistone complex*, are thereafter never replaced. One cannot doubt that if this remarkable conclusion about the genetic material is substantiated, it must be accorded great significance in the theoretical analysis of cell organization.

The temporal stability of DNA (that is, its non-renewal) is well established. With mature retinal cells, this is readily demonstrated. It is sufficient simply to expose them to the specific DNA precursor, H^3-thymidine. No nuclear incorporation of radioactivity is recorded (Maraini and Franguelli, 1962; Schultze and coworkers, 1961; Sidman, 1961)--unless the DNA has been lesioned (see below). Alternatively, if the DNA is made radioactive by providing the labeled precursor during the mitotic cycles which precede differentiation, the radioactivity is still retained, apparently undiminished, weeks or months later (Sidman, 1961; see also Bennett and coworkers, 1960).

There are firm indication that the histones may be immune from replacement in the nervous system. In the brain, "the data suggest a metabolic stability of histones which corresponds to that of the deoxyribonucleic acid" (Piha and coworkers, 1966; see also Balhorn and coworkers, 1972; Byvoet, 1966; Hancock, 1969). These studies corroborate investigations which indicate that histone is only produced in dividing cells, just before mitosis. In the retina, there is so far no evidence which bears upon this question.

RENEWAL AND REPAIR

The non-renewal of DNA is far from being simply a metabolic oddity of no particular significance. On the contrary, it represents a serious challenge to the concept that cell survival is dependent upon the renewal of cell constituents. The conclusion that "the gradual accumulation of defects provoked by a variety of environmental hazards as well as by functional excitation is...prevented by the continual reconstruction of the cell" (Young, 1976) is supported by reports of retinal damage by visible radiation. When the intensity of radiation entering the eye is raised slightly beyond that present in the normal energy environment, structural damage

to the retina (particularly the visual cells) becomes so severe that it can readily be detected by histological examination (Anderson and coworkers, 1972; Friedman and Kuwabara, 1968; Ham and coworkers, 1978; Kuwabara, 1970; Kuwabara and Gorn, 1968; Lawwwill and coworkers, 1977; Marshall and coworkers, 1972; Noell and coworkers, 1966; Sperling, 1977; Sperling and Johnson, 1974). If slightly higher than normal levels of radiant energy can provoke lesions as profound as this, then surely at normal energy levels molecular lesions must also occur--lesions, however, which are too small for microscopic detection. In other words, *retinal cells suffer molecular damage during the normal visual process.*

If there were no mechanism for disposal of these denatured molecules, their accumulation would soon kill the cells. However, not only do the cells survive throughout a long lifetime, they remain essentially free of any senescent changes (Young, 1976). This capacity to "grow old without aging" can be ascribed to the *renewal* processes which dominate the chemical activities of these cells. Damaged molecules are generally not retained for more than a few days, because most of the visual cell molecular constituents--damaged or undamaged--do not persist for longer than this. No matter what its age, the visual cell is largely an assemblage of recently synthesized molecules, *except for the DNA* (and possibly the histones). The non-renewal of DNA stands out as a glaring exception, intensified by its status as the key molecule in the organization of the cell.

This theoretical impasse has been overcome by the remarkable discovery that DNA can be *repaired.* Several mechanisms are now known by means of which regions of DNA which have been damaged can be restored to their original condition (Cleaver, 1974; Evans, 1975; Hanawalt, 1975; Painter, 1974; Williams, 1976). Best documented in nucleated cells is the process of excision repair (Fig. 6) in which certain proteins in the NHCP fraction detect the localized denaturation of the DNA double helix, excise a portion of the DNA strand containing the lesion, and replace it with the correct sequence of nucleotides using the same elegant mechanism employed in DNA synthesis: complementary base-pairing with the undamaged sister strand. It is a variation on the fundamental chemical reaction in living systems--the replication of DNA.

I have recently obtained evidence which suggests that retinal nuclei can repair lesioned DNA. When retinas are incubated in vitro in the presence of H^3-thymidine after being exposed to ultraviolet radiation (254 nm), the nuclei in some cells in all retinal layers incorporate the labeled molecule into DNA (Fig. 7). The nuclei in unirradiated retinas do not take up the radioactive DNA precursor. Incorporation of H^3-thymidine in the nuclei of the irradiated cells is interpreted as evidence of the replacement of patches of nucleotides which have been removed during the process of excision repair. This appears to support the contention that the non-*renewal* of DNA is compensated for by specific *repair* mechanisms.

It seems important to emphasize the distinction between renewal and repair. The processes are fundamentally different. Renewal denotes the repeated replacement of molecules brought about by their continued synthesis and balanced destruction. Renewal is incessant, whether or not any denaturation of cell structure has taken place. If damage *does* occur, the lesioned molecules will soon be replaced as a result of the ongoing renewal process. The restitution of visual cell outer segments which have been damaged by radiation, retinal detachment, dietary deficiency, hibernation or cold stress is readily accomplished through *renewal*, which rebuilds the outer segments *continually.* In contrast, molecular *repair* is *not* a continuing

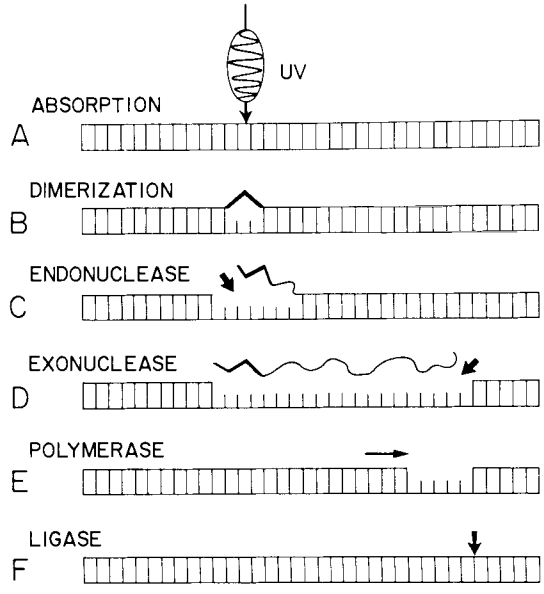

Fig. 6. Diagram of DNA excision repair after UV radiation damage. Absorption of UV photons by DNA (A) induces covalent linkage of adjacent pyrimidines. The thymine-thymine dimer is most readily formed (B). Resulting distortion of the helical structure is recognized by an endonuclease which incises the DNA strand (C). An exonuclease then excises the damaged strand by making a second incision (D). Approximately 100 nucleotides are removed. Then a DNA polymerase mediates the linkage of the replacement nucleotides, complementary to those in the sister strand (E). (This can be detected by autoradiography). Lastly, a ligase seals the backbone with a terminal covalent bond (F).

process. It is a specific sequence of chemical reactions which takes place only after a lesion has appeared in an existing molecule. These reactions do not replace the entire molecule. Instead, they restore the original distribution of atoms in the denatured region of the molecule. Restitution of structure through renewal is clearly *the primary mechanism for ridding the living system of errors.* Molecular repair has been demonstrated for only one molecule, DNA, the only molecule known (with considerable certainty) not to be renewed.

When examined from this point of view, the evidence that histones may also be temporally stable takes on added significance. Because of the intimate bonding of histone to the DNA double helix, molecular lesions which denature the histones might have serious consequences. Without the possibility of replacement of such damaged molecules, or restitution of original structure through repair, the histones would represent a peculiarly vulnerable as well as crucially situated part of the molecular organization of the cell. Another theoretical impasse seems to be emerging: Two molecules, DNA and histone, linked together physically and functionally, synthesized simultaneously and thereafter not renewed--both essential to cell survival, but only one with a known mechanism for the elimination of lesions.

GENETIC REGULATION

The interrelationship of histone and DNA forms the central concept of current

theories about the nature of genetic regulation--the process by which part of the total genetic information contained in the DNA can be selectively expressed. When firmly bonded to histone, DNA is totally inert; no RNA synthesis is possible. In order for particular genes to be derepressed, the ionic bonds between the negatively charged double helix and the positively charged groups on the surface of the histones must be loosened.

Fig. 8 depicts the relationship of DNA and histone in chromosomes according to the recently developed concept of the *nucleosome* (Bradbury, 1975; Finch and Klug, 1977; Kornberg, 1977; Noll, 1977; Olins, 1977; Oudet and coworkers, 1977; Simpson and coworkers, 1977; Thomas and Butler, 1977; Worcel, 1977). This model is recent in origin, but appears to be accepted by virtually all researchers currently investigating the molecular organization of chromosomes.

According to this model, the DNA double helix is wrapped around the outside of core particles called nucleosomes. Each nucleosome consists of two each of four types of histone proteins (H2A, H2B, H3, H4), forming an octamer. (The remaining histone, H1, is involved in the process of chromosome cross-linking and condensation. Compared to the four nucleosome histones, it is significantly more variable in structure as well as distinctive in its metabolism). The model provides a satisfactory explanation for the uniformity of amino acid sequence observed in the nucleosome histones in all nucleated cells. No other proteins have been so rigidly conserved during the course of evolution (Bailey and Dixon, 1973; DeLange and coworkers, 1969; Patthy and Smith, 1973; Panyim and coworkers, 1971). Changes in primary structure of these histones apparently cannot be tolerated because of the requirement for matching of complementary surfaces of the eight units in each nucleosome, and for binding the nucleosome to the DNA double helix. Each nucleosome is associated with 200 base-pairs of DNA, but only 140 of these are in contact with the nucleosome surface. (This represents about 1.75 turns of the DNA around the nucleosome). When the DNA-nucleosome chain is fully extended, it produces a beads-on-a-string appearance (Fig. 9). This is believed to occur in portions of the DNA containing genetically active regions. Compaction is achieved by ascending orders of superhelical arrangements which culminate in dense clumps of "heterochromatin", considered to be genetically inactive (Brown, 1966).

The ionic bonds between DNA and histone must be broken if the genetic information is to become accessible, because the two strands of the double helix must be partially unwound and separated so that RNA polymerase can transcribe the base sequence of one of the strands. This separation is also a necessary prelude to DNA replication and DNA excision repair.

A mechanism for modifying the DNA-histone bonds has been discovered. It involves the reversible enzymatic alteration of the DNA-binding groups on the surface of the nucleosome. Histones are subjected to several post-synthetic modifications, including methylation, phosphorylation and acetylation. Reversible acetylation, in particular, has been clearly demonstrated to be related to changes in genetic activity (Allfrey, 1971, 1977; Bonner and coworkers, 1977; Johnson and coworkers, 1974; MacGillivray and Rickwood, 1974; Pogo and coworkers, 1968). There are histone-acetylating enzymes (acetyl coenzyme A is the acetate donor) as well as deacetylases which can remove the acetyl groups from histones. These enzymes are located in the nucleus, where they form part of the NHCP fraction. The reversible acetylation reaction is very selective. It affects mainly the lysine residues in histones H3 and H4, forming ε-N-acetyllysine (Allfrey, 1977; Simpson and coworkers, 1977). Addition of the acetate group to the nitrogen atom modifies the surface conformation and eliminates the positive charge (Adler and coworkers, 1974), breaking the ionic bond with DNA (Fig. 9).

These changes, in themselves, are insufficient for the initiation of RNA synthesis. For RNA transcription to occur, additional chemical changes are required. These seem to involve NHCP proteins in specific conformations induced by protein phosphorylation (Allfrey, 1974; Chiu and Hnilica, 1977; Kleinsmith and coworkers, 1976; Stein and coworkers, 1976, 1977).

I have obtained evidence of acetylation in the nuclei of retinal cells. Autoradiographic analysis shows that 30 to 60 minutes after injection of H^3-acetate in adult mice, the nuclei of all the retinal cells are radioactive (Fig. 10).

This was first observed in frog retinas by Schettino (1978), who also showed that extraction of lipid and RNA did not significantly decrease the nuclear labeling. In confirming and extending these results in the mammalian retina, I have found that DNAse digestion also fails to diminish perceptibly the content of nuclear radioactivity. However, if histones are extracted from tissue sections prior to autoradiography, there is a slight decline in nuclear labeling. This result suggests that there may be a continuing replacement of acetate groups on the surface of nucleosomes in retinal cells. Histones may be stable, but the nucleosomes which they form seem to be the site of incessant, reversible atomic modification.

Even after digestion of DNA and RNA and the extraction of histones, however, most of the acetate radioactivity remains in the nucleus in these experiments. This indicates that the non-histone proteins may also undergo acetylation reactions. Here current nuclear theory offers no explanation, because the possible acetylation of the non-histone proteins of the nucleus has yet to be investigated.

CONCLUSION

The preliminary histochemical and autoradiographic results mentioned in this report seem clearly to indicate that the theory of nuclear metabolism and genetic regulation which currently is undergoing rapid development may be applied to the retina with noteworthy predictive value. Although the chemical activities of retinal nuclei have received scant attention so far, these initial experiments suggest that this unexplored field may prove to be a rich source of information centered on key molecules in the organization of the cell. New knowledge of nuclear chemistry is likely to influence importantly our ideas concerning the overall chemistry of the retina, including the integration and control of its functional, renewal and rhythmical aspects.

ACKNOWLEDGMENT

This report is respectfully dedicated to George Wald. The important assistance of Mirdza Lasmanis and Zoja Trirogoff is gratefully acknowledged. My research is supported by grants EY 00095, EY 02277 and EY 00444 from the United States Public Health Service.

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Neurochemistry Vol. 1, pp. 143-150.
Pergamon Press Ltd. 1980. Printed in Great Britain.

STUDIES ON PUTATIVE NEUROTRANSMITTERS IN THE DISTAL RETINA

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ABSTRACT

Histochemical and cytochemical studies of the retinas of the teleosts Carassius carassius and Eugerres plumieri revealed that acetylcholinesterase, besides its main localization in the inner retinal layers, can be also found in the outer plexiform and inner nuclear layers, and between the apposed processes of horizontal cells of a given layer. Similar studies of catecholamine (CA) containing cells in Cyprinus carpio and Eugerres plumieri have identified cells with bodies located in the inner plexiform and nuclear layers, where amacrine cells are found, and which send processes to both plexiform layers. As a part of our search for the possible role of cholinergic and catecholaminergic systems in the lateral spread of horizontal cell activity, the effects of CA's and acetylcholine (ACh) were studied in the perfused retina of Eugerres plumieri. Dopamine (0.2 mM) and other CA's slightly depolarize horizontal cells while markedly increasing their response to local illumination and decreasing their response to the surround. ACh(10mM) plus BW anticholinesterase (1 mM) produced similar effects on most of the cells studied. Hexamethonium (cholinergic antagonist), interferes with the ACh but not with the catecholamine effects. The effects of both types of agents are suppressed by the α-adrenergic blocking phentolamine (0.5 mM).

KEYWORDS

Fish retina; catecholaminergic cells, ACh activity, horizontal cell responses, dopamine effects, ACh effects.

INTRODUCTION

Morphological studies of the neuronal circuitry in the distal vertebrate retina have revealed many of the complex interconnections between photoreceptors and second order cells, and between the latter (Stell and Lightfoot, 1975; Stell, 1978). The synaptic region, the outer plexiform layer, represents an important region of cellular interactions essential for the integration of nervous signals relating to the visual phenomena. The mechanisms, including the neurotransmitters involved and their actions, are as yet little understood. Many laboratories are pursuing studies on the involvement of different substances with apparent neurotransmitter properties in such cellular interrelations, and their metabolism under different physiolo-

gical conditions. The present report summarizes recent studies on the localization and pharmacological effects of components of catecholaminergic and cholinergic systems in the distal retina.

LOCALIZATION STUDIES

Histochemical Localization of Catecholamines

Previous studies of catecholamine containing neurons in the retina have been summarized by Ehinger (1976). Such neurons are found in the innermost part of the inner nuclear layer and a few are distributed among ganglion cells. In certain species, the former send processes towards both inner and outer plexiform layers, and this may indicate that such neurons perform an important function in the interactions between the two synaptic regions.

The use of a modification of the "Faglu" water-stable fluorophores method originally described by Furness, Costa and Wilson (1977; see Nakamura, 1979; Negishi and others, 1979) has permitted the confirmation of the above mentioned anatomical findings in the retinas of different teleosts, including fresh water (Cyprius carpio, Tribolodon trakonensis and Serrasalus notatus) and marine fishes (Eugerres plumieri, Mugil brasiliensis, Mugil curema, Mugil cephalus and Centropomus subsp.). Figure 1 illustrates the typical morphology of the catecholamine-containing interplexiform cells (Dowling and Ehinger, 1975, 1978; Dowling, Ehinger and Hedden, 1976).

Histochemical and Cytochemical Localization of Acetylcholinesterase

A large number of studies on the histochemical localization of AChE in the retina have been summarized by Nichols and Koelle (1968), and Stell (1972), Neal (1976). The cells which contain this enzyme, a possible indicator of the presence of cholinergic systems, are the amacrine cells and some ganglion cells and, thus, the cholinergic system would be restricted to the inner retina. Microgasometric measurements performed by Drujan and Svaetichin (1972) on isolated retinal cells of Eugerres plumieri also show that only amacrine, ganglion and bipolar cells have detectable AChE activity.

However, Drujan, Díaz Borges and Brzin (1979), using modified copper thiocholine cytochemical method (Brzin, Tennyson and Duffy, 1966) were able to detect a precipitate in the outer retina. The AChE is localized on the membrane of horizontal cell processes, at the places where they contact bipolar cell dendrites and photoreceptor endings, forming a characteristic synaptic complex (Fig. 2A), and also at the lateral contacts between horizontal cells of a given layer and their ascending processes (Fig. 2B).

PHARMACOLOGICAL STUDIES

The isolated retina of the teleost (Eugerres plumieri) was kept in a chamber and perfused with solutions of varying concentrations of different drugs. Intracellular recording permitted the continuous monitoring of horizontal cell electrical activity during changes of the perfusion solutions. Light stimuli consisted of alternating spots and annuli, both centered on the site of recording. In this fashion it was possible to evaluate the effects of the applied drugs upon the responses elicited by direct activation of the cell through the receptors feeding into it (spot), and upon the responses elicited through lateral spread from distant regions (annulus). Details of these techniques are given elsewhere (Laufer and Negishi, 1978; Negishi and Drujan, 1978 and 1979b).

A characteristic effect on horizontal cell responses can be observed following addition of different catecholamines to both fish (Negishi and Drujan, 1978, 1979a and b)

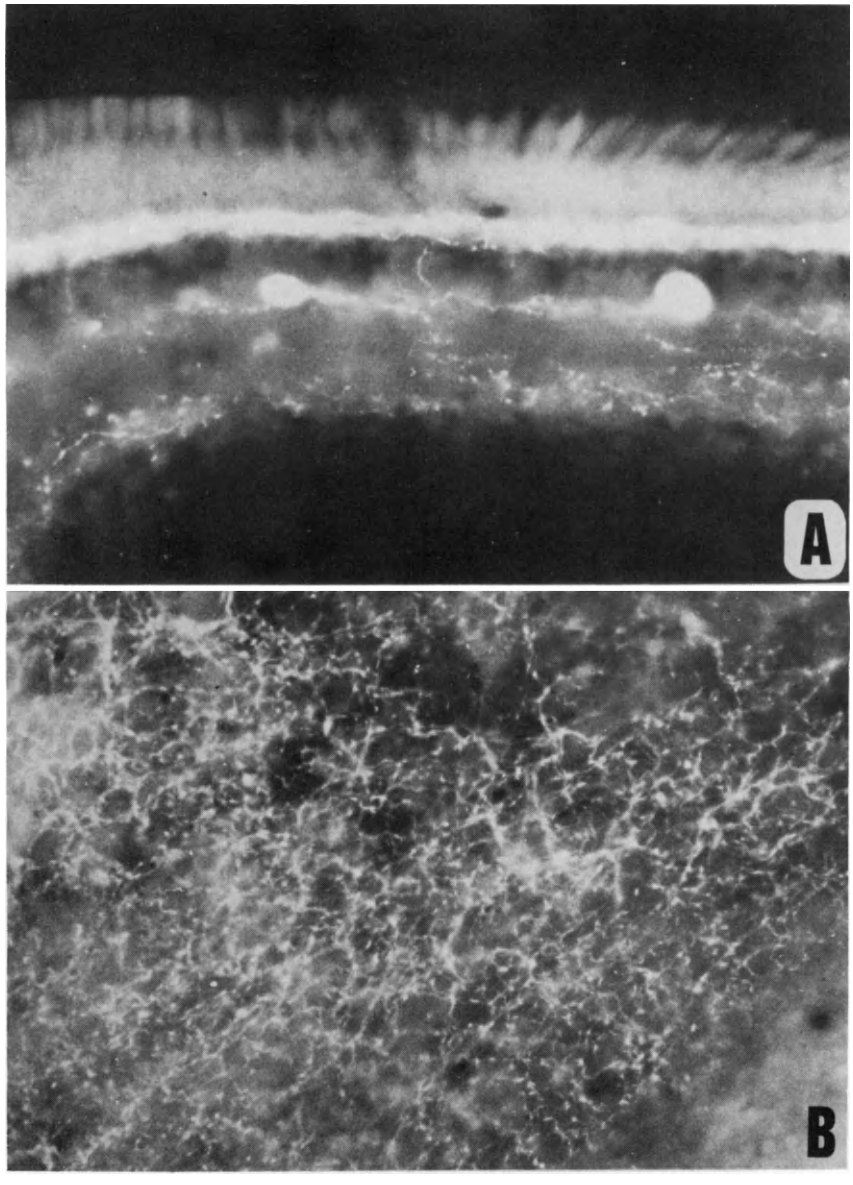

Fig. 1. Fluorescence micrographs of the fish retina. A. Radial section, showing the somata of two dopaminergic cells located at the innermost cellular row of the inner nuclear layer. They send fine processes towards both the outer part of the inner nuclear layer and the inner plexiform layer. The diameter of the larger fluorescent cell body is about 10 µm. B. Tangential section showing fine fiber network surrounding the horizontal cells.

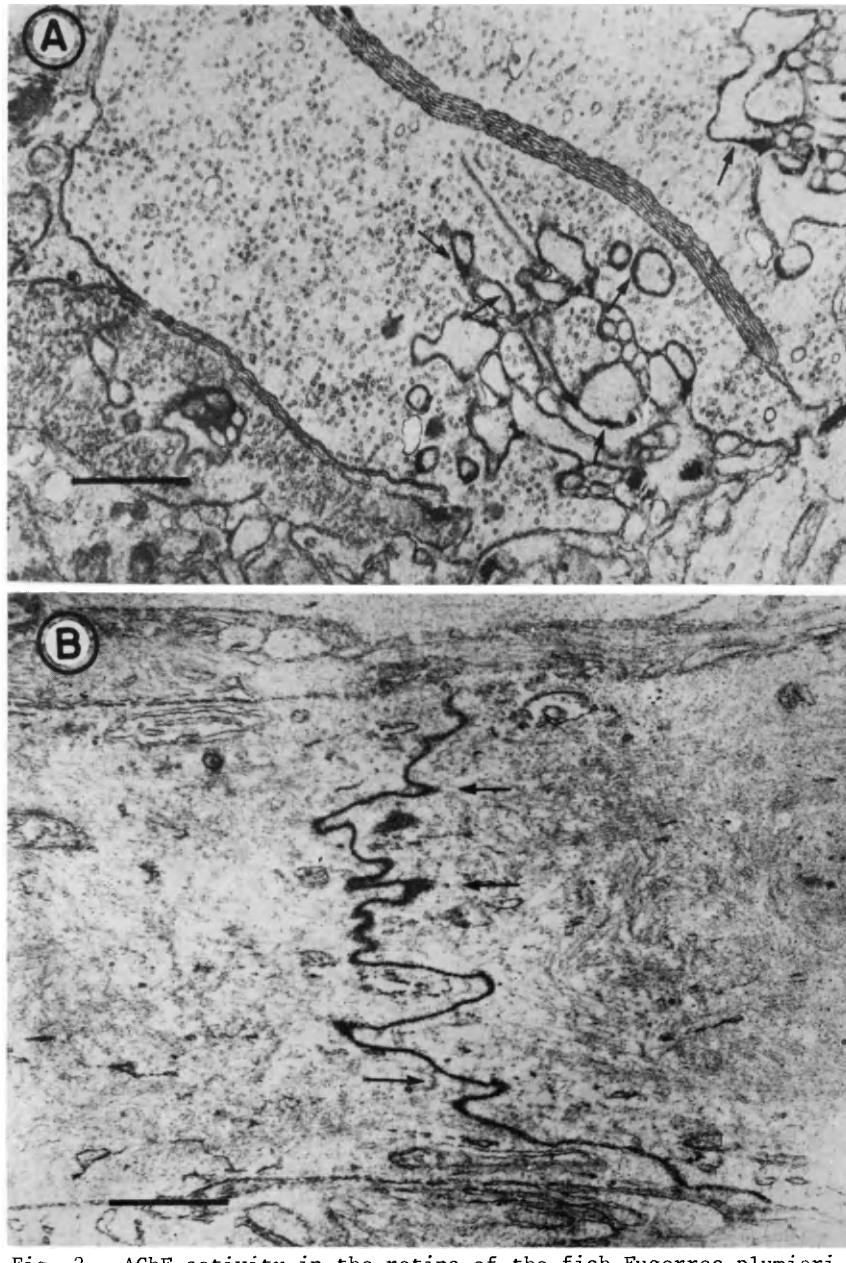

Fig. 2. AChE activity in the retina of the fish Eugerres plumieri. A. Reaction deposits in the synaptic region of the receptor cells. B. Activity of the enzyme at the lateral contact between the horizontal cells of a given layer.

Figure 3 summarizes the sites where AChE was found to be present in the outer retina.

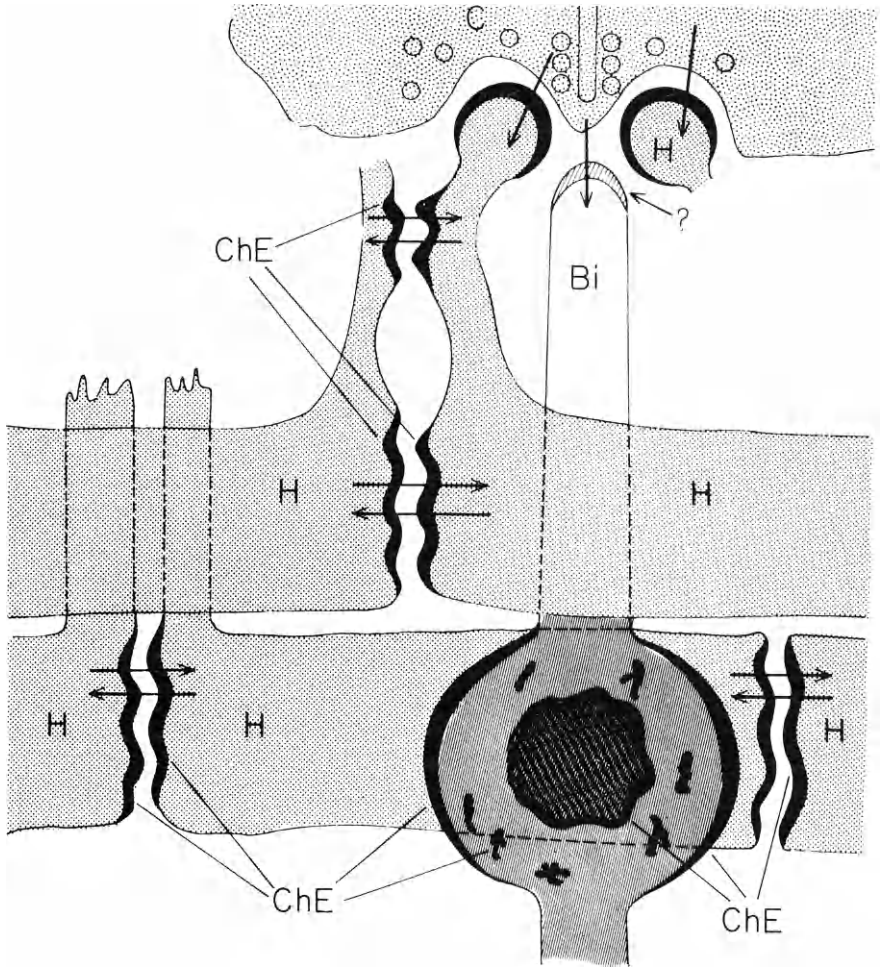

Fig. 3. General scheme of the localization of AChE in the outer retina. (H-horizontal cell, Bi-bipolar, ChE-colinesterase, R-receptor).

and turtle (Laufer, unpublished observations) retinas. It consists of a marked increase in the responses to the center flash and a simultaneous reduction of the responses to the annulus. These changes are usually, but not always, accompanied by depolarization of the cell. The effect is reversible, dose-dependent, and can be observed with dopamine, noradrenaline, and adrenaline, but not with an equimolar dose of L-DOPA. Figure 4 (A and B) illustrates these characteristic effects, produced by perfusion during 10 sec with a 200 μM solution of dopamine. In B, after 10 min perfusion with control Ringer solution the two responses have almost returned to their initial amplitudes. These effects of DA could be blocked by previous perfusion with 500 μM phentolamine.

Acetylcholine added to the perfusate produces a qualitatively similar result, although much higher doses are required and the effect is smaller, and the recovery

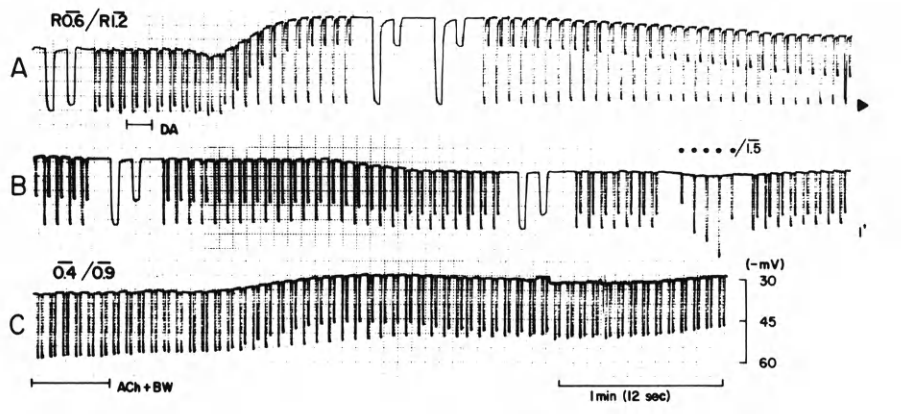

Fig. 4. The results obtained in the same cell by successive application of dopamine (DA, 200 µM) and a mixture of acetylcholine (ACh, 10 mM) plus the cholinesterase inhibitor BW (1 mM) show that the same qualitative effect is produced, namely, depolarization of the cell, increase of the center response and reduction of the surround response.

much faster. In Fig. 4C a perfusate containing 10 mM ACh, together with 1 mM of the AChE blocker BW was used for 30 sec and, again, an increase of the response to the center flash and a decrease of the response to the annulus is observed, with recovery after 2 to 3 min. These effects could be reduced by previous perfusion with 500 µM hexamethonium, as well as by 500 µM phentolamine (Negishi and Drujan, 1979c).

Figure 5 illustrates the above mentioned effects and their blockade by phentolamine. Typical effects can be seen, in response to both ACh (A) and dopamine (B). Phentolamine was perfused for 5 min prior to record D and, then, subsequent application of either agent was ineffective. Our tentative interpretation of this blocking effect by both drugs is that the effect of acetylcholine is mediated by a catecholaminergic mechanism, probably in the interplexiform layer where cells have been shown to contain DA.

In conclusion, both cholinergic and catecholaminergic systems appear to be present in the outer retina of several species. The study of the actions of compounds which belong to the two systems reveals that they have, at least qualitatively, similar effects. Furthermore, the results point to a direct effect on lateral integration of activity in the case of catecholamines, but an indirect effect of acetylcholine, via its action on interplexiform catecholaminergic cell.

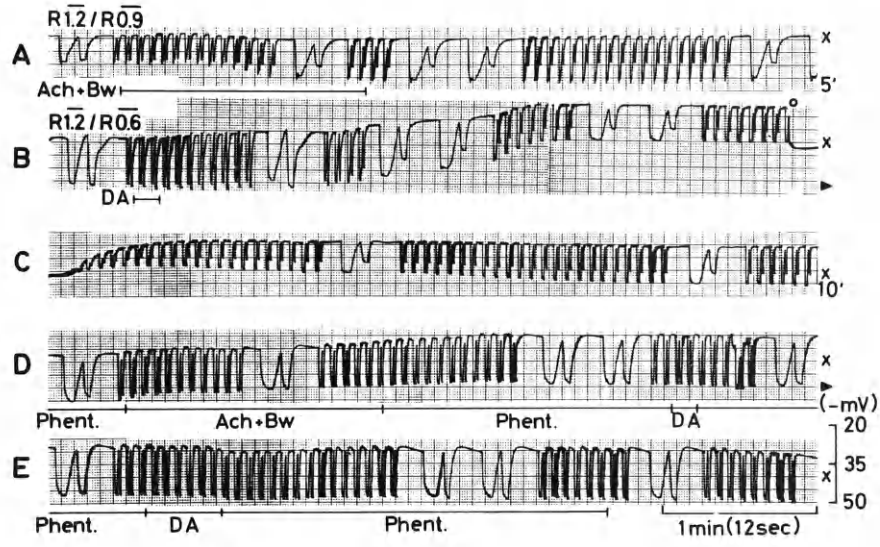

Fig. 5. The application of the adrenergic α-blocker phentolamine (Phent, 0.5 mM) interferes with both the effects of ACh plus BW and dopamine (DA).

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Neurochemistry Vol. 1, pp. 151-165.
Pergamon Press. Ltd. 1980. Printed in Great Britain.

THE LOCALIZATION AND METABOLISM OF GLUTAMATE, ASPARTATE AND GABA IN THE RAT RETINA

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ABSTRACT

Radiolabelled glucose and glutamine have been used to study the metabolism of the neuroactive amino acids glutamate, aspartate and GABA in the rat retina. Endogenous amino acids have been measured by double label dansylation. Significant increases in the concentrations of all three amino acids have been found on light-adaptation.

In vitro, glutamine is metabolized alongside glucose and, at 600 μM (the concentration in the rat vitreous), is a major precursor of the neuroactive amino acids. GABA is formed principally in the amacrine and inner plexiform layers of the tissue, whereas, in photoreceptor cells, glutamate and aspartate are heavily labelled. Evidence has been obtained for decreased turnover of all three amino acids on light stimulation. The data are consistent with a role for glutamate and/or aspartate as photoreceptor neurotransmitters. Glucose utilization is reduced in the light-stimulated retina.

KEYWORDS

Rat retina; photoreceptors; glucose and glutamine metabolism; light stimulation; aspartate; glutamate; GABA.

INTRODUCTION

Species differences in the homeostasis of γ-aminobutyric acid (GABA) in retinas are now well-recognized and were seen initially when uptake sites were compared by autoradiography - GABA entering predominantly, if not solely, into the glial cells of Müller in rat and primate retinas, and into various neurones in such species as frog, pigeon, chicken and goldfish (for reviews see Lam, 1975; Neal, 1976; Voaden, 1976; 1979). The differences can also be detected metabolically (Voaden, Lake and Nathwani, 1977). In both groups, however, a growing body of evidence attests to possible role(s) for GABA in retinal neurotransmission, associated with the functioning of higher order neurones (Voaden, 1979).

Glutamate and aspartate are less well studied as regards the inner retinal layers, but these amino acids have, for a long time, been favoured candidates to be photo-

receptor neurotransmitters (Neal, 1976; Voaden, 1979).

LOCALIZATION

All three amino acids exist in the retina at about the same levels as in the brain (Table 1). In the few species investigated (monkey, rat, frog and rabbit), GABA has been found to be most concentrated in the amacrine, inner plexiform and ganglion cell layers of the tissue (Fig. 1; Voaden, 1978; 1979), whereas glutamate and aspartate are present at higher levels in photoreceptor cells. However, distribution of these latter is more species variable. In the monkey the highest concentration of both is in the ganglion cell layer (Berger and colleagues, 1977), whereas in the rat it has been found more distally (Kennedy, Neal and Lolley, 1977; Morjaria and Voaden, 1979a).

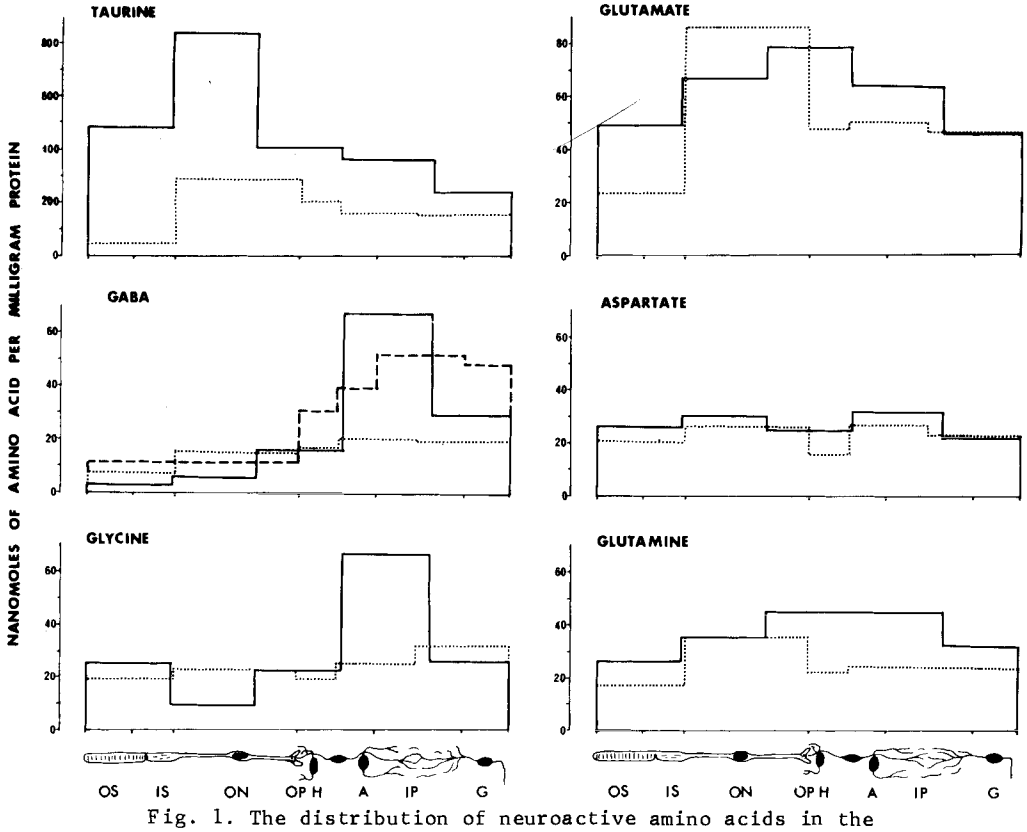

Fig. 1. The distribution of neuroactive amino acids in the rat retina.
The values have been obtained from Graham (1974) (— — —); Kennedy, Neal and Lolley (1977) (·····); and Morjaria and Voaden (1979a) (———). Recalculations have been based on 12% protein and 83% water contents for the retina. The retinal layers are : OS - photoreceptor outer segments; OP - outer plexiform layer; H - horizontal cell rich portion of the inner nuclear layer; A - amacrine cell rich portion of the inner nuclear layer; IP - inner plexiform layer; G - ganglion cell and nerve fibre layer.
Reproduced from Voaden (1979).

TABLE 1 The Concentrations of Glutamate, Aspartate and GABA in Light- and Dark-Adapted Retinas

Species		Glutamate D	Glutamate L	Aspartate D	Aspartate L	GABA D	GABA L
Rat	1.	4.7	5.9*	2.2	2.9*	2.5	2.9*
	2.	4.4	4.7	1.5	1.4	1.9	1.9
Mouse	3.	3.7	4.6*	1.2	1.4	1.4	1.7*
Chicken	2.	4.2	4.6	0.4	0.4	3.4	3.7
	4.	3.1	3.6	0.5	0.5	2.6	2.5
Frog	2.	4.0	3.9	0.8	0.7	2.3	2.7*
	5.	2.8	2.7			1.5	2.6*
Goldfish	2.	1.7	1.7	0.3	0.3	1.4	2.0*
	6.	3.8	3.9			1.7	3.2*
Marine fish	7.	2.8	3.1	0.3	0.2	1.1	1.2 1.4*
Brain		7.8 - 12.5		1.5 - 2.7		0.8 - 2.3	

D - dark-adapted; L - light-stimulated or adapted. Results are expressed as μmol / g wet wt. Recalculations have been based on a retinal water content of 85%, and protein of 10%.
*$p < 0.05$
Brain values are from McIlwain and Bachelard (1971), and retina from : 1. Table 2; 2. Starr (1973); 3. Cohen, McDaniel and Orr (1973); 4. Pasantes-Morales and colleagues (1973); 5. Graham, Baxter and Lolley (1970); 6. Lam (1972); 7. Van Gelder and Drujan (1978).

Effects of Light

Differences between light- and dark-adapted retinas have been sought in a range of species (cf. Table 1). The endogenous GABA level is increased in light-stimulated frog, goldfish, marine fish (flickering illumination only), mouse and, in our hands, rat retinas (see also Table 2), but is unaltered in the chicken. In contrast, in studies on whole retinas, changes in glutamate and/or aspartate have only been observed in the rodents. However, a rise in glutamate concentration has been detected in light-stimulated photoreceptor cells of the frog (Graham, Baxter and Lolley, 1970).

In the rat, the differences in all three amino acids are enhanced further when the animals are exposed to strong fluorescent light for 18 hr (Table 2; Oraedu, Voaden and Marshall, 1979). At the stage these retinas were analysed they appear normal, but the photoreceptor cells do go on to develop typical 'light-damage' lesions, even when the animals are returned to a normal environment. Nevertheless, increases have also been seen following 6 and 12 hr exposure periods, and these do not lead to overt symptoms (Oraedu, Marshall and Voaden, unpublished). In addition, we have never observed lesions in the inner retinal layers. The changes, although perhaps extreme are probably physiological, therefore. The concentration of GABA

decreases on continued exposure to the light (Oraedu, Voaden and Marshall, 1979).

TABLE 2 Aspartate, Glutamate and GABA in the Albino Rat Retina

Amino Acid	Dark-Adapted 48 hr (n=15)	Light-Adapted (n=60)	Fluorescent light+ 18 hr (n=18)
Aspartate	3.3±0.2	4.3±0.1***	5.9±0.2***
Glutamate	7.1±0.3	8.9±0.2***	11.1±0.6***
GABA	3.7±0.2	4.3±0.1*	5.3±0.3***

*$p < 0.05$; ***$p < 0.001$.
Amino acids were estimated by double label dansylation (Morjaria and Voaden, 1979a). Results are expressed as nmol/3 mm dia. disc retina±SEM (approximate wet wt. 1.5 mg).
+Data taken from Oraedu, Voaden and Marshall (1979). A photographic light box was placed on top of the cage and temperature was maintained below 25°C with a fan. Two animals were exposed at one time.

The above data supports the observation of changes in amino acid levels in normal light- as compared with dark-adaptation. It is, therefore, anomalous that Starr (1973) did not observe them in his study on rats. However, Cohen, McDaniel and Orr (1973) noted erratic changes in endogenous amino acid levels in mice, and both Starr (1973) and Neal (1976) have commented on the inconsistency of GABA uptake in light- as compared with dark-adapted rat retinas - increases occasionally being observed in the light-adapted tissue. Seasonal variation has been postulated by both authors as a possible explanation; differences appearing in the winter. The results from dark-adapted rats, shown in Table 2, were obtained in the summer months. Alternatively the extent of dark-adaptation may be relevant as the present results were obtained after 48 hr, whereas Starr (1973) and Cohen, McDaniel and Orr (1973) dark-adapted animals for 18-24 hr and 90 min respectively.

To localize further the reactive pools of amino acids, retinas of rats exposed for 18 hr to the light source have been bisected at approximately the outer plexiform layer (cf. Morjaria and Voaden, 1979a), and the two sections analysed. The results (Table 3) showed significant increases in aspartate, glutamate and GABA in the photoreceptor cells, and of glutamate and GABA in the inner retinal layers.

Glutamate and aspartate as photoreceptor neurotransmitters. A considerable body of evidence supports the conclusion that photoreceptor cells release their neurotransmitters in the dark and that this release is curtailed on light-stimulation (eg. Neal, 1976; Voaden, 1979). Equally it has been postulated many times that glutamate and/or aspartate might be the neurotransmitter(s). Most recently neurophysiological studies on goldfish (Kleinschmidt and Yazulla, 1978) and skate retinas (Wu and Dowling, 1979) have supported this contention - the latter providing strong evidence for aspartate being the likely compound in this species.

The present observations of raised glutamate and aspartate in rat photoreceptor cells on light stimulation are consistent with a curtailment of release, and, therefore, with the suggestion that one or other might be the neurotransmitter. In

TABLE 3 Endogenous Amino Acids in the Retinas of Rats Exposed for 18 hr to Fluorescent Light.

		Light-Adapted control (n=8)	Fluorescent Light+ 18 hr (n=8)
100 μm Photoreceptor Section	Aspartate	1.7±0.3	3.3±0.5*
	Glutamate	3.3±0.5	4.8±0.5*
	GABA	0.4±0.06	0.7±0.06**
Inner Retinal Layers	Aspartate	2.9±0.3	3.3±0.4
	Glutamate	5.7±0.5	6.9±0.7*
	GABA	2.8±0.3	5.1±0.4***

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.
Results are expressed as the mean±SEM and represent nmol / 3mm dia. disc of retina (estimations were done on 4 mm dia. discs and the data recalculated, cf. Table 2).
Animals were exposed as described in Table 2 and then bisected using the technique of Arden and Ernst (1971). Amino acids were estimated by double label dansylation.
+Data taken from Oraedu, Voaden and Marshall (1979).

addition, in uptake studies with extracellular concentrations of ^{3}H-glutamate or ^{3}H-aspartate of less than 5 μM, and thus with high-affinity uptake predominating (cf. Neal and White, 1978; White and Neal, 1976), a significant amount of label can be detected, by autoradiography, in rat photoreceptor cells (Marshall and Voaden, unpublished) - adding further support to the above premise.

GABA in photoreceptor cells. A small concentration of GABA has consistently been observed in the photoreceptor cell layer of the rat and other species (Fig. 1; Table 1; Voaden, 1978) - Morjaria and Voaden (1979a; cf. Fig.1) finding 7% of the total retinal GABA in an 80 μm section of this layer (Fig. 2). As well as photoreceptors, this fraction would also contain about 15% of the Müller cell cytoplasm of the tissue (Rasmussen, 1972). If all of the GABA was associated with this, and the amino acid had an even distribution through these cells, they would then contain approximately 45% of the total in the tissue. The GABA profile, shown in Fig. 1 argues against this as Müller cell cytoplasm reaches its peak contribution at the inner limiting membrane (Rasmussen, 1972). It is, therefore, probable that some GABA is associated with photoreceptor cells themselves (cf. Voaden, 1978). About 5% of the glutamate decarboxylase activity of the rat retina has also been found in this layer (Graham, 1974; Morjaria and Voaden, 1979a) and histochemistry has provided evidence for the presence, in photoreceptor inner limbs, of GABA-α-oxoglutarate transaminase (Hyde and Robinson, 1974; rat) and succinic semialdehyde dehydrogenase (Moore and Gruberg, 1974; salamander). Thus the GABA bypath may be present. It must be noted, however, that little or no glutamate decarboxylase or GABA-α-oxoglutarate transaminase activity has been found by Sarthy and Lam (1979) in isolated turtle photoreceptor cells and, with glutamate as precursor, no GABA synthesis detected (Sarthy and Lam, 1978). Species differences may exist.

METABOLISM

An increase in the concentration of a compound could result from increased synthe-

sis or decreased release. To investigate this radioactive precursors are needed, and in the following the effects of light stimulation on the metabolism of ^{14}C-glucose and ^{14}C-glutamine by rat retinas have been studied. Glucose is considered the main substrate of the retina, providing a source not only of energy but also of carbon atoms for the synthesis of other molecules (Lolley, 1969). Glutamine, which is a major metabolic product of neuroactive amino acids taken up by Müller cells (Riepe and Norenburg, 1977; Starr, 1975a; Voaden, Lake and Nathwani, 1977; White and Neal, 1976), is also readily available as a substrate. It is present in vitreous at 200-600 μM (Coull and Cutler, 1978; Morjaria and Voaden, 1979a) and autoradiography suggests that it can enter most, if not all cells of the rat retina (Voaden and colleagues, 1978).

Glucose and glutamine contribute equally in terms of carbon atoms, to the pools of glutamate and aspartate in the dark-adapted retina, whereas glutamine may be a better precursor of GABA (Table 4; Morjaria and Voaden, 1979a). The presence of glutamine has no effect on the entry of carbon atoms from glucose (Morjaria and Voaden, 1979a).

TABLE 4 The Relative Incorporation of Label from ^{14}C-Glucose and ^{14}C-Glutamine into Amino Acids in the Isolated, Dark-Adapted Rat Retina.

		Relative Specific Activity*	
		^{14}C-glucose +glutamine	^{14}C-glutamine +glucose
80 μm Photoreceptor Section	Aspartate	9.7	8.1
	Glutamate	23.7	20.3
	GABA	5.0	7.8
Inner Retinal Layers	Aspartate	7.2	6.6
	Glutamate	16.6	16.6
	GABA	4.5	8.4

*Specific activity of the amino acid relative to that of the precursor at the beginning of the incubation. For ^{14}C-glucose this was 11.77 X 10^{-3} dpm / nmol and for ^{14}C-glutamine 55.5 X 10-3 dpm / nmol. Glucose was present in both media at 5.5 mM and glutamine at 600 μM. Retinas were incubated for 30 min at 37°C with the labelled precursors and then 3 mm dia. discs of the tissue sectioned as described in Fig. 2. The labelled products were then isolated (Morjaria and Voaden, 1979a). The concentration of endogenous amino acids was estimated by double label dansylation in retinas incubated as above, but with non-radioactive substrates (cf. Table 7).

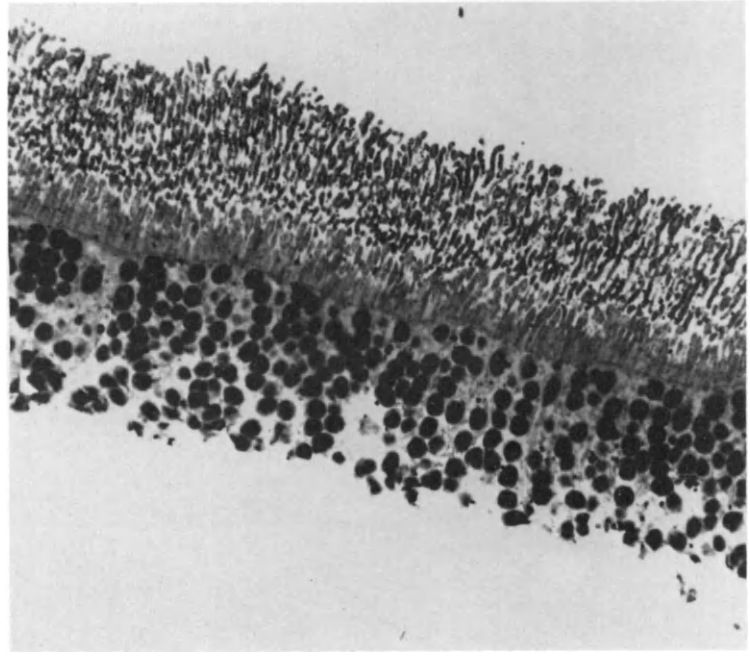

Fig. 2. An 80 μm section of rat photoreceptor cells (outer and inner segments plus nuclei) obtained by the technique of Arden and Ernst (1971).
A 3.0 mm dia. disc of retina was placed, photoreceptors downwards, into a 3.5 mm dia. well, 80 μm deep, drilled in a block of aluminium. The block was frozen and the protruding tissue sliced away. The above section was recovered from the well, and processed for light microscopy.

TABLE 5 Metabolism of ^{14}C-glucose and ^{14}C-glutamine by Light-Stimulated and Dark-Adapted Rat Retinas.

Amino Acids	^{14}C-glucose+glutamine D	L	^{14}C-glutamine+glucose D	L
Aspartate	25±3	23±0.3	62±3	32±3***
Glutamate	99±10	99±3	394±10	273±5***
Glutamine	14±1	12±0.3	720±21	700±18
GABA	18±2	15±1	157±13	107±3*
Total tissue Radioactivity	669-16	389-23***	1379-15	1145-13***

*$p < 0.05$; ***$p < 0.001$. D - dark-adapted 48 hr; L - light-stimulated (see page 8).
Results are expressed as the mean±SEM (n=4) and represent dpm X 10^{-3} / mg protein. Glucose was present in both media at 5.5 mM and glutamine at 600 μM. Retinas were incubated for 30 min at 37°C with ^{14}C-glucose (30 μCi/ml) or ^{14}C-glutamine (15 μCi/ml).
Data taken from Voaden and Morjaria (1979).

TABLE 6 The Metabolism of ^{14}C-Glutamine by Photoreceptor and Inner Retinal Layers of the Isolated Rat Retina.

		Dark-Adapted dpmX10^{-3} / amino acid	RSA	Light-Stimulated dpmX10^{-3} / amino acid	RSA
I	Aspartate	6.7± 0.9	8.1	3.3± 0.4**	3.2
	Glutamate	29.9± 2.8	20.3	18.4± 1.8**	9.9
	Glutamine	34.6± 3.2	34.3	35.7± 8.0	42.9
	GABA	4.9± 0.4	7.8	3.8± 0.7	6.8
	Total Radioactivity	80.5± 7.9		65.1± 1.2	
II	Aspartate	9.5± 0.9	6.6	4.8± 0.7***	3.5
	Glutamate	46.0± 4.1	16.6	29.4± 4.1*	10.3
	Glutamine	77.6± 6.3	41.1	60.7± 6.8	33.4
	GABA	28.7± 1.6	8.4	16.6± 1.3***	4.5
	Total Radioactivity	178.4±13.0		120.2±13.4*	

*$p < 0.05$; **$p < 0.02$; ***$p < 0.01$; I - 80 μm photoreceptor section; II - inner retinal layers.
Results are expressed as the mean±SEM (n=4) and represent radioactivity per 3 mm dia. disc retina. Experimental conditions were as in Tables 4 and 5. RSA = specific activity of the amino acid relative to that of the precursor at the beginning of the incubation.
Data taken from Voaden and Morjaria (1979).

Effects of Light

Approximately 95% of the photoreceptor cells of the rat retina are rods (Cicerone, Green and Fisher, 1979). Although they are highly insensitive to red light, they are saturated by very low levels of shorter wavelength illumination (λ_{max} rhodopsin 500 nm). For the present experiments, therefore, daylight was reduced to $\ll 0.15$ μwatts / sq. metre. In terms of human vision this corresponds to the low mesopic range. Eight to ten week-old female albino Wistar rats were used throughout the study. They were dark-adapted for 48 hr before use and were then killed and the retinas dissected out under a dim red light. Incubations were done either in darkness or in the reduced daylight and the relevant lighting maintained until the tissue was frozen (for bisection) or added to a protein precipitant.

As shown in Table 5, when the metabolism of ^{14}C-glucose was followed under these conditions and the retinas processed after only a 2 min wash to avoid loss of soluble compounds, there was a 40% reduction in total tissue radioactivity on light stimulation, but no change in the entry of label into the amino acids. In contrast, when ^{14}C-glutamine was metabolised, light caused a significant decrease in the labelling of aspartate, glutamate and GABA. There was also an 18% reduction in the total tissue radioactivity, accounted for by the reduced label in the amino acids.

In previous in vivo studies with the glucose analogue 2-deoxy-glucose, Morjaria and Voaden (1979b) have also obtained results which imply a decrease in the rate of glucose consumption by light- as compared with dark-adapted rat retinas (the former being maintained in normal room lighting). The decrease (38%) occurred in the photoreceptor cell layer.

To localize further the changes in amino acid metabolism, retinas, incubated with the radioactive precursors, were sliced to a depth of 80 μm from the photoreceptor side (cf. Fig. 2). Table 6 shows that with ^{14}C-glutamine as precursor, light reduced the entry of the label by 51% and 39% respectively into aspartate and gluta-

TABLE 7 Endogenous Amino Acids in Isolated Rat Retinas Incubated with both Glucose and Glutamine as Substrates.

	80 μm Photoreceptor Section		Inner Retinal Layers	
	D	L	D	L
Aspartate	1.5 ± 0.2	1.8 ± 0.2	2.6 ± 0.4	2.5 ± 0.3
Glutamate	2.7 ± 0.2	3.4 ± 0.4	5.0 ± 0.6	5.1 ± 0.8
Glutamine	1.8 ± 0.2	1.5 ± 0.3	3.4 ± 0.4	3.3 ± 0.4
GABA	1.1 ± 0.1	1.0 ± 0.1	6.2 ± 1.0	6.7 ± 0.8

D - dark-adapted 48 hr; L - light-stimulated (see page 8). Results are expressed as the mean ± SEM ($n \geq 7$) and represent nmol / 3 mm dia. disc retina. Retinas were incubated at 37°C for 30 min with 5.5 mM glucose and 600 μM glutamine. Discs of tissue were then sectioned as described in Fig. 2. Endogenous amino acids were measured by double-label dansylation (Morjaria and Voaden, 1979a).

mate in the photoreceptor layer, and into these amino acids and GABA in the inner retinal layers. When the endogenous amino acids were analysed in retinas treated similarly but incubated with unlabelled substrates, there were no significant differences between the light- and dark-adapted tissue (Table 7), although trends towards the changes seen between freshly-isolated light- and dark-adapted retinas (Table 2) were apparent. These results imply that changes occur in amino acid turnover on light-stimulation, followed later by adjustment in the tissue levels of the endogenous amino acids.

Increased tissue levels combined with decreased entry of label would in turn imply decreased turnover of glutamate and aspartate in photoreceptor cells and of glutamate, aspartate and GABA in the inner retinal layers of the rat retina on light stimulation. The changes in photoreceptor metabolism are consistent, therefore, with a role for glutamate and/or aspartate in photoreceptor transmission (cf. page 4). As the inner retinal sections would have contained photoreceptor terminals, it is not possible to say definitely whether or not the changes observed here are also associated with photoreceptor function. However, results from a more detailed profile of the entry of label from ^{3}H-glutamine into these amino acids suggests not (Morjaria and Voaden, 1979a). The profile also suggests that the principal location of GABA formation from glutamine is in the amacrine/inner plexiform layers of the tissue. This is consistent with (1) the position of labelled cells seen after a pulse and chase study with ^{3}H-glutamine, in which GABA was the principal end product (Voaden and colleagues, 1978), (2) the location of endogenous pools of GABA in the rat retina (Fig. 1) and (3) the tentative localization of glutamate decarboxylase to amacrine synaptic terminals (Wood, McLaughlin and Vaughn, 1976;

TABLE 8 The Metabolism of ^{14}C-Glucose by Photoreceptor and Inner Retinal Layers of the Isolated Rat Retina.

		Dark-Adapted dpmX10^{-3} / amino acid	RSA	Light-Stimulated dpmX10^{-3} / amino acid	RSA
I	Aspartate	1.7±0.2	9.7	1.3±0.2	5.9
	Glutamate	7.4±1.0	23.7	6.8±1.4	17.3
	Glutamine	0.6±0.1	2.9	0.7±0.1	4.0
	GABA	0.7±0.2	5.0	0.8±0.1	7.0
	Total Radioactivity	19.3±1.9		16.4±2.6	
II	Aspartate	2.2±0.3	7.2	1.9±0.2	6.3
	Glutamate	9.8±1.6	16.6	8.6±1.2	14.2
	Glutamine	2.0±0.3	5.0	1.6±0.2	4.0
	GABA	3.3±0.7	4.5	2.7±0.4	3.4
	Total Radioactivity	38.3±6.5		35.1±2.9	

I - 80 μm photoreceptor section; II - inner retinal layers. Results are expressed as the mean±SEM (n=4) and represent radioactivity per 3 mm dia. disc retina. Experimental conditions were as in Tables 4 and 5. RSA = specific activity of the amino acid relative to that of the precursor at the beginning of the incubation.
Data taken from Voaden and Morjaria (1979).

cf. also Graham, 1974). It thus seems probable that changes in GABA turnover are occurring here. Although the present results suggest that this is decreased turnover, the lowered specific activities might also result from the release of newly-formed, more highly-labelled pools that have not equilibrated with the general tissue stores. The preferential release of neuroactive amino acids derived from glutamine as compared with those from glucose has been found in studies on rat cortical synaptosomes (Bradford, Ward and Thomas, 1978), pigeon optic tectum (Reubi, Van den Berg and Cuenod, 1978) and rabbit hippocampus (Hamberger and colleagues, 1979). As yet, no effects of light stimulation on endogenous GABA release from the rat retina have been found (Coull and Cutler, 1978; Kennedy and Neal, 1978; Starr, 1975b), although Bauer and Ehinger (1977) reported an increased output of preloaded labelled GABA from the rabbit retina. Species differences undoubtedly exist in the response of retinal GABA systems to light as Starr (1975b), using ^{14}C-glucose as the radiolabelled precursor and working with the frog retina *in vivo*, found increased entry of label into GABA, combined with raised endogenous levels, suggesting increased turnover; less label entered or remained in glutamate. In the same study no effects were seen in the rat, perhaps because ^{14}C-glucose was used as the precursor (cf. Table 5).

Little is known of the function(s) of GABA in the rat retina. It is not possible, therefore, to link the changes observed here with a physiological role. However, Graham (1974) has provided evidence suggesting that this amino acid may be involved with light-adaptation over the scotopic visual range.

Retinas incubated with ^{14}C-glucose were also bisected : the results are shown in Table 8. Again, as with the data in Table 5, entry of label into the amino acid pool is not significantly altered by light-stimulation. However, because of slight changes in labelling of the photoreceptor layer, combined with subtle alterations in the endogenous amino acids there (Table 7), the specific activities of glutamate and aspartate are decreased - about half as much as when ^{14}C-glutamine was the precursor. Complex changes may occur in glucose metabolism on light-stimulation of photoreceptors (eg. Cohen and Noell, 1965). Consequently, it cannot be concluded that glucose and glutamine are here labelling separate pools of the amino acids. However, the data for the 'inner retinal sections' (Tables 6 and 8) do suggest the existence of separate pools. The profiles of GABA production from ^{14}C-glucose and ^{3}H-glutamine through the rat retina are very similar, but differences exist in those for glutamate and aspartate (Morjaria and Voaden, 1979a).

In contrast to the data shown in Table 5, there was no evidence for a decrease in total tissue radioactivity, on light stimulation, in retinas incubated with ^{14}C-glucose and then processed for slicing. The non-amino acid, 'light-sensitive', glucose derivatives may, therefore, have been washed from the tissue.

Less ATP may be needed in the light-stimulated photoreceptor because of cessation of the sodium current that flows between the inner and outer limb of the dark-adapted cell, and the consequent decrease in Na^+/K^+ ATPase activity in the inner limb. It has also been proposed that the tricarboxylic acid cycle (TCAC) will be inhibited by a build-up of 6-phosphogluconate, occurring because of stimulation of the pentose phosphate pathway (Cohen and Noell, 1965). It is significant, therefore, that oxygen uptake is depressed in photoreceptors subjected to steady illumination (Hanawa and Kuge, 1961; Sickel, 1972), and possible that the 'light-sensitive' derivatives observed here (Table 5) are TCAC intermediates.

Under similar conditions succinoxidase activity in photoreceptor inner limbs may increase (Epstein and O'Connor, 1966). It is, therefore, of interest that the GABA bypath may be present there (cf. page 5), forming a route by which carbon

atoms could enter the TCAC at the level of succinate. However, in the present studies no significant changes were observed in GABA turnover in photoreceptor cells on light stimulation (Tables 6 and 8).

The present results emphasize the potential importance of glutamine in retinal metabolism and show changes in the homeostasis of glutamate, aspartate and GABA in the rat retina in response to light. In particular, the data obtained is consistent with a role for glutamate and/or aspartate as photoreceptor neurotransmitters.

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Neurochemistry Vol. 1, pp. 167-181.
Pergamon Press Ltd. 1980. Printed in Great Britain.

GABA AND DOPAMINE RECEPTOR BINDING IN RETINAL SYNAPTOSOMAL FRACTIONS

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ABSTRACT

Binding sites for a variety of agonists and anatagonists of GABA and dopamine were demonstrated in retina subcellular fractions. These sites exhibited the binding kinetics and pharmacologic specificity consistent with known properties of the physiological receptors for GABA and for dopamine. Important differences noted between brain and retinal receptors will be discussed.

KEYWORDS

GABA receptor; dopamine receptor; spiroperidol; subcellular fractionation; adenylate cyclase.

INTRODUCTION

The current interest in neurotransmitters of the retina has been generated in part by recent advances in techniques for analyzing transmitter systems. Previously the retinal transmitter systems were studied extensively and almost exclusively by autoradiographic and electrophysiological methods. Using these techniques, most of the neurotransmitter compounds in brain have been implicated in retinal function. These include dopamine, gamma aminobutyric acid (GABA), acetylcholine, serotonin, glycine glutamate, aspartate and others which are less well-established such as taurine (for a review, see Graham, 1974). Autoradiography has been used to identify cell types, presumably the presynaptic neuron, which takes up these compounds or their precursors. Electrophysiology has to some degree identified cells, presumably the postsynaptic neurons, which respond to these neurotransmitters.

Biochemical analyses can be highly useful as a complement to these studies. Methods are now available for determining uptake, storage, release and receptor binding characteristics of these transmitters in vitro. Pharmacological sensitivity can be carefully determined and comparisons made between retina and other neuronal tissues such as brain, spinal cord and sympathetic ganglia.

One of the major problems in the biochemical studies involving retinal neurotransmitters has been the relatively small amounts of retinal tissue which could be obtained in a fresh condition. In the studies described herein, one approach has been

to develop techniques which can accommodate small sample sizes. A second approach has been the use of bovine retinas which have not previously been well-characterized, but which we were able to obtain in large quantities in a very fresh state from a local slaughter house.

Receptor binding assays are a relatively new concept in the field of neurochemistry, however, they are now in common use by many laboratories. The concept is a simple one based on the expected characteristics of the physiologically relevant receptor site for each neurotransmitter. (For a review, see Yamamura, Enna and Kuhar, 1978). Receptor sites should exhibit a very high affinity for the appropriate neurotransmitter and should have limited capacity (saturation). Established agonists and antagonists for the transmitter should inhibit receptor binding at concentrations which are consistent with their actions in vivo. The affinity and saturation characteristics are usually determined by generating a "binding curve", which is based on individual measurements of the amount of neurotransmitter bound by a given amount of tissue in the presence of different concentrations of the neurotransmitter. Ideally, the amount bound increases as the concentration of the ligand increases up to a point at which the receptor sites become saturated. The amount bound at the saturation point can be used to determine the approximate number of receptor sites; the concentration of ligand at which saturation occurs can be used to determine the approximate affinity of the receptor site. Scatchard, Lineweaver-Burke or Hill plots are routinely used to analyze data from binding curves and to establish K_m (affinity) and B_{max} (number of receptors).

Binding of neurotransmitters to a variety of other sites (uptake sites, membrane-bound enzyme sites, non-specific binding sites, etc.) is of major concern in these studies since these other sites usually far exceed the number of the receptor sites. In order to obviate this problem, binding of radioactively labeled transmitter is determined both in the presence and absence of saturating concentrations of unlabeled transmitter. Binding which occurs in the presence of excess unlabeled transmitter is thought to be "non-specific" since it is nondisplaceable, i.e., non-saturating. The amount of non-specific binding is subtracted from the total amount bound in the absence of excess transmitter. The resulting difference is termed specific binding and should be equivalent to receptor binding. However, the specific binding must be further verified by analyzing the ability of various concentrations of specific agonists and antagonists to inhibit the binding. The concentrations of agonist or antagonist required to inhibit transmitter binding to the receptor by fifty percent (IC_{50}) is usually determined and compared to other in vivo systems. Inhibitory constants (K_i) can also be determined by taking into account the concentration of transmitter used in determining the IC_{50} and thus perhaps represents a better form by which to make comparison (Chang and co-workers, 1975).

Specific receptor assays have been developed for the GABA receptor and the dopamine receptor in brain (Enna and Snyder, 1975; Fields and co-workers, 1977; Creese and co-workers, 1977). We have modified these techniques by miniaturization which has allowed the characterization of dopamine and GABA receptors in retinal tissue.

As previously reported in brain, receptors for GABA and dopamine in retina are highly concentrated in membranes from synaptosomal fractions. Thus most of the studies reported here have utilized synaptosomal fractions rather than whole retinal homogenate. Most of the small conventional sized synaptosomes from retina or brain can be obtained in a P_2 fraction which sediments during centrifugation at 15,000 xg for 12 min (Cotman and Matthews, 1971). In brain, larger particles such as nuclei and unbroken cells are usually sedimented in a P_1 or debris pellet using lower speeds (5,000 xg for 10 min.) prior to the centrifugation step for the P_2 fraction. One

peculiar aspect of retinal subcellular fractionation is that synaptosomes from photoreceptor cells are unusually large (3-5 μ in diameter) and thus sediment in the P_1 or "debris" pellet. We have devised a modified fractionation procedure for the retina in which the large, photoreceptor cell synaptosomes (PCS) are sedimented in a separate fraction intermediate between the P_1 and P_2 fractions (Redburn, 1977; Redburn and Thomas, in press).

Although the PCS fraction contains more contaminants than the P_2 fraction, it is enriched in synaptosomes; in particular those from photoreceptor cells. These two retinal synaptosomal fractions have been characterized as to the uptake, storage and release properties associated with specific retinal neurotransmitters (Redburn, 1979).

This fractionation technique may represent at least a crude separation of synaptosomes of the inner plexiform layer (P_2) from those of the outer plexiform layer (PCS). Some data support this suggestion. In some cases where a specific neurotransmitter is known to be restricted to a single plexiform layer the synaptosomal fractions also show a high degree of specificity. The appropriate fraction displays biochemical characteristics for that neurotransmitter. For example, autoradiography and histofluorescence shows dopamine to be present only in the IPL of rabbit retina and only the P_2 or IPL fraction from rabbit retina shows uptake, release and receptor binding activity for dopamine. In other cases, such as GABA and acetylcholine, also thought to be restricted to the IPL rabbit retina (Masland and Livingston, 1976; Graham, 1972), results are less clear since the PCS fraction does display some GABAnergic and cholinergic properties (Redburn and co-workers, 1978).

DOPAMINE RECEPTORS IN RABBIT AND BOVINE RETINA

Considerable evidence has been published in support of dopamine as a retinal neurotransmitter. In rabbit retina, fluorescence and autoradiography studies show convincingly that a specific subset of amacrine cells contain dopamine and are able to synthesize and degrade it (Dowling and Ehinger, 1978). These same cells also concentrate exogenously applied dopamine and release it upon light stimulation (Kramer, 1971). Dopaminergic amacrine cells appear to make pre and postsynaptic contact exclusively with other amacrine cells via conventional sized, non-ribbon containing synapses.

Biochemical analysis of pre and postsynaptic markers for dopaminergic systems provide additional insight into the molecular physiology of this important retinal transmitter substance. In rabbit, the dopamine system is largely associated with a specific synaptosomal fraction which is highly enriched in synaptosomes from amacrine and perhaps to a lesser extent from horizontal and bipolar cells. A high affinity uptake system for dopamine is enriched in this fraction as is dopamine-sensitive adenylate cyclase (see below) and Ca^{++} dependent, K^+ stimulated release mechanisms (Thomas and co-workers, 1978). We now report the concentration of high affinity receptor sites for dopamine in an identical fraction using a (^{3}H)-spiroperidol binding assay (Fields and co-workers, 1977; Creese and colleagues, 1977).

Spiroperidol is a butyrophenone neuroleptic whose affinity for dopamine receptors in brain tissue is greater than that of any other known drug (Burt and others,1977). In addition, (^{3}H)-spiroperidol has been found to label dopamine receptors *in vivo* and *in vitro* (Creese and co-workers, 1977). Due to the relatively large amount of material available from beef sources, a more complete pharmacologic study of the retinal dopamine receptor was made using bovine retina. However, results suggest

that rabbit and bovine retinal dopamine systems share many kinetic and pharmacologic properties.

The binding characteristics of bovine and rabbit retina appear to be similar to those previously reported for brain (Creese and co-workers, 1977; Fields and colleagues, 1977). The binding of (^{3}H)-spiroperidol was saturable at n Molar concentrations. Scatchard analysis revealed only a single dissociation constant of approximately 0.3 nM which compares to 0.25 nM reported for the highly dopaminergic brain region, the striatum.

The ability of a variety of receptor antagonists to inhibit (^{3}H)-spiroperidol binding indicated a high degree of pharmacological specificity associated with the receptor. (See Table 1).

Potent antipsychotic drugs such as fluphenazine and haloperidol which are also potent dopamine antagonists were able to displace receptor binding at n Molar concentrations. Agonists such as dopamine and the inactive (-) stereoisomer of the antagonist (+) butaclamol were less potent displacers.

Calculated K_i values for these displacers in retina were roughly equivalent to those reported in caudate and anterior pituitary from rat brain (Fields and colleagues, 1977). However, the K_i values in brain were slightly lower and ranged from 1.2-6.7 nM with (+) butaclamol being the most active displacer. In retina, K_i values ranged from 3 to 8 nM with (+) butaclamol being the least active displacer. Despite these differences, the pharmacological specificity of the retinal receptor, based on these studies, appears to be remarkably similar to the striatum receptor.

TABLE 1 Inhibition of (^{3}H)-Spiroperidol Binding to Rat Brain and Bovine Retina

The concentrations of drugs required to inhibit specific binding by 50% (IC_{50}) were determined from log probit plots and converted to K_i values according to the equation $K_i = IC_{50}/(1+c/K_d)$ where c is the concentration of (^{3}H)-spiroperidol and K_d its dissociation constant. (Chang and co-workers, 1975).

Drugs	*Brain, K_i (nM)		Retina, K_i (nM)	
	Caudate	Anterior Pituitary	OPL	IPL
Haloperidol	6.7	4.0	5.2	3.1
Fluphenazine	2.4	2.6	6.6	3
(+) Butaclamol	1.4	1.2	8	4
(-) Butaclamol	20,000	12,000	>1,000	> 1,000
Dopamine	36,000	9,000	>1,000	> 1,000

* From Creese and colleagues (1977).

Receptor density was also calculated by Scatchard analysis and equalled approximately 190 pmol/gm protein. Reported values for human, bovine and rat caudate range from approximately 300-500 pmol/gm protein; whole rat brain receptor density is roughly 150 pmol/gm protein (Fields and others, 1977).

Specific binding activity at 0.5 nM (^{3}H)-spiroperidol binding has been reported for a number of structures (Creese and co-workers, 1977) and although based on a single measurement rather than a complete analysis such as B_{max} or receptor density calculations, it does provide a useful comparison of the relative concentrations of dopamine receptors. By this comparison, bovine retina synaptosomes contain a receptor concentration roughly equivalent to frontal cortex but one-third that of the striatum (Table 2). This finding is in good agreement with a brief report by Rosenfeld and others (1979) in which (^{3}H)-spiroperidol binding in bovine, monkey and rabbit retina was about one-third that detected in striatum.

Receptor sites have clearly been shown to be concentrated in synaptosomes fractions and are presumably located on postsynaptic membranes which remain attached to presynaptic endings during synaptosome formation. However, receptor sites are also found in other subcellular fractions such as the so-called "debris" pellet, presumably associated with the plasma membrane of cell body fragments and in the microsomal fraction presumably associated with dendritic processes. Thus, the exact source of the dopamine receptor described in this study cannot be identified with certainty. However, it is clear that dopamine receptors in the rabbit retina are most concentrated in a synaptosomal fraction highly enriched in conventional sized synaptosomes from IPL. In addition presynaptic markers for dopamine, i.e., uptake and release systems, are also concentrated in this fraction.

TABLE 2 Specific Binding of (^{3}H)-Spiroperidol to Membrane Fractions From Rabbit Retina and From Regions of Rat Brain

Tissue Source	f mole/mg protein
Rabbit retina homogenate	57
Rabbit retina OPL synaptosomes	50
Rabbit retina IPL synaptosomes	88
Rat corpus striatum*	546
Rat frontal cortex*	132
Rat hypothalamus*	42
Rat cerebellum*	12

* From Fields and co-workers (1977)

DOPAMINE RECEPTORS IN GOLDFISH RETINA

Unlike most mammalian retinas examined, dopaminergic neurons of certain teleost and primate retinas appear to be a class of interplexiform cells which make synaptic contacts in both outer and inner plexiform layers (Dowling and Ehiner, 1978; Sarthy and Lam, 1979). In the goldfish retina, electron microscopic and auto-

radiographic studies indicate that most presumptive dopaminergic neurons are interplexiform cells which contact some cone horizontal cells and bipolar cell dendrites in the outer plexiform layer (Dowling and Ehinger, 1978; Sarthy and Lam, 1979). Unfortunately, fractionation procedures developed for mammalian retinas have proved unsuccessful in goldfish retina. Thus our studies of dopamine receptors have only utilized membranes from whole retinal homogenate.

Washed membranes from goldfish retinal homogenates bound a variety of dopamine agonists and antagonists with high affinities and with characteristics similar to those reported for the brain and mammalian retina.

Scatchard analysis of (^{3}H)-spiroperidol binding revealed only a single dissociation constant of approximately 0.23 nM which is similar to 0.3 nM in bovine retinal synaptosomes and for homogenates from the highly dopaminergic brain region, the striatum. Receptor density was also calculated by Scatchard analysis and equalled approximately 37 pmol/gm protein which is considerably less than values of 300-500 pmol/gm protein reported for human, bovine and rat caudate homogenates (Fields and co-workers, 1977).

Based on specific binding activity at 0.5 nM (^{3}H)-spiroperidol, the relative concentrations of dopamine receptors in goldfish, rabbit and bovine retinal homogenates are remarkably similar (37, 57 and 48 pmol/gm protein respectively). Washed membrane fractions from goldfish brain also demonstrated a high affinity binding site for (^{3}H)-spiroperidol which was displaced by 0.1 μM (+) butaclamol. The apparent K_d was 0.85 and the B_{max} was 66 pmol/gm protein as compared to a K_d of 0.8 nM and a B_{max} of 152 pmole/gm protein in rat brain (Fields and co-workers).

The calculated inhibition constants (K_i) for selected dopamine agonists and antagonists in the goldfish retina were similar to those reported for caudate and anterior pituitary from rat brain and mammalian retina. In the retina, K_i values ranged from 0.9 to 13 nM with fluphenazine being the most active. As with rabbit and bovine retina, the pharmacological specificity of the goldfish retinal dopamine receptor appears to be remarkably similar to that in the striatum.

Although butyrophenones such as spiroperidol and (+)butaclamol have a very high affinity for the dopamine receptor, there are reports that at higher concentrations they also bind to serotonin receptors (Leysen and others, 1978). In the experiments reported here, low concentrations were used for both the ligand, (^{3}H)-spiroperidol, and the displacer, (+)butaclamol, which should assure that most of the displaceable binding measured was associated with the dopamine receptor. As a further test, an additional compound ADTN, which binds only to dopamine receptors, was used as displacer in the retina. The amount of (^{3}H)-spiroperidol displaced by 10 nM ADTN and 0.1 μM (+) butaclamol were similar. These data suggest that most of the displaceable (^{3}H)-spiroperidol binding measured under our conditions was associated with the dopamine receptor.

DOPAMINE-STIMULATED ADENYLATE CYCLASE IN RETINA

In the brain, there is considerable evidence that some postsynaptic actions which are stimulated by dopamine-receptor interaction are in fact mediated via the activation of adenylate cyclase (Kebabian and co-workers, 1972). It has been suggested that dopamine receptors can be divided into two classes, one which is associated with adenylate cyclase (D_1); the other which is not (D_2)(Kebabian and Calne, 1979).

Most dopamine agonists and antagonists such as (^{3}H)-spiroperidol bind both classes of receptors. In an attempt to further characterize the dopamine receptor, retinal adenylate cyclase activity was analyzed in the presence and absence of dopamine in a retina containing dopaminergic-amacrine cells (rabbit) and in a retina containing dopaminergic-interplexiform cells (goldfish).

In the rabbit retina, dopamine produced a dose dependent increase in cyclic AMP formation (Table 3). A fifty percent increase in enzyme activity was observed with 100 μM dopamine which is very similar to that seen in brain (Clement-Cormier and others, 1974; Thomas and co-workers, 1978; Clement-Cormier and Redburn, 1978). Other dopamine agonists such as apomorphin, N-methyldopamine (epinine) and 2 amino-6,7, dihydroxy-1,2,3,4-tetrahydronaphthalene (ADTN) were also effective stimulators of adenylate cyclase.

The dose response curve for dopamine-stimulated adenylate cyclase activity in homogenates of the goldfish retina indicated a half maximal increase in enzyme activity with 2 μM dopamine and a maximum response with 10 μM dopamine. The absolute number of picomoles of cyclic AMP formed above basal levels in response to dopamine stimulation, 25 pmol/mg protein/min, is comparable to that seen in rabbit retina (34 pmol/mg protein/min). Based on this calculation, it would appear that goldfish and rabbit retinas have roughly the same number of receptor-cyclase complexes, i.e., D_1 dopamine receptor sites.

TABLE 3 Effect of Dopamine Agonists on Adenylate Cyclase Activity in Subcellular Fractions of the Rabbit Retina

	Concn (μ M)	Adenylate cyclase activity (pmoles cAMP/ mg protein/ 1 min)		
		Homogenate	P_1 fraction	P_2 fraction
Rabbit:				
Control		39.05	37.75	100
Dopamine	100	73.50	38.25	15.075
Apomorphine	100	70.00	37.40	15.803
Epinine	100	73.20	38.01	16.015
ADTN	100	82.00	37.67	18.000
Goldfish:				
Control		2.5		
Dopamine	100	25.00		

TABLE 4 Calculated Inhibition Constants for Various Drugs on Dopamine-Stimulated Cyclic AMP Production*

Drug	Rabbit P_2 K_i (M)	Goldfish Homogenate K_i (M)
Fluphenazine	5.5×10^{-9}	2×10^{-9}
Chlorpormazine	5×10^{-8}	-
Thioridazine	1×10^{-7}	-
(+) Butaclamol	4.5×10^{-8}	4×10^{-8}
(-) Butaclamol	$> 10^{-6}$	10^{-5}
(α) Fluphenthixol	1.0×10^{-9}	-
(β) Fluphenthixol	$> 10^{-6}$	-

* Reported K_i values were calculated from the relationship $IC_{50} = K_i (I + S/K_m)$, where S is the concentration of dopamine (100 μM) and K_m is the concentration of dopamine required for half-maximal stimulation of adenylate cyclase activity (5 μM).

Pharmacological studies with both intact and broken cell preparations reveal that the response to dopamine can be competitively inhibited by neuroleptic drugs, with the phenothiazines being more potent than the butyrophenones (Clement-Cormier and others, 1975). The data on dopamine-sensitive adenylate cyclase in homogenates of the goldfish retina reveal a pharmacological profile similar to that reported for brain and mammalian retina.

The phenothiazine, fluphenazine, was the most potent inhibitor of dopamine activation of adenylate cyclase in homogenates of the goldfish retina with a calculated K_i of 2×10^{-9} M (Table 4). The enzyme activity exhibited stereoselectivity to (-) and (+) butaclamol with the (+) isomer being the active inhibitor. As observed with mammalian whole brain homogenates, no dopamine stimulation of cyclase was observed in the goldfish brain homogenates with concentrations of dopamine up to 1 mM.

One major finding in these studies was the unusually low basal activity of adenylate cyclase in the goldfish retina (2.5 ± 0.5 pmol/gm/min) as compared to goldfish brain (15 ± 1.0 pmol/gm/min) or rabbit retina (39.1 ± 3.9 pmol/gm/min). Because the basal activity is so low, the rather modest level of dopamine stimulated enzyme activity observed actually represents a tenfold increase over basal levels. This percent increase in enzyme activity in the presence of dopamine represents the highest reported for any dopaminergic system to date in a broken cell preparation (Clement-Cormier and colleagues, 1975). Thus, under our experimental conditions most of the adenylate cyclase in goldfish retina is functionally coupled to

dopamine binding sites; in rabbit retina approximately equal amounts are coupled and uncoupled.

GABA RECEPTOR BINDING IN BOVINE RETINA

Unlike other neurotransmitter receptors, the GABA receptor in retina and brain exhibits high affinity binding which is enhanced by a variety of solubilizing treatments (Enna and Snyder, 1978; Mohler, 1979; Redburn and co-workers, 1979). Pretreatment with a detergent, Triton X-100 and, extensive washing; or inclusion of sodium perchlorate, a chaotropic agent, in the assay mixture significantly enhances specific, high affinity binding of (^{3}H)GABA to retinal synaptosomal membranes. Maximal effects were noted at 0.05% Triton or 100 mM $NaClO_4$. The treatments appear to enhance GABA binding at sites which have the affinity and pharmacologic specificity expected of the physiologically relevant GABA receptor.

However, Scatchard analysis of (^{3}H)-GABA binding revealed a striking difference between effects of Triton and perchlorate treatment particularly in the P_2 fraction (Table 5). After Triton treatment the P_2 fraction contained 10 pmol/mg protein (B_{max}) of a binding site with an affinity of 350 nM. A second, higher affinity site (K_d=38 nM) seen in the Triton treated PCS fractions, was virtually absent in the Triton treated P_2 fraction. Perchlorate treatment gave opposite results: only higher affinity sites were observed (K_d=14 nM, B_{max}=1.20 pmol/mg protein) with virtually no binding present at the lower affinity site (K_d 200-400 nM). Scatchard analysis of PCS fractions revealed that both sites were present after Triton or perchlorate treatment; however, a somewhat higher B_{max} was noted for the 20 nM site after Triton as compared to perchlorate.

TABLE 5 Kinetic Constants of ^{3}H-GABA Binding in Bovine Retina Derived from Scatchard Analysis

	Site 1		Site 2		Site 3	
	B_{max}	K_d (nM)	B_{max}	K_d (nM)	B_{max}	K_d (M)
Triton Treated						
PCS	2.38	38	9.32	345		
P_2	(variable) $\leq$0.6	38	10.0	380		
Perchlorate Treated						
PCS	0.90	38	11.53	291	22.9	3.49
P_2	1.28	14	0		125	5.24
Untreated						
PCS					42.5	11.2

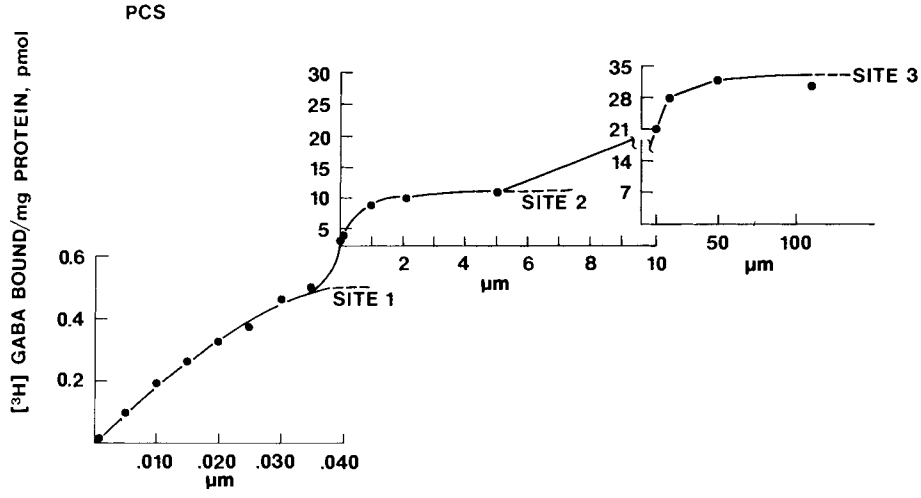

Fig. 1. Specific (^{3}H)-GABA binding in PCS fractions of bovine retina. Data are means of duplicate determinations on each of three separate preparations. Apparent points of saturation are noted as sites 1, 2 and 3.

Binding curves using a wide range of ligand concentrations further emphasizes the differences between the two fractions and treatments (Figs. 1 and 2). Binding in the PCS fraction in the presence of perchlorate exhibited a complex profile with three apparent points of saturation, one at ∿40nM, a second at ∿1 μM, and a third at ∿20 μM. These correspond to the sites revealed by Scatchard analysis; two receptor sites with apparent K_d's of 38 nM and 291 nM, as described above; and a third site of 3 μM. The two higher affinity sites correspond to those observed with Triton treatment (see Table 5). In the P_2 fraction only two sites are apparent, both in the binding curve and the Scatchard plot; a single high affinity with K_d 14nM, and a much lower affinity site with a K_d of 5 μM. There is virtually no binding at the 200 nM site.

The much lower affinity sites seen in both fractions (K_d=3-5 μM) had apparent dissociation constants similar to that reported for the GABA uptake site (Hutchison and co-workers, 1973). Since 100 mM sodium was present in the perchlorate treated samples, this binding may reflect Na-dependent binding to the uptake site. Nipocotic acid, a potent inhibitor of GABA uptake, was a potent inhibitor of (^{3}H)-GABA binding at the 3 μM site in both synaptosomal fractions. Maximum inhibition was observed with ∿1μ M nipocotic acid; however, the maximum amount displaced was never more than ∿75% of the amount displaced by unlabeled GABA. This may be due to the fact that at 3 μM ligand concentration, some portion of the binding is associated with nipocotic acid-insensitive receptor binding sites. When the ligand concentration was kept below the K_d of the receptor binding site (10 nM) nipocotic acid at 10 μM displaced less than 15% of the binding displaceable by unlabeled GABA.

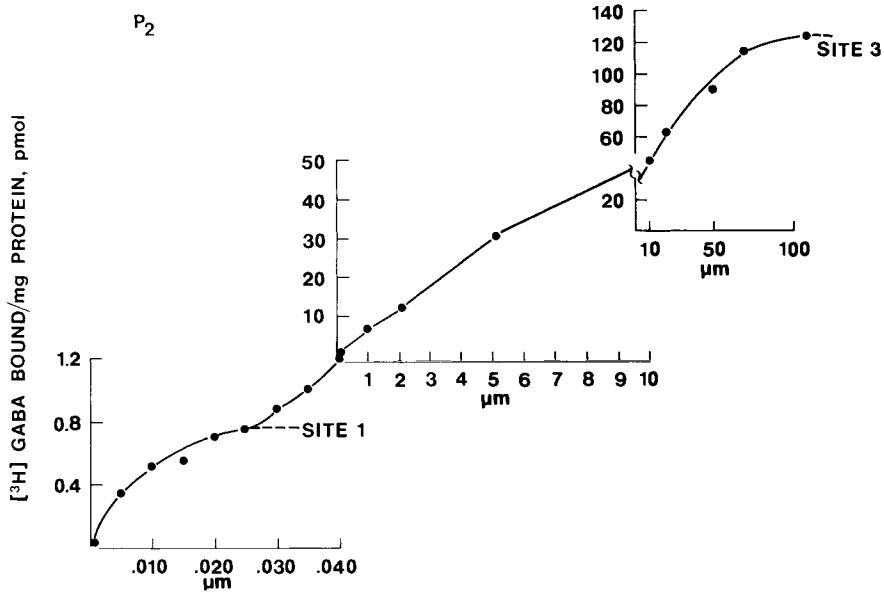

Fig. 2. Specific (^{3}H)-GABA binding in P_2 fractions of bovine retina. Data are means of duplicate determinations on each of three separate preparations. Apparent points of saturation are noted as sites 1 and 3.

Pharmacological specificity of the perchlorate and Triton enhanced binding sites in retina was similar to that previously reported for Triton treated brain fractions (Enna and Snyder, 1975). GABA receptor agonists, muscimol and THIP, were potent inhibitors of (^{3}H)-GABA binding when ligand concentration was 10 nM or 50 nM (Table 6). The antagonists bicuculline, and imidazole acetic acid were significantly less potent.

(^{3}H)-GABA binding in the presence of perchlorate offered a major advantage over the assay requiring pretreatment with Triton in that much less tissue was required. Seventy to eighty percent of the original protein in P_2 and PCS fractions was lost after treatment with 0.05% Triton. Since perchlorate was added during the assay itself, no protein was lost due to pretreatment and washing procedures. An additional advantage of the perchlorate treatment is that variability among different preparations was reduced significantly which may also be due to the lack of extensive washing procedures which are necessary after Triton treatment.

These data suggest that the bovine retinal GABA receptors are similar to brain in that they appear to exist in at least two states based on differing affinities for GABA, one having a higher affinity (20-40 nM), the other a lower affinity (200-400 nM). Although the affinities and pharmacological specificity remain relatively constant, the number of higher and lower affinity sites (B_{max}) varies according to the solubilizing treatments used. This phenomenon has been well described in brain tissue (Toffano and co-workers, 1978). So-called type B membranes obtained from unfrozen, non-Triton-treated synaptosomal fractions from cerebral cortex or cerebellum

exhibit binding only at the 200 nM site. Type A membranes obtained from the same source but freeze-thawed, pre-treated with Triton and washed extensively, exhibits binding at both the 20 nM and the 200 nM site. Both type A and type B membranes are obtained from retina; however, treatment conditions differ from those described for brain and they vary between the two different retinal synaptosomal fractions. Two binding sites (type A membranes) are observed in the PCS fraction after Triton or perchlorate treatment, similar to brain fractions. In contrast, the P_2 fraction shows only type B activity (binding at the 200 nM site) after Triton treatment. Of even more interest, is the binding observed in the P_2 fraction after perchlorate treatment which is almost exclusively limited to the 20 nM site, and therefore, different from either Type A or Type B activity. This is apparently the only tissue described to date which exhibits binding at the 20 nM site in the absence of binding at the 200 nM site. This retinal fraction is unique in that Triton treatment favors the lower affinity site exclusively, perchlorate treatment favors the higher affinity site exclusively.

TABLE 6 Inhibition of (^{3}H)-GABA Binding to Bovine Retinal Fractions

IC_{50} µM

Perchlorate

	PCS	P_2
(+) Bicuculline	1.5	3.0
Imidazole acetic acid	0.25	0.70
Muscimol	0.003	0.005
THIP	0.028	0.015

K_I µM

	Perchlorate		Triton	
	PCS	P_2	PCS	P_2
(+) Bicuculline	1.19	1.75	2.30	2.03
Imidazole acetic acid	0.20	0.41	0.30	0.26
Muscimol	0.024	0.0030	0.0026	0.0035
THIP	0.022	0.011	-	-

The exact mechanisms of action of sodium perchlorate on the affinity of the GABA receptor is unclear. Like the detergent action of Triton X-100, the chaotropic action of perchlorate is nonspecific in nature. Nonetheless, both of these agents apparently allow the direct analysis of binding characteristics of the GABA receptor. One reasonable suggestion is that these agents remove some endogenous substance which enhibits the binding of (^{3}H)-GABA to the receptor. Toffano and colleagues

(1978) suggest that this substance, GABA modulin, is a membrane protein which modulates the affinity of the receptor for GABA. Others suggest that membrane phospholipids may regulate the receptor (Johnson and Kennedy, 1978). Still others report that a significant proportion of the "endogenous inhibitory substance" is actually endogenous GABA or GABA-like molecules such as taurine, imidazole acetic acid, β-alanine or homocarnosine (Greenlee and co-workers, 1978). The data presented here is consistent with the hypothesis for an endogenous inhibitory substance; however, no attempt has been made to identify the exact nature of this substance in retina. The differences in the P_2 synaptosomal fraction after perchlorate vs. Triton treatment may allow a careful analysis of the factors which favor the lower affinity GABA receptor conformation as seen after Triton treatment; in comparison to factors which favor the higher affinity state as seen with perchlorate treatment.

SUMMARY

Receptor binding studies have been successfully completed in a variety of retinal subcellular fractions. The dopamine receptor has been demonstrated in retinas of three different species. The receptor in goldfish retina is of particular interest since it represents a tissue unusually rich in D_1 receptors, i.e. those which are linked to adenylate cyclase.

The GABA receptor has been kinetically and pharmacologically defined in bovine retina. Important findings include the somewhat unusual sensitivity of the retinal GABA receptor to various solubilizing agents such as Triton and perchlorate. A potentially useful system for further study may exist in the P_2 fraction of bovine retina. After Triton treatment, the receptor appears to exist in a single affinity state (∿200nM). After perchlorate treatment the receptor also appears to exist in a single affinity state but with a ten-fold lower K_d(20nM). In view of the controversial theories regarding endogenous regulators of GABA receptor affinity, this system would seem ideal for studying conditions which favor the higher affinity state (perchlorate) vs. conditions which favor the lower affinity state (Triton).

ACKNOWLEDGEMENT

We are indebted to Mr. Al Tykla, Mr. Ernest Pollard and the Blue Ribbon Packing Company for their cooperation in furnishing bovine retinas for this research. We wish to thank Ms.Cheryl K. Mitchell for excellent technical assistance with binding assays and Ms.Barbara Bate for typing this manuscript. This research was supported gy NEI Grant EYO-1655-3 and Research Career Development Award 1 KO4 EY 00088-2.

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Neurochemistry Vol. 1, pp. 183-190.
Pergamon Press Ltd. 1980. Printed in Great Britain.

RETINAL ORGANIZATION: NEUROTRANSMITTERS AS PHYSIOLOGICAL PROBES

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ABSTRACT

Neurons in the goldfish retina which use GABA, dopamine and glycine as neurotransmitters have been tentatively identified. Our studies show that H1 cone horizontal cells, which receive input predominantly from red-sensitive cones, fulfill most of the criteria to be identified as GABA-ergic. In contrast, all the other types of horizontal cells in this retina are not GABA-ergic. Additionally, at least some Ab pyriform amacrine cells, which are sustained and red-depolarizing with synaptic terminals contacting center-depolarizing bipolar cells in sublamina b of the inner plexiform layer, may be GABA-ergic. Although identifications of putative dopaminergic and glycinergic neurons are far less complete, our results and those of other investigators suggest that: (1) one type of interplexiform cell (I1) may be dopaminergic; (2) another type of interplexiform cell (I2), which is morphologically distinct from I1, may be glycinergic; and (3) at least some sustained, red-hyperpolarizing amacrine cells with synaptic terminals in sublamina a of the inner plexiform layer may also be glycinergic. Furthermore, we show in this paper how the use of these neurotransmitters as physiological probes lead to further understanding of transmitter-specific pathways and functional organization of the retina.

KEYWORDS

Retinal organization; neurotransmitters; GABA; dopamine; glycine; neuronal pathways; autoradiography; immunocytochemistry; *Carassius auratus*.

INTRODUCTION

The vertebrate retina has been used extensively as a model system to study the mechanisms by which sensory information is transmitted and processed in the central nervous system. Retinal organization has in the past been studied using mainly morphological and electrophysiological techniques, with little emphasis on the biochemical aspects of synaptic transmission. Thus, although we now possess a fairly detailed understanding of the morphology, synaptic connections and physiological responses of many retinas, the synaptic chemistry, in particular the identities of neurotransmitters used by different retinal cells types, is still largely unknown. Using a variety of techniques, we have attempted to identify retinal neurotransmitters and use them as an additional tool to examine retinal organiza-

tion. We chose the goldfish for many of our studies because the morphology, electrophysiology and synaptic organization of this retina have been studied systematically and in detail by a number of investigators. In this paper, we describe the identifications of probable GABA-ergic, glycinergic and dopaminergic neurons in the goldfish retina and we show how the localizations of such neurons lead to further understanding of transmitter-specific pathways and functional organization in the retina.

MATERIALS AND METHODS

Adult goldfish (*Carassius auratus*; 5 to 7 inches long) were used for most of our studies. All the experimental procedures have been described in detail elsewhere: (1) light and electron microscope autoradiography of ^{3}H-labeled neurotransmitter uptake (Lam and Steinman, 1971; Marc and others, 1978). (2) Isolation of identified single cells from the retina (Lam, 1972a, 1976). (3) The syntheses of neurotransmitter candidates from labeled precursors and the activities of transmitter-synthesizing enzymes in the retina and isolated cells (Lam, 1972a, b, 1975, 1976). (4) Purification of L-glutamic acid decarboxylase (GAD) from catfish brain and the production of antibodies against catfish GAD (Su, Wu and Lam, 1979). (5) Immunocytochemical localization of GAD-containing cells in the retina (Lam and others, 1979; Brandon, Lam and Wu, 1979). (6) Biochemical studies of high-affinity uptake mechanisms for GABA, glycine and dopamine and Ca^{++}-dependent, K^{+}-stimulated release of these transmitter candidates from the retina (Sarthy and Lam, 1979).

RESULTS

Identification of GABA-ergic Neurons

It is generally accepted that most of the following criteria must be met before a substance can be considered the neurotransmitter for a neuron: (1) The substance should be present in the presynaptic cell. This substance is usually accumulated in the cell by a specific high-affinity uptake mechanism, synthetic pathway, or both. (2) Upon physiological stimulation, this substance should be released from the presynaptic terminals and the release should probably be Ca^{++}-dependent. (3) This substance applied exogenously should elicit in the postsynaptic cell a direct, specific and reversible response which is quantitatively similar to that elicited by the endogenous transmitter. In general, the effects of known agonists and antagonists of the transmitter candidate on the postsynaptic cell are also tested. (4) The action of the substance should be effectively inactivated following its stimulation of the postsynaptic receptors. This inactivation mechanism is usually mediated by degradation or clearance.

As a first step to study the GABA system in the goldfish retina, we showed that this retina contains high GABA concentrations which range from 1.6mM in the dark-adapted retina to 3.1 mM in the light-adapted retina (Lam, 1972b). In addition, retinas incubated with ^{14}C-glutamic acid synthesize ^{14}C-GABA and the accumulation of this newly synthesized GABA is also influenced by light-stimulation of the retina. Lastly, goldfish retinas contain significant levels of GAD and GABA-glutamate transaminase (GABA-T), the enzymes responsible for GABA synthesis and degradation respectively (Lam, 1972b).

We next examined by autoradiography whether certain retinal neurons selectively accumulate exogenous GABA. We showed that when goldfish retinas were incubated with Ringer's solution containing 1 to 10μM ^{3}H-GABA, the label was localized only to certain horizontal and amacrine cells (Lam and Steinman, 1971). In addition, this accumulation was greatly enhanced by appropriate light-stimulation of the retina. More recently, Marc and others (1978) showed by electron microscope autoradiography that in the goldfish retina: (1) of the four types of horizontal

cells (H1, H2, H3 and RH) which receive inputs predominantly from red-, green-, blue-sensitive cones and rods respectively (Stell and Lightfoot, 1975), only the H1 cone horizontal cells possess the high-affinity GABA uptake mechanism; and (2) of the many types of amacrine cells, at least some of the sustained, red-depolarizing amacrine cells (type Ab pyriform cells; Famiglietti, Kaneko and Tachibana, 1977; Murakami and Shimoda, 1977) which are both pre- and post-synaptic to red-sensitive, center-depolarizing bipolar cells, selectively take up GABA. Furthermore, the accumulation of ^{3}H-GABA in H1 horizontal cells is increased by stimulating the retina with red, green or blue light during incubation and decreased by darkness. In contrast, ^{3}H-GABA accumulation in Ab amacrine cells was enhanced by incubating the retinas in darkness or under green or blue lights, but was suppressed by incubation under red light.

The function of the high-affinity mechanism for GABA uptake is not clear. One possible role may involve the removal and therefore inactivation of GABA from the synaptic clefts following its release and stimulation of the postsynaptic GABA receptors. Additionally, an efficient GABA uptake system into GABA-ergic neurons may decrease the requirement for continual GABA synthesis during repeated stimulation of the cell.

Although these studies, especially the influence of light stimulation on GABA accumulation, pointed to GABA as a transmitter candidate for H1 cone horizontal cells and Ab pyriform amacrine cells, it remained to be shown that these cells have the ability to synthesize significant amounts of GABA, contain high levels of the enzyme GAD and can release GABA upon appropriate stimulation of the retina. A direct way to determine the GABA-synthesizing capacity of these cells is to dissociate the retina into single cells, identify and select each cell under visual observation and measure the ability of these cells to synthesize GABA as well as other retinal transmitter candidates from exogenously supplied precursors (Lam, 1972a; 1976). Using this method, we showed that isolated axons from cone horizontal cells synthesize significant amounts of GABA but not ACh, catecholamines or serotonin (Lam, 1975, 1976).

We also measured the GAD activity in these isolated axons. Our autoradiographic study of ^{3}H-GABA uptake indicated that probably no more than 20% of all the retinal neurons are GABA-ergic. Thus, if all the cone horizontal cell axons (H1, H2 and H3) are GABA-ergic, the specific activity of GAD in these axons should be approximately 5 times that found in the whole retina. However, Lam (1976) found that the specific activity of GAD in these axons was 1.33 ± 0.47 μmoles GABA formed/hr/g wet weight, only about 1.6 times higher than that in the whole retina (0.86 ± 0.22). If we assume that only axons of H1 cells which take up GABA have GAD activity, since 40 to 50% of all cone horizontal cells are H1 cells, a corrected specific activity of GAD in H1 axons would be about 3 μmoles GABA formed/hr/g wet weight, or 3 to 4 times greater than that found in the whole retina. This value is comparable to the expected activity of GABA-ergic retinal neurons and also to that found in inhibitory axons of the lobster neuromuscular junction (about 5.3 μmoles GABA formed/hr/g wet weight, calculated from Hall, Bownds and Kravitz, 1970), which has been shown to use GABA as the neurotransmitter.

Another direct way to localize GABA-ergic neurons is to visualize the GAD-containing neurons by immunocytochemistry. This method has been successfully used to localize probable GABA-ergic neurons in several parts of mammalian nervous system (Wu, Matsuda and Roberts, 1973; Saito, 1976; Wu, 1976; Ribak, Vaughn and Saito, 1978; Brandon, Lam and Wu, 1979). Unfortunately, the available antibodies were against mouse GAD and do not cross-react with GAD from goldfish brain and retina (Saito, 1976; Wu and Lam, unpublished data). Thus, prior to immunocytochemical studies of GAD in the goldfish retina, we first purified GAD from catfish brain and obtained antibodies against this enzyme by injecting it into rabbits (Su, Wu

and Lam, 1979). The purity of the injected enzyme and the specificity of the antibody were each confirmed by several criteria (Su, Wu and Lam, 1979). Our immunocytochemical studies showed that of all the cells in the retina, GAD is localized only in H1 horizontal cells and at least some Ab pyriform amacrine cells (Lam and others, 1979). Thus, in the goldfish retina, the high-affinity mechanism for GABA uptake and the enzyme for GABA biosynthesis are present in the same neurons.

The release of GABA from H1 horizontal cells was studied autoradiographically (Marc and others, 1978) by first cutting a retina into halves and incubating them with ^{3}H-GABA for ten minutes under red light. Each hemiretina was then incubated separately for an additional five minutes in unlabeled Ringer's solution, one under a red flickering light and the other in darkness. They were then fixed with glutaldehyde and processed for autoradiography. Grains counts of these autoradiographs showed that H1 cells from the hemiretina incubated in darkness contained 75% less label than those incubated under red light. Thus, darkness, which depolarizes H1 horizontal cells (Kaneko, 1970) also elicits release of GABA from H1 cells. In addition, using the method described by Sarthy and Lam (1979), we have shown that ^{3}H-GABA taken up by H1 horizontal cells and Ab amacrine cells can be released by raising the K^{+} concentration in the medium to 56mM, and that this release is probably Ca^{++}-dependent because it is blocked by 10mM exogenous Co^{++} (Chin and Lam, in preparation).

Finally, recent electrophysiological studies indicate that in the goldfish retina, GABA may be involved in the feedback synapse between the horizontal cells and cones (S.M. Wu, personal communication; M. Murakami, personal communication). In the retina of another teleost, the channel catfish, which has only two types of photoreceptors: rods and red-sensitive cones (Naka, 1977), autoradiographic and electrophysiological studies showed that feedback synapses from cone horizontal cells to cones may use GABA as a neurotransmitter and that a function of this feedback is to improve the frequency response of the system (Lam, Lasater and Naka, 1978).

Taken together, our studies of the GABA system in the goldfish retina show that: (1) H1 horizontal cells which receive input predominantly from red-sensitive cones, are GABA-ergic. (2) H2, H3, and RH horizontal cells are, however, not GABA-ergic. (3) At least some Ab pyriform amacrine cells, which are sustained, red-depolarizing, are probably GABA-ergic. (4) By analogy with the catfish retina, a function of the negative feedback from horizontal cells to photoreceptors is to improve the frequency response of the retina.

Identification of Dopaminergic Neurons

Goldfish retinas have been shown to synthesize dopamine but not noradrenalin or octopamine from exogenous tyrosine and to contain activity of tyrosine hydroxlase, the rate-limiting enzyme for dopamine synthesis (Lam, 1976). Recent fluorescence, electron microscopic and neurophysiological studies (Dowling and Ehinger, 1978; Hedden and Dowling, 1978) show that in this retina, most of the putative dopaminergic neurons belong to a class of interplexiform cells, which are retinal neurons that extend their processes widely in both the outer and inner plexiform layers (Cajal, 1893; Gallego, 1971; Dawson and Perez, 1973; Boycott and others, 1975 Kolb and West, 1977). These results are supported by our autoradiographic studies on the uptake of ^{3}H-dopamine in the goldfish retina (Marc and others, 1978; Sarthy and Lam, 1979).

The dopaminergic system in this retina has been characterized further by examining biochemically the mechanisms of dopamine uptake and release (Sarthy and Lam, 1979). We showed that the uptake of dopamine is a Na^{+}- and temperature-dependent process

which can be explained kinetically by the presence of a single high-affinity mechanism with a K_m of 0.26 μM and a V_{max} of 66 x 10^{-12} mole/min/mg protein. This uptake is inhibited at least 70% by the centrally acting drugs benztropine and diphenylpyraline, and by a combination of the metabolic inhibitors cyanide and iodoacetate. In addition, ^{3}H-dopamine taken up by the dopaminergic interplexiform cells can be released by raising the K^+ concentration in the medium to 56mM (Sarthy and Lam, 1979). This release is probably Ca^{++}-dependent as it is inhibited by 10mM Co^{++} or 20mM Mg^{++} in the medium.

Our results, together with those of Dowling and his coworkers, indicate that in the goldfish retina, at least one type of interplexiform cell is probably dopaminergic. Furthermore, no other cells in this retina appear to be dopaminergic. Electron microscope autoradiography of ^{3}H-dopamine uptake are in progress to determine the dopaminergic pathways in this retina (Marc and Lam, in preparation). In addition, we have also begun studies on the distribution and properties of the post-synaptic dopamine receptors (Redburn, Clement-Cormier and Lam, in preparation) and the effects of light stimulation on dopamine accumulation.

Identification of Glycinergic Neurons

Our studies of putative glycinergic neurons in the goldfish retina have been confined to autoradiographic and biochemical analyses of glycine uptake and release. We have shown by autoradiography that at least two distinct populations of neurons in this retina possess a specific mechanism for the accumulation of exogenous glycine (Marc and others, 1978; Marc, Lam and Stell, 1979). These neurons have been tentatively identified by electron microscope autoradiography and physiological studies as (1) a type of sustained, red-hyperpolarizing amacrine cell with synaptic terminals predominantly in sublamina a of the inner plexiform layer (Marc and others, 1978) and (2) a type of interplexiform cell with cell bodies in the middle of the inner nuclear layer with numerous processes radiating from the somas (Marc, Lam and Stell, 1979). The glycine-accumulating interplexiform cell, which we have designated as I2 cells, are morphologically distinct from the dopamine-accumulating interplexiform cells, which we have designated as I1 cells.

Kinetically, glycine uptake in this retina can be explained by the presence of two affinity systems: a 'high-affinity' mechanism with a K_m(H) of 8.08 μM and a V_{max}(H) of 9.12 pmoles/min/mg protein, and a 'low affinity' mechanism with a K_m(L) of 0.63 mM and a V_{max}(L) of 430 pmoles/min/mg protein (Chin and Lam, in preparation). Although both the high and low affinity mechanisms are temperature- and Na^+-dependent, they can be readily distinguished physiologically: the low-affinity mechanism is inhibited more than 90% by 5mM of serine, alanine and proline in the medium, whereas the high-affinity mechanism is not affected by these or all other amino acids tested. This result suggests that the low-affinity uptake for glycine goes through system A of the neutral amino acid transport system which is present in most tissues to transport glycine and certain amino acids for metabolic purposes. To determine the affinity by which glycine was taken up by the glycine-accumulating neurons, retinas were incubated with 1 to 5μM ^{3}H-glycine in the presence or absence of 5mM serine, alanine and proline. Autoradiographic studies showed that glycine was taken up specifically into these neurons even in the presence of excess serine, proline and alanine. This result indicates that the glycine-accumulating neurons possess a specific high-affinity mechanism for glycine uptake. In addition, we have shown both biochemically and autoradiographically that the ^{3}H-glycine accumulated by these neurons can be released by a Ca^{++}-dependent, K^+-depolarization of the retina (Chin and Lam, in preparation).

Recent electrophysiological studies (S.M. Wu, personal communication) indicate that glycine may be involved in a sign-inverting synapse which mediates the dep-

clarizing responses to red flashes in a subpopulation of C-type horizontal cells. This finding is in agreement with our autoradiographic study which shows that the glycine-accumulating interplexiform cells (I2) make synaptic contacts with dendrites of certain horizontal cells in the outer plexiform layer (Marc, Lam and Stell, 1979). In contrast, dopamine-accumulating interplexiform cells (I1) have been shown to contact predominantly the somas and not dendrites of mainly H1 horizontal cells (Dowling and Ehinger, 1978; Sarthy and Lam, 1979; Marc and Lam, in preparation).

In both *Necturus* and rabbit retinas, there is electrophysiological and pharmacological evidence that certain amacrine cells may be glycinergic (Ames and Pollen, 1969; Miller, Dacheux and Frumkes, 1977; Caldwell and Daw, 1978). In the goldfish retina, much more evidence is required before the glycine-accumulating neurons can be considered glycinergic. In addition, we have as yet not determined the synaptic connections of the glycine-accumulating amacrine cells and those of I2 cells in the inner plexiform layer. This is because the two populations of ^{3}H-glycine-labeled processes are difficult to distinguish by autoradiography alone. We hope that, similar to the light-stimulation dependence of ^{3}H-GABA uptake, conditions can be found to label only one type of glycine-accumulating neuron at a time so that its synaptic pathways can be determined.

CONCLUSIONS

There are several general implications of our studies on the synaptic chemistry and organization of the goldfish retina. First, for all three classes of retinal neurons that we have examined: horizontal, interplexiform and amacrine cells, each class of cells uses more than one neurotransmitter. Furthermore, within a single class of neurons, the various cell types that contact different neurons may use different transmitters. For instance, our study of putative glycinergic neurons has led to the demonstration of a second type of interplexiform cell (I2) which may be glycinergic and which is morphologically and biochemically distinct from the probable dopaminergic interplexiform cells (I1). Our results also imply that in other parts of the central nervous system, sub-populations of a class of neurons having similar functional architecture may use different transmitters.

The physiological significance, if any, for various sub-populations of a class of retinal neurons to use different transmitters is unknown. A simple-minded conjecture is that since the synaptic density in certain regions of the retina, especially the plexiform layers, is very high, the existence of many different transmitter-specific pathways would ensure interactions to occur only among appropriately wired neurons, and decrease the probability of non-specific stimulation by other transmitters in the area due to diffusion and incomplete inactivation. Another speculation is that the diversity of transmitters within a class of retinal neurons may be of importance for guiding or specifying the precise connections between two classes of neurons during retinal development. For example, in the goldfish retina, each horizontal cell type receives input predominantly from a different type of photoreceptors. Although the mechanism underlying this specificity is unknown, detailed analyses of the emergence of different transmitter systems during retinal development may shed light on whether such specificity is related to the transmitter-specific pathways in the adult retina. To this end, we have utilized some of the techniques and findings described in the present paper to examine the development of transmitter systems in the retina (Hollyfield and others, 1979 and the following paper). We chose *Xenopus laevis* because its development has been precisely staged (Nieuwkoop and Faber, 1957) and because many aspects of retinal differentiation in this animal have been characterized (Saxen, 1954; Hollyfield, 1971; Hollyfield and Rayborn, 1978). Since a retinal neuron using GABA, dopamine or glycine as a neurotransmitter possesses specific mechanisms for its uptake, synthesis (except for glycine) and release, we

first examined the appearance and maturation of these three properties during retinal development. The results of this work are presented in the following paper.

Finally, in spite of numerous attempts to identify the transmitters used by the other cone horizontal cells, H2 and H3, in the goldfish retina, there are still no likely candidates. These cells do not synthesize ACh, catecholamines or serotonin from their precursors (Lam, 1975, 1976), do not contain GAD (Lam and others, 1979) and also do not selectively accumulate GABA, dopamine, glycine, aspartic acid, glutamic acid or taurine (Marc and others 1978; Marc and Lam, unpublished data). In addition, our studies with isolated cells and intact retinas also indicate that several other retinal cell types do not use any known or suspected candidates as transmitters. A search for the neurotransmitters of these cells is therefore especially challenging.

ACKNOWLEDGMENTS

We thank Ms. Patricia Cloud for typing the manuscript. This work has supported by research grants from the Retina Research Foundation (Houston), Merck Company Foundation and U.S. National Institutes of Health (EY02423 to DMKL, EY02576 to REM and NS13224 to JYW). DMKL is a recipient of a Research Career Development Award from the U.S. National Eye Institute.

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THE RELEASE OF ACETYLCHOLINE AND AMINO ACIDS FROM THE RABBIT RETINA IN VIVO

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ABSTRACT

Rabbits were anaesthetised with urethane and an eye-cup was prepared by removing the cornea, iris, lens and vitreous. The retina was loaded with |^{3}H|choline by filling the eye-cup with medium containing labelled choline and eserine. The retina was washed and then 10 min samples of medium from the eye-cup were assayed for |^{3}H|ACh, and in some experiments, endogenous amino acids. The ERG was routinely recorded.

Exposure of the dark adapted retina to flashes of light (3Hz) produced a striking increase in the efflux of |^{3}H|ACh. This light evoked release of |^{3}H|ACh was reduced by the presence of GABA in the eye-cup, an effect which was abolished by picrotoxin. Both picrotoxin and bicuculline caused striking increases in the spontaneous resting release of |^{3}H|ACh.

Flashes of light (3Hz) did not alter the efflux of glutamate, glutamine, alanine, glycine or GABA from the dark adapted retina. However, the release of taurine was greatly increased and the release of aspartate was significantly reduced (by more than 50%), a result consistent with the suggestion that aspartate but not ACh or taurine may be a photoreceptor transmitter substance.

KEYWORDS

Retina; transmitter substances; ACh, light evoked release; amino acid release; retinal ACh-GABA interactions; photoreceptor transmitter.

INTRODUCTION

The presence of ACh in the retina together with, in some species, exceptionally high levels of ChAc activity, strongly suggest that cholinergic synapses occur in the retina (for reviews, see Graham, 1974; Neal, 1976a). There is also much evidence to suggest that certain amino acids such as GABA, glycine, taurine, aspartate and perhaps glutamate may be retinal synaptic transmitter substances. (For review, see Neal, 1976b). However, much of this evidence is indirect and there have been

few reports on the effects of light stimulation on the release of these putative transmitters from the retina (Pasantes-Morales and co-workers, 1974; Ehinger and Lindberg-Bauer, 1976; Masland and Livingstone, 1976; Bauer and Ehinger, 1977; Coull and Cutler, 1978).

The demonstration of the release of a substance, in response to appropriate physiological stimulation is an important criterion in establishing a transmitter role for a substance and with this in mind, we have studied the effect of light on the release of both $|^3H|$ACh and endogenous amino acids from the retina. Preliminary reports on some of the work have already been published (Massey and Neal, 1978; 1979; Neal, Collins and Massey, 1979).

METHODS

Eye-Cup Preparation

Rabbits were anaesthetized with urethane (1.5g/kg i.p.) and the head was clamped so that one eye faced upwards. This eye was sutured to a ring for support and then the cornea, iris and lens were removed. The vitreous was removed by gentle traction with tissue "wicks" using a dissection microscope. Any over-vigorous traction caused immediate retinal detachment and preparations in which detachment or haemorrhage occurred were rejected. The eye-cup preparation is shown diagramatically in Fig. 1 and is similar to that described by Kramer (1971) using cats.

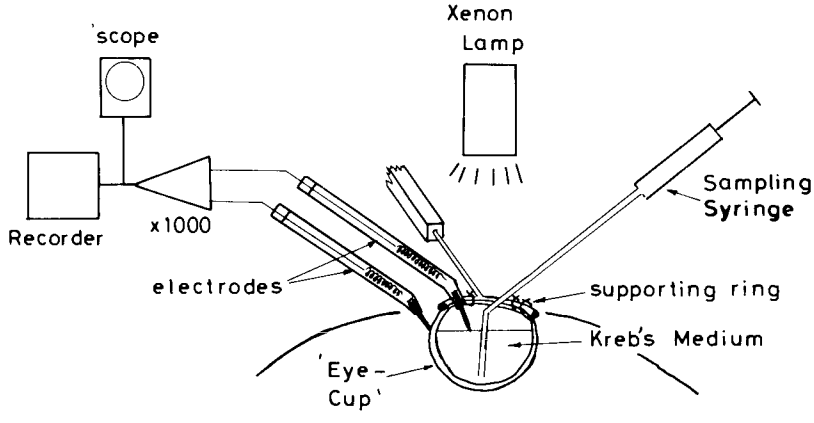

Fig. 1. Diagram of rabbit eye-cup preparation. The electrodes, supporting ring, and sampling syringe were mounted on micromanipulators.

The eye-cup was filled with Krebs bicarbonate Ringer (500µl) containing $|^3H|$choline (10µM) and this solution was left in contact with the retina for 30 min. (Previous experiments indicated that retinae quickly accumulate $|^3H|$choline by sodium dependent, high affinity transport processes and that this uptake is associated with a high degree of $|^3H|$ACh formation. (Neal and Gilroy, 1975; Atterwill and Neal, 1978). The medium containing $|^3H|$choline was removed, and the retina was washed for 60 min by continuous irrigation with fresh medium containing eserine sulphate (30µM). Eserinized medium (500µl) was then placed in the eye-cup using a syringe mounted on a micromanipulator and the medium was replaced at 10 min intervals. The $|^3H|$ACh and $|^3H|$choline in the resulting samples were separated by high

voltage electrophoresis and measured by liquid scintillation counting (Potter and Murphy, 1967). In some experiments, the endogenous amino acids released into the medium were also assayed, using a sensitive double-label dansylation method (Clark and Collins, 1976).

Electroretinography

The ERG was recorded in all experiments using low resistance electrodes. These were balsa "wicks" soaked in saline/agar and connected to the differential amplifier (7A22) of a tektronix oscilloscope (7403N) via chlorided silver wires. The ERG was recorded on a Mingograph ink writing recorder. One electrode just dipped into the medium in the eye-cup and the other was placed in contact with the back of the globe. (Fig. 1).

Light Stimulation

Most experiments were performed in dim red light and the retina was dark adapted for 60 min before samples were collected from the eye-cup. The retina was stimulated for 10 min periods by flashes of light from a xenon lamp (Devices photic stimulator, 3182). The retinal illuminance was increased twice during the period of stimulation in an attempt to obtain the maximum firing rate of ganglionic cells (Masland and Ames, 1976). The characteristics of the Devices photic stimulator result in a frequency dependent illuminance. At 3Hz, which was the frequency used most often, the retinal illuminance was started at 1.6 lux, this was increased to 5.2 lux at 3 min and to 16 lux at 6 min.

RESULTS

Effect of Light Stimulation on the Release of $|^3H|$Ch and $|^3H|$ACh

In the dark adapted retina there was a steady spontaneous resting release of radioactivity. Electrophoretic analysis of the samples revealed that about 80% of the spontaneous release was due to $|^3H|$Ch and only 20% was $|^3H|$ACh (Fig. 2); no other radioactive metabolites, such as betaine or phosphorylcholine were detected.

Light stimulation of the retina at a frequency of 3Hz caused a prompt increase in the release of radioactivity (approximately 2 times the resting release) which was almost entirely accounted for by an increase in the efflux of $|^3H|$ACh (Fig. 2). Thus, light stimulation increased the release of $|^3H|$ACh by 4.10 ± 0.29 (mean $\pm$ S.E.M., n = 20) times the spontaneous resting release while the efflux of $|^3H|$Ch increased by only 1.02 ± 0.02 (mean $\pm$ S.E.M., n = 20) times the resting release.

This light evoked release was not apparent if an ERG could not be recorded but in a functional eye-cup preparation it could be repeated as many as 8 times after a 30 minutes recovery period. In the subsequent experiments only the results with $|^3H|$ACh are described since no significant changes in the efflux of $|^3H|$Ch occurred.

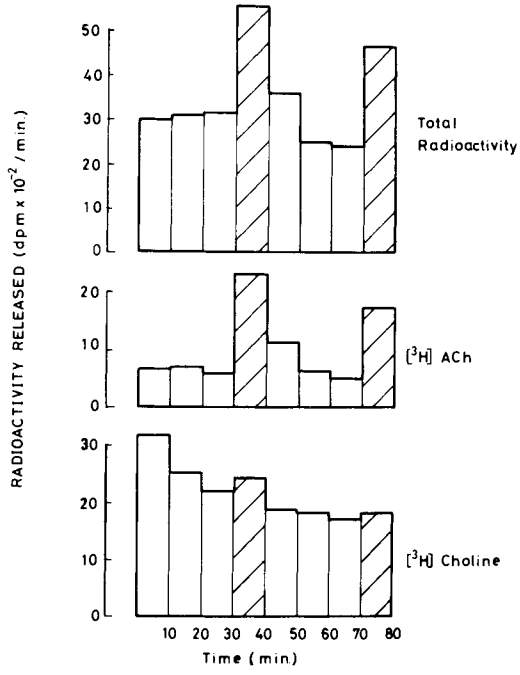

Fig. 2. Typical experiment illustrating the effect of light flashes (3Hz) on the release from the dark adapted retina of total radioactivity (top), $|^{3}H|$ACh (centre), and $|^{3}H|$choline (bottom). The hatched columns indicate stimulation with light flashes (3Hz).

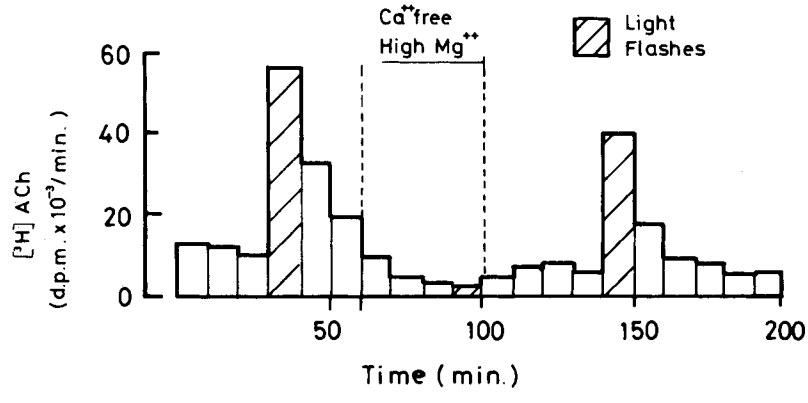

Fig. 3. Typical experiment showing that the evoked release of $|^{3}H|$ACh produced by light flashes (3Hz) was abolished when the retina was exposed to calcium free medium containing $MgCl_2$ (20mM) This experiment was repeated 4 times with the same result. The mean values are given in the text.

Effect of Calcium Free Medium on the Light Evoked Release of $|^3H|$ACh.

The light evoked release of $|^3H|$ACh was calcium dependent. Thus, when the medium in the eye-cup was replaced with calcium free medium containing 20mM magnesium chloride, the evoked release of $|^3H|$ACh was abolished being only 0.78±0.05 (mean ± S.E.M., n = 4) times the resting release (Fig. 3). On return to normal medium, the light evoked response recovered.

Exposure of the retina to calcium free, high magnesium medium produced striking changes in the ERG. At the lowest light intensity the ERG was completely abolished whilst at the two higher intensities, the isolated a-wave clearly remained. On returning to ordinary Krebs medium, a normal series of ERGs could again be recorded.

Effect of Stimulus Frequency on Light Evoked $|^3H|$ACh Release

The effect of different stimulation rates on the release of $|^3H|$ACh is illustrated in Fig. 4 and is summarized in Fig. 5. The light evoked release of $|^3H|$ACh increased with frequency from 0.5 to 3Hz but no further increase was observed at 10Hz, whilst at 30Hz the evoked release was not significantly different from the spontaneous resting release.

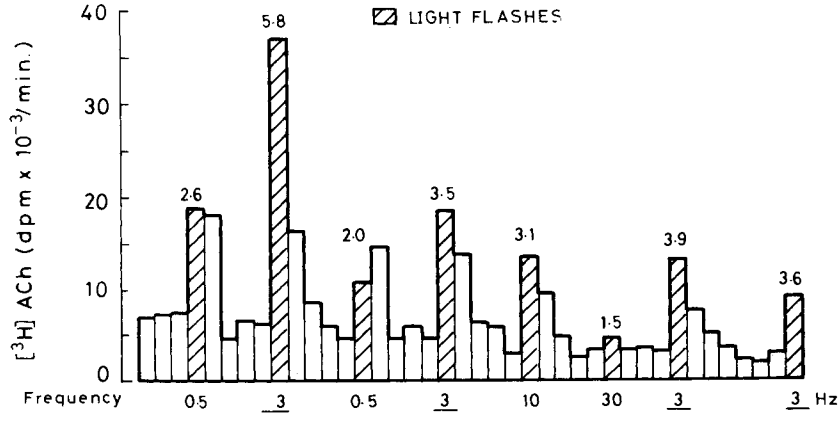

Fig. 4. Typical experiment showing the effect of frequency on the light evoked release of $|^3H|$ACh. Where the same frequencies were repeated in an experiment, the mean evoked release was calculated and used as one result in Fig. 5.

These results indicate that the evoked release of ACh is significantly affected by changes in the stimulus frequency. However, it should be noted that due to the characteristics of the photic stimulator, which delivered short pulses of fixed duration regardless of the frequency, the total amount of light falling upon the retina increased with stimulation frequency. Thus, at the higher stimulation rates, increasing light adaptation would be expected to decrease retinal sensitivity and consequently tend to flatten the ACh release-frequency curve. This suggestion is supported by ERG recordings which revealed that up to 2Hz the shape of b-wave was unchanged but a reduction in amplitude was evident at 4Hz, except for the first response. The second response was particularly small, noticeable throughout this

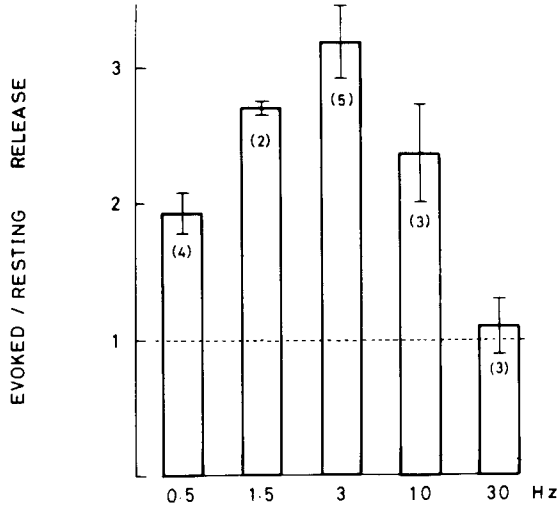

Fig. 5 Summary of effect of frequency on the light evoked release of $|^3H|$ACh. Each result is the mean ± S.E.M. of the number of separate experiments shown in parentheses. The result for each experiment was itself the mean of 1 to 4 determinations. The results at 0.5, 1.5 and 3Hz were all significantly different from one another ($p<0.05$ at least). The release at 10 Hz was not significantly different from that at 3Hz.

series of experiments, as previously described, but not explained, by Armington (1974). As the stimulus frequency was increased the amplitude of the b-wave was further reduced until it was barely visible at 16Hz and absent at 30Hz. These two higher frequencies still produced a normal first response as well as a small negative off response.

Effect of Light Adaptation on Evoked $|^3H|$ACh Release

In four separate experiments, the spontaneous resting release of $|^3H|$ACh was apparently not affected by continuous background illumination of 1 to 5 log units greater than the flashing light stimulus. However, the light evoked release of $|^3H|$ACh produced by flashes (3Hz) was reduced by 34% (mean of 2 experiments) when the background illumination was approximately 1 log unit greater than the stimulus, and was abolished when the background illumination was raised a further 4 log units. In the latter case, the usual 3Hz light evoked release returned after 30 min dark adaptation.

Effect of GABA on Evoked $|^3H|$ACh Release

There is much inferential evidence that GABA may be a transmitter in the retina (Neal, 1976b) and as GABA may be involved in presynaptic inhibition (Davidson and Southwick, 1971) for which there is strong anatomical evidence in the retina (Dowling, 1968; Sosula and Glow, 1970), we have examined the effects of GABA and GABA anatagonists on the release of $|^3H|$ACh.

Exposure of the dark adapted retina to GABA (1mM) did not affect the spontaneous resting release of ACh but the light evoked release (3Hz) was reduced by more than 50% (Fig. 6). Thus, the evoked release in controls = 4.62 ± 0.569, whilst in the presence of GABA the evoked release = 2.18 ± 0.274 times the resting release, mean $\pm$ S.E.M. of 4 separate experiments, $p<0.05$. The normal response returned when the medium containing GABA was replaced with normal medium (Fig. 6).

In contrast to its inhibitory effect on ACh release, GABA produced a striking in-

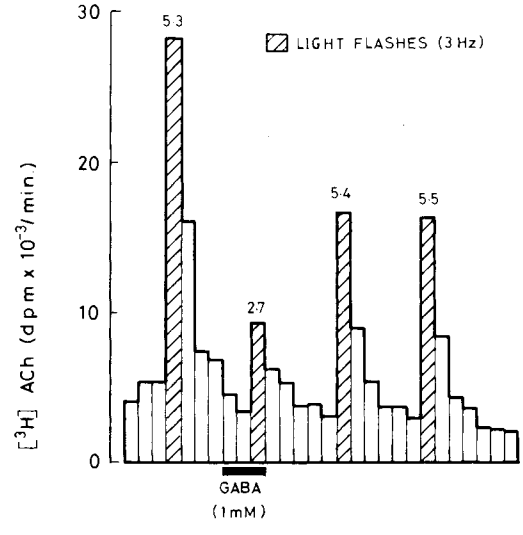

Fig. 6. Effect of GABA (1mM) on $|^3H|$ACh release evoked by light flashes (3Hz).

crease in the amplitude of the ERG. This was particularly noticeable at lower light intensities when the amplitude of the b wave was sometimes double that seen in the absence of GABA. This effect of GABA on the ERG has been reported previously (Starr, 1975).

Effect of GABA Antagonists on Evoked $|^3H|$ACh Release.

Exposure of the dark adapted retina to picrotoxin (20μM) (Fig. 7) or bicuculline (5μM) (Fig. 8) caused striking increases in the spontaneous resting release of $|^3H|$ACh to 4.1 times and 5.0 times the previous resting releases respectively (Each result the average of 3 experiments). The drugs did not increase the release of $|^3H|$choline from the retina.

The light evoked release (3Hz) of $|^3H|$ACh in the presence of either bicuculline or picrotoxin was approximately the same as that seen in the absence of the drugs, i. e. about 3 times greater than that seen in the two collection periods immediately before stimulation. However, compared with the spontaneous release in normal medium

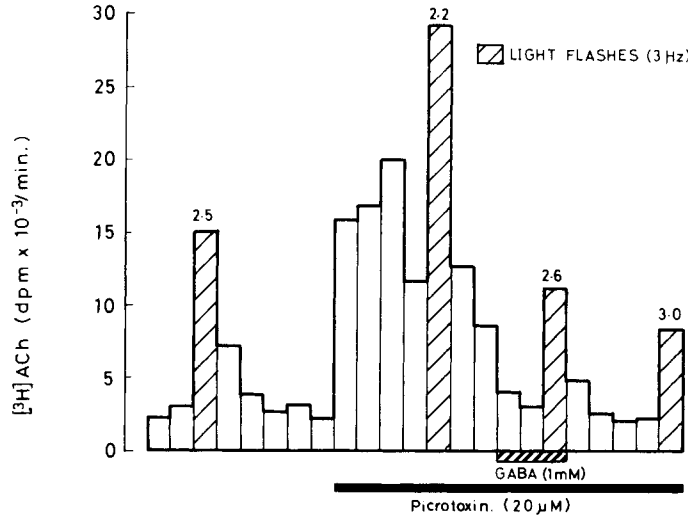

Fig. 7. Experiment showing effect of picrotoxin on $|^3H|$ACh release. The GABA antagonist greatly increased the spontaneous resting release of $|^3H|$ACh and abolished the inhibitory effect of GABA on the light evoked release of $|^3H|$-ACh.

containing no GABA antagonist, the light evoked release of $|^3H|$ACh was up to 12 times the resting release, the highest seen in this series of experiments.

Picrotoxin decreased the amplitude of the ERG. Initially, this trace appeared qualitatively similar to the ERG recorded in calcium free medium as the a-wave was

relatively unaffected. However, the c-wave persisted and at high intensities a small b-wave could be seen. Paradoxically, bicuculline did not alter the ERG.

In three experiments, 1mM GABA was applied to the retina in the presence of 20μM picrotoxin. Even at this low concentration, picrotoxin abolished the inhibitory effect of GABA on the light evoked release of $|^3H|$ACh (Fig. 7). The effect of GABA on the ERG was also abolished.

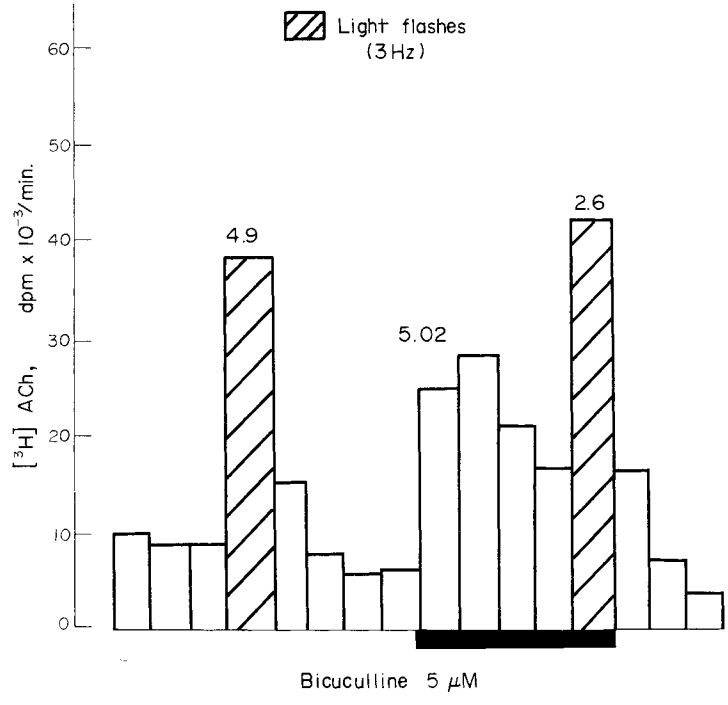

Fig. 8. Effect of bicuculline on spontaneous and light evoked release of $|^3H|$ACh.

In contrast to the GABA antagonist, the glycine antagonist, strychnine (1μM) had no effect on either the spontaneous or light evoked release of $|^3H|$ACh.

Effect of Light Stimulation on the Release of Endogenous Amino Acids

In six experiments, the endogenous amino acids in some of the samples were assayed in addition to measuring $|^3H|$ACh.

The effect of light flashes on the release of taurine, aspartate and $|^3H|$ACh is shown in Figs. 9 and 10. The efflux of taurine was strikingly increased, the mean release during the period of stimulation being 4.5 times greater than the spontaneous resting release. The release of $|^3H|$ACh was also increased by light flashes,

the evoked release being 4.2 times the resting release (Fig. 10). In contrast to both taurine and ACh, the efflux of aspartate was reduced by more than 50% by flashes of light (Fig. 9) whilst the efflux of glutamate, glutamine, alanine, glycine and GABA was not significantly affected (Fig. 10).

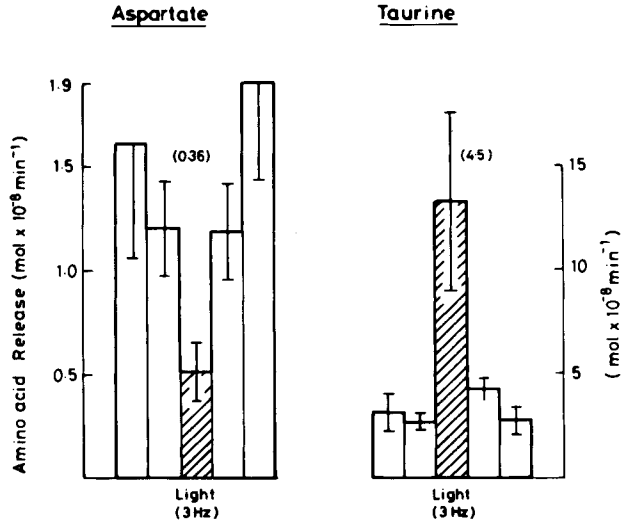

Fig. 9. Effect of light flashes (3Hz) on the release of aspartate and taurine from the dark adapted retina. Each result is the mean ± S. E.M. of 6 experiments. (The numbers in parentheses indicate the stimulated release/ mean of the 2 prestimulation releases).

DISCUSSION

Light Evoked Release of ACH

The present experiments demonstrate for the first time a light evoked release of ACh from the retina in vivo and provide strong evidence for ACh as a retinal synaptic transmitter substance. These experiments confirm and extend the previous work of Masland and Livingstone (1976) who reported a light evoked release of $|^3H|$ACh from the isolated rabbit retina. It is generally considered essential to demonstrate that the release of a substance is calcium dependent before considering it seriously as a transmitter substance and we have shown that the light evoked release of ACh from the retina was reversibly abolished by calcium free medium. Exposure of the retina to calcium free medium also abolished the ERG. except the a-wave which is thought to be a photoreceptor response (Penn and Hagins, 1969; Brown,1968; Dowling, 1970), indicating that inhibition of synaptic transmission across the retina (Dowling and Ripps, 1973; Cervetto and Piccolino, 1974) is associated with the abolition of the light evoked release of ACh.

The light evoked release of ACh significantly increased with frequency in the range 0.5 to 3Hz but above 10Hz, the evoked release diminished in parallel with the amplitude in the ERG. These results are somewhat difficult to interpret since in-

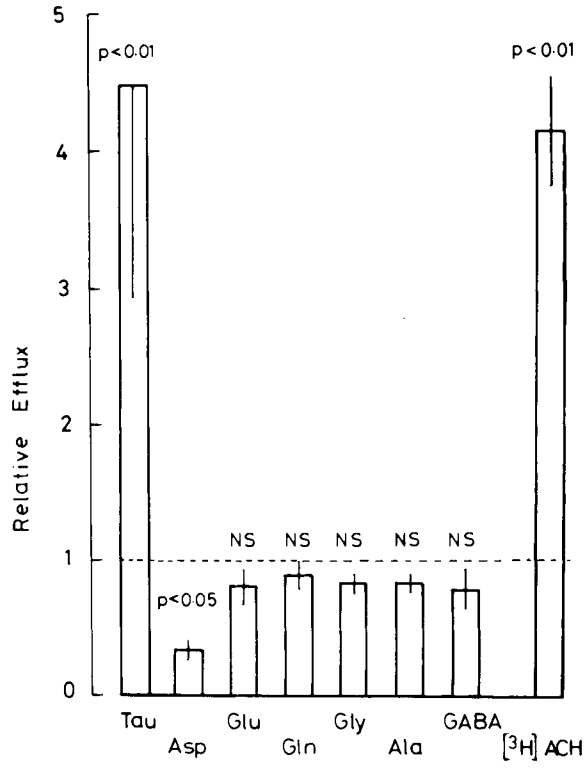

Fig. 10. Effect of light flashes (3Hz) on the release of endogenous amino acids and $|^{3}H|$ACh from the dark adapted retina. Each result is the mean $\pm$ S.E.M. of 6 experiments and is expressed as the relative efflux (light evoked release/mean of the 2 collection periods immediately preceding stimulation).

creasing the frequency of our photic stimulator also increased the total amount of light falling on the retina during the 10 min stimulation period. Thus, the reduction in the evoked release of ACh at higher frequencies was probably due to increasing light adaptation of the retina. Exposure of the dark adapted retina to continuous light of intensity approximately 1 log unit greater than the stimulus at 3Hz did not increase the spontaneous resting release of ACh, although stimulation with light flashes (3Hz) still evoked a large release of ACh. Although these results must be regarded with caution, they do allow certain tentative conclusions in regard to the identity of the cholinergic neurones. The fact that the efflux of ACh from the retina is increased at all implies that the cholinergic neurones are depolarized by light. As the distal neurones of the retina, i.e. the photoreceptors, horizontal and some bipolar cells, respond to light stimulation with hyperpolarizations (Werblin and Dowling, 1969; Kaneko, 1970) and ganglion cells are always postsynaptic (Dubin, 1970), this strongly suggests that the cholinergic neurones are amacrine cells or depolarizing bipolars. Furthermore, it appears that a flashing

stimulus is necessary for the light evoked release of ACh from the retina (as for dopamine release, Kramer, 1971), suggesting that the cholinergic neurones release ACh in response to the ON and/or OFF portion of the stimulus.

Cells giving a sustained (depolarizing) response to light should exhibit a prolonged release of neurotransmitter during continuous light stimulation whereas transient cells, which give a brief response and then return to near their resting potential after the onset of continuous light, should release little neurotransmitter. However, a flashing stimulus, causing repeated depolarizations should evoke a maximum release from transient cells, as observed in these experiments, indicating that the cholinergic neurones may be transiently responding cells.

Photoreceptor, horizontal and both classes of bipolar cells give sustained responses to light but some amacrine and ganglion cells respond with transient depolarizations (Werblin and Dowling, 1969; Kaneko, 1970). Although the sustained release of $[^3H]$ACh from a small population of depolarizing bipolar cells may have gone unnoticed against the background release during continuous light stimulation, it seems probably that the majority of cholinergic neurones may be transiently responding amacrine cells, which respond best to the onset and termination of a stimulus (Hedden and Dowling, 1978).

The suggestion that a major proportion of the cholinergic neurones in the retina is a sub-population of amacrine cells is consistent with regional distribution studies of cholineacetyl transferase (ChAT) which have shown that the enzyme is highly localized in the inner plexiform layer (Ross and McDougal, 1976), and with autoradiographical studies in the chicken retina, in which the cellular uptake sites of $[^3H]$choline were identified as a subpopulation of amacrine cells and a small proportion of bipolar cells (Baughman and Bader, 1977). The retina appears to possess both nicotinic and muscarinic receptors (Straschill and Perwein, 1973; Masland and Ames, 1976; Negishi, Kato, Teranishi and Laufer, 1978; Hruska, White, Azari and Yamamura, 1978) and studies of the binding sites of α-bungarotoxin-horseradish peroxidase suggest that the nicotinic receptors occur mainly in the inner plexiform layer (Vogel, Maloney, Ling and Daniels, 1977). The suggestion that some amacrine cells are cholinergic is also supported by studies of the histochemical localization of AChE (Nichols and Koelle, 1968).

GABA Inhibition of Light Stimulated ACh Release

The light evoked release of $[^3A]$ACh was inhibited by GABA, but the spontaneous release was not affected, suggesting the possibility of inhibitory GABAergic neurones presynaptic to the cholinergic neurones in the retina. The possibility that GABA merely blocked transmission in the outer plexiform layer is unlikely since the amplitude of the ERG was enhanced by GABA. The suggestion that GABA interacts with cholinergic neurones in the inner plexiform layer is consistent with recent studies which have shown that glutamate decarboxylase (GAD), the synthesising enzyme for GABA, is localized in this layer (Brandon, Wu and Lam, 1978) and also with much evidence suggesting that GABA may be an amacrine cell transmitter substance (Neal, 1976b). However, it should be noted that species variation may be important, because although in the rabbit retina, the GABAergic neurones appear to be mostly restricted to the proximal half of the retina, in other, non-mammalian retinae, GABA may also be a transmitter at horizontal cell synapses (Marc and co-workers, 1978; Lam and Steinman, 1971). In the cat $[^3H]$GABA is accumulated by interplexiform cells as well as amacrine cells (Nakamura, McGuire and Sterling, 1978)

GABA is thought to be a major inhibitory transmitter in the central nervous system (Krnjevic, 1974; Curtis and Johnston, 1974) and may have a presynaptic as well as

postsynaptic effect (Davidson and Southwick, 1971). Although there is strong anatomical evidence for presynaptic inhibition in the inner plexiform layer of the retina (Dowling, 1968; Sosula and Glow, 1970) our present experiments cannot differentiate between these two possibilities.

The GABA antogonists, picrotoxin and bicuculline, both abolished the inhibitory effect of GABA on the light evoked release of $|^3H|$ACh, but only picrotoxin antagozed the enhancing effect of GABA on the ERG, possibly reflecting some difference in the mechanism of the two antagonists. More importantly, both antagonists caused a large increase in the efflux of $|^3H|$ACh in the absence of light stimulation implying that there is a constant dark release of GABA which inhibits cholinergic neurones in the inner plexiform layer.

This suggestion is supported by intracellular recording from amacrine cells in the mudpuppy whose transient responses to light were enhanced by bicuculline and picrotoxin (Miller, Dacheux and Frumkes, 1977). Some amacrine cells receive input from both types of bipolar cells (Miller and Dacheux, 1976) and hyperpolarizing bipolar cells are thought to maximally release an excitatory transmitter in the dark (Dacheux, Frumkes and Miller, 1979; Frumkes and Miller, 1979). Thus a possible mechanism for the dark release of GABA could be the dark depolarization of GABAergic cells receiving input from hyperpolarizing bipolar cells (Miller, Dacheux and Frumkes, 1977).

Further evidence for amacrine cell modulation by GABA comes from studies of the proximal negative response (PNR), thought to reflect the activity of transient amacrine cells (Burkhardt, 1970). In frog retinae, this response was depressed by GABA, but enhanced by picrotoxin and bicuculline, which also removed the suppressive effect of a larger flashed spot suggesting that the surround inhibition may be GABA mediated (Burkhardt, 1972; Mooney, 1978). From the effects of bicuculline on the intensity/response curve of the PNR, Mooney (1978) has suggested that GABA may be involved in the transformation from sustained to transient signals which occurs in the inner retina.

GABA is inhibitory to ganglion cells (Noell, 1959; Straschill and Perwein, 1969; Ames and Pollen, 1969) while picrotoxin and bicuculline enhanced their responses (Miller, Dacheux and Frumkes, 1977) but abolished their directional sensitivity, thought to result from asymmetrical lateral inhibition by amacrine cells (Dowling, 1970) suggesting a role for GABA in this mechanism (Wyatt and Daw, 1975). Physostigmine also excited, but abolished the directional sensitivity of rabbit ganglion cells, which then responded to stimulation in the null direction, in a manner indistinguishable from the effect of picrotoxin (Ariel and Daw, 1978). The suggestion that retinal cholinergic neurones are tonically inhibited by GABA and thus excited by picrotoxin provides a simple explanation for this last observation, but the same effect could arise postsynaptically at the ganglion cells where ACh and GABA are excitatory and inhibitory respectively (Masland and Ames, 1976; Miller, Dacheux and Frumkes, 1977). These experiments cannot differentiate between the two mechanisms although it is possible that they may be complementary.

Glycine is also probably an inhibitory transmitter in the inner plexiform layer since $|^3H|$glycine was accumulated by amacrine cells (Ehinger and Falck, 1971; Bruun and Ehinger, 1974; Voaden, Marshall and Murani, 1974; Nakamura, McGuire and Sterling, 1978) and it was inhibitory to ganglion cells (Ames and Pollen, 1969; Miller, Dacheux and Frumkes, 1977). The light stimulated release of both $|^3H|$glycine (Ehinger and Lindberg-Bauer, 1976) and endogenous glycine (Coull and Cutler, 1978) has also been demonstrated.

However, it is unlikely that glycine is inhibitory to the cholinergic neurones

since strychnine, a potent glycine antagonist, did not affect the release of $|^3H|$-ACh, in contrast to the GABA antagonists picrotoxin and bicuculline. This interpretation, and the above discussion on GABA, depends critically on the specificity of the antagonists employed. Frumkes and Miller (1979) showed that at 10^{-5}M or less strychnine blocked glycine, but not GABA, and at 10^{-3}M or less picrotoxin and bicuculline were GABA, but not glycine antagonists and Mooney (1978) showed a similar selectivity on the PNR at a concentration of 10^{-5}g/ml (30μM for strychnine and 20μM for N-methyl bicuculline). As the antagonist concentrations used in these experiments were considerably lower (1μM, 5μM and 20μM for strychnine, bicuculline and picrotoxin, respectively) this assumption seems to be justified.

Furthermore, the concentration of strychnine, although low, should be sufficient to block glycine activity since the affinity constant of $|^3H|$strychnine binding was 0.03nM, about 300 times more potent than glycine (Young and Snyder, 1973).

Effect of Light on Amino Acid Release

The most interesting finding in our study of amino acid release from the retina was that flashes of light caused a significant decrease in the release of aspartate. This confirmed a previous experiment in which we found that light flashes (10Hz) produced a striking decrease in the release of aspartate from the rat retina *in vitro* (Neal, 1976b). A later study with the rat retina failed to confirm this reduction in aspartate release (Kennedy and Neal, 1978) but this was probably due to the high frequency of stimulation (30Hz) used in these experiments. This suggestion is supported by our studies of $|^3H|$ACh release, where we have found that light flashes at 30Hz do not evoke a release of ACh, although in the same preparation, large increases in ACh release are produced by flashes at 3Hz and 10Hz (Massey and Neal, 1979).

In darkness, vertebrate photoreceptors are partially depolarized and it has been suggested that there is a continuous release of an excitatory transmitter substance from their pedicles which maintains the horizontal cells and some bipolar cells in a depolarized state. Flashes of light hyperpolarize the photoreceptor cells and the resulting reduction in the release of excitatory transmitter causes hyperpolarization of the second order neurones (for refs see Neal, 1976b and Wu and Dowling, 1978). Recently, it has been suggested on the basis of electrophysiological experiments that aspartate was the most likely candidate for this depolarizing photoreceptor transmitter (Wu and Dowling, 1978). This suggestion in strongly supported by the present study, since only the release of aspartate was reduced by stimulation of the retina by light.

The increase in endogenous taurine release from the rabbit retina evoked by flashes of light agrees with previous reports, which have described an evoked release of both labelled and endogenous taurine from the isolated chicken retina (Pasantes-Morales and co-workers, 1974). It is not known whether the light evoked release of taurine from the retina is calcium dependent but it seems probable that this is so since both the light evoked release of labelled taurine (Pasantes-Morales and co-workers, 1974) and the potassium evoked release of endogenous taurine from the retina have both been shown to be highly Ca dependent.

The consistent demonstration of taurine release in response to light supports the suggestion that the amino acid might be a retinal transmitter substance. However, the cellular origin of neither the endogenous nor the labelled taurine released from the retina is known. A high proportion of retinal taurine is present in photo-

receptors (Orr, Cohen and Lowry, 1976). These cells also accumulate exogenous taurine (Lake, Marshall and Voaden, 1978) although glial cells in the retina have also been shown to accumulate the amino acid (Ehinger, 1973). It is difficult to escape from the conclusion that the most likely cells from which taurine is released by light stimulation are photoreceptors, although release from other cells which accumulate taurine (certain amacrine, bipolar and glial cells) cannot be excluded.

In the present study we did not detect any change in the efflux of endogenous glycine from the retina in response to light. This result is in agreement with a previous study using the chicken retina (Pasantes-Morales and co-workers, 1974) but a light evoked release of glycine from the perfused rat eye has been reported (Coull and Cutler, 1978). Also, flashes of light have been shown to increase the release of labelled glycine from the cat and rabbit retina (Ehinger and Lindberg-Bauer, 1976).

Although GABA is generally believed to be a retinal transmitter substance, it has proved remarkably difficult to demonstrate its release. Endogenous GABA release was not increased by light stimulation in the present study nor by potassium depolarization of the rat retina (Neal, 1976b). In agreement with these results, the efflux of labelled GABA from the chicken retina was not increased significantly by potassium depolarization (Pasantes-Morales and co-workers, 1974). However, a small light evoked increase in labelled GABA from the rabbit retina has been reported (Bauer and Ehinger, 1977). The reasons for these disagreements are at present unknown and will only be resolved by further experiments.

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Neurochemistry Vol. 1, pp. 209-229.
Pergamon Press Ltd. 1980. Printed in Great Britain.

RETINAL INDOLEAMINE ACCUMULATING NEURONS

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ABSTRACT

A previously unknown set of neurons, characterized by their ability to accumulate indoleamines, has been identified in the retina of Cebus monkeys, rabbits, cats, pigeons, chicken, goldfish and lampreys. They are not demonstrable with presently available techniques in humans, Cynomolgus monkeys, cows, pigs and rats. The neurons are called indoleamine accumulating neuron and form a subset of amacrine cells, distinguishable from all other subsets of amacrines with known transmitter. By electron microscopy they have been shown to be contacted by bipolar cells in the dyad arrangement and to form reciprocal contacts on the bipolar cells. A procedure is available for destroying selectively the processes of the indoleamine accumulating neurons.

The indoleamine accumulating neurons do not show any formaldehyde induced fluorescence in the normal animals or in animals in which the 5-hydroxytryptamine concentration in the brain has been elevated pharmacologically, and the 5-hydroxytryptamine concentration in the normal retina is too low to make it a likely neurotransmitter. What little is present is presumably in blood platelets in most cases (chicken may be an exception). The rate limiting enzyme in the 5-hydroxytryptamine synthesis, tryptophan hydroxylase, is not detectable in the retina. 5-hydroxytryptamine does not elicit any increase in retinal cyclic AMP. There is an energy dependent, high affinity uptake system for indoleamines but the effect of various inhibitors is different from that on the uptake into brain tissue. Several lines of evidence thus disfavour 5-hydroxytryptamine as a retinal neurotransmitter. Nevertheless, the active uptake of indoleamines suggests that the transmitter of the indoleamine accumulating neurons is an indole which, however, at present remains unidentified.

KEYWORDS

Retina; indoleamines; uptake; uptake inhibitors; fluorescence microscopy; electron microscopy; autoradiography; amacrine cells.

INTRODUCTION

Acetylcholine (Neal, 1976; Masland and Livingstone, 1976; Baughman and Bader, 1977; Massey and Neal, 1979), dopamine (Ehinger, 1978), glycine and γ-aminobutyric acid (Voaden, 1976; Ehinger, 1978) have been shown to be neurotransmitters in the retina;

all in the amacrine cells. There are no accurate counts available on the percentage of amacrine cells likely to use these transmitters, but even a rough estimate based on the densities of these amacrine cells in various micrographs published on the rabbit retina suggests that all transmitters have not yet been found. Considering that the retina is in fact a part of the brain it is to be suspected that whichever substance is a brain neurotransmitter may also be a retinal one. 5-hydroxytryptamine is an acknowledged CNS neurotransmitter, which already early was reported present in the vertebrate retina in significant concentrations (Levene, 1962; Welsh, 1964). However, neither Häggendal and Malmfors (1965) nor we (unpublished) were ever able to see any formaldehyde induced fluorescence ascribable to endogenous 5-hydroxytryptamine in the retina of a number of species, and Häggendal and Malmfors (1965) found no detectable amounts with their chemical assay (suggesting less than 50 ng/g wet weight). Nevertheless, Hauschild and Laties (1973) reported a cell type in chicken retina with the formaldehyde induced fluorescence typical of indoleamines. The cell type appeared only during a restricted period immediately after the hatching. In addition, certain enzymes involved in the metabolism of 5-hydroxytryptamine have been demonstrated in the retina, e.g. 5-hydroxytryptophan decarboxylase (Baker and Quay, 1969; Smith, 1973) and monoamine oxidase (Graham, 1974; Smith, 1973).

Since, finally, 5-hydroxytryptamine was also reported to affect the firing of retinal ganglion cells (Ames and Pollen, 1969; Straschill, 1968; Straschill and Perwein, 1969, 1975), it seemed as a likely candidate for being one of the missing retinal neurotransmitters, the more so since it is present in high concentrations in the pineal gland which is at times regarded as developmentally related to the retina. The work summarized here represents various attempts to see if 5-hydroxytryptamine is a retinal neurotransmitter. We have detected a special set of neurons in the retina of many animals, and named them indoleamine accumulating neurons and we have shown that they make synaptic connections which differ from the dopaminergic neurons which also can be identified in the electron microscope, but we have not been able to show that 5-hydroxytryptamine is a likely retinal neurotransmitter. Instead, our evidence disfavours it as transmitter, forcing us to guess some related substance may be the actual transmitter.

METHODS

Animals. The following animals were used: ordinary and albino rabbits weighing 1-2 kg; adult cats; Cynomolgus monkeys weighing 1.5-2 kg; chick embryos (White Leghorn) 13, 16 and 19 days, newborn chicken (Derko, a hybrid between Rhode Island and White Plymouthrock); chicken (Derko) 6-14 weeks; adult pigeons; and goldfish of 10-15 cm length. Human retinas were obtained from two eyes which were enucleated because of malignant melanoma. The retinas were taken from parts well away from the tumour. The ages of the patients were 38 and 67 years.

Fluorescence histochemistry. It was performed according to the standard Falck-Hillarp technique (see Falck and Owman, 1965 or Björklund, Falck and Owman, 1972). The tissues to be analysed were freeze-dried and then exposed to formaldehyde of controlled humidity and temperature whereby the fluorophores are formed. After this procedure the tissues were embedded in paraffin wax and sectioned. Indoleamines are characterized by their yellowish and catecholamines by their greenish fluorescence in the fluorescence microscope with the most commonly used filter settings.

Electron microscopy. The dopaminergic neurons were first removed from rabbit retinas with the procedure of Ehinger and Nordenfelt (1977). The remaining indoleamine accumulating neurons were then labelled by injecting 25 or 50 µg 5,6-dihydroxytryptamine intravitreally. Four hours later the eyes were fixed in 2% O_sO_4 and processed for electron microscopy according to standard procedures (Dowling and Ehinger,

1978b).

Incubations. Pieces of retina and slices from hypothalamus and nucleus caudatus were incubated in a buffer prepared according to Ames (1965). The tissue was equilibrated with the buffer for 10 min and then labelled 5-hydroxytryptamine or dopamine together with the drugs to be tested were added. The incubation was terminated after 10 min by transferring the tissue to an ice cold buffer without additives and it was washed for 20 min. After it was weighed the tissue was dissolved in Soluene® and the radioactivity was measured in a liquid scintillator as were samples from the incubation medium.

Tryptophan hydroxylase activity. This enzyme was determined in the retina and the brain according to Kuhar, Roth and Aghajanian (1971) using the method of trapping $^{14}CO_2$ released from L-|1-^{14}C| tryptophan. The tissue was homogenized in oxygenated 0.1 M Tris acetate, pH 8.1. L-|1-^{14}C| tryptophan was added and the mixture was incubated in small sealed flasks for 15 min at 21°C. The evolved CO_2 was captured in center wells containing ethylene glycol monomethyl ether and monoethanolamine. The reaction was terminated by adding perchloric acid and the flasks were then incubated for one more hour at 37°C to capture all CO_2. The ethylene glycol monomethyl ether and monoethanolamine mixture was then transferred to scintillation vials and the radioactivity was measured. Modifications of these standard conditions were made by adding co-factors as proposed by Ichiyama and co-workers (1970) and Gal and Patterson (1973).

Aromatic amino acid decarboxylase activity. This enzyme was assayed with 3,4-dihydroxyphenylalanine (DOPA) as substrate by trapping the $^{14}CO_2$ evolved from DL-3,4-dihydroxyphenyl |1-^{14}C|alanin. Assay conditions were identical with the standard assay system for tryptophan except for the adding of pyridoxal phosphate.

5-hydroxytryptamine. It was measured both by the radioenzymatic method of Saavedra, Brownstein and Axelrod (1973) and the high pressure liquid chromatography method of Hansson and Rosengren (1978a).

Dopamine. It was assayed with the high pressure liquid chromatography procedure of Hansson and Rosengren (1978b).

Cyclic AMP. It was measured in the intact retina as described by Bucher and Schorderet (1974a) and Schorderet (1975, 1977). In short, the tissue was equilibrated with a buffer for 40 min before being transferred to glass homogenizers containing fresh buffer, theophylline, EDTA and the pharmacological agent to be tested. The incubation was terminated after 10 min by transferring the homogenizers into boiling water. After homogenization and centrifugation the supernatant was analysed for the content of cyclic AMP with a commercial AMP assay kit obtained from the Radiochemical Centre, Amersham, United Kingdom.

RESULTS AND COMMENTS

Morphology of Indoleamine Accumulating Neurons

The normal retina treated for fluorescence microscopy with the Falck-Hillarp technique displays dopamine containing cell bodies at the junction of the inner nuclear layer and the inner plexiform layer in all species investigated so far (for reviews see Stell, 1972; Rodieck, 1973; Graham, 1974; Ehinger, 1976, 1978). They are situated among the amacrine cells and their number is estimated to constitute approxima-

tely 5 to 10 per cent that of the amacrines.

In the normal rabbit retina three sublayers of dopaminergic terminals can be seen. The outermost sublayer is most and the innermost sublayer least pronounced. The fluorescence of the structures described can be slightly enhanced without the appearance of new structures if a small amount (1-5 µg) of a catecholamine (dopamine, α-methyldopamine or α-methylnoradrenaline) is injected intravitreally four hours before processing the retina. However, with somewhat higher doses of a catecholamine (10-50 µg) or with a low to moderate dose (5-50 µg) of an indoleamine[1] (5-hydroxytryptamine, 6-hydroxytryptamine, 5,6-dihydroxytryptamine or 5,7-dihydroxytryptamine), it was found that the number of fluorescent perikarya increased 2- to 3-fold (Ehinger and Florén, 1976). These newly visualized perikarya represent the indoleamine accumulating neurons. They have in part a similar distribution to that of the dopaminergic perikarya, but additional perikarya are seen further out in the inner nuclear layer. These additional perikarya are of the same size or often slightly smaller than the other perikarya. At times fluorescent perikarya are also seen in the ganglion cell layer. The processes of all these cell bodies ramify in three sublayers in the inner plexiform layer. The innermost sublayer is now the best developed one and the middle sublayer is least developed (Fig. 1).

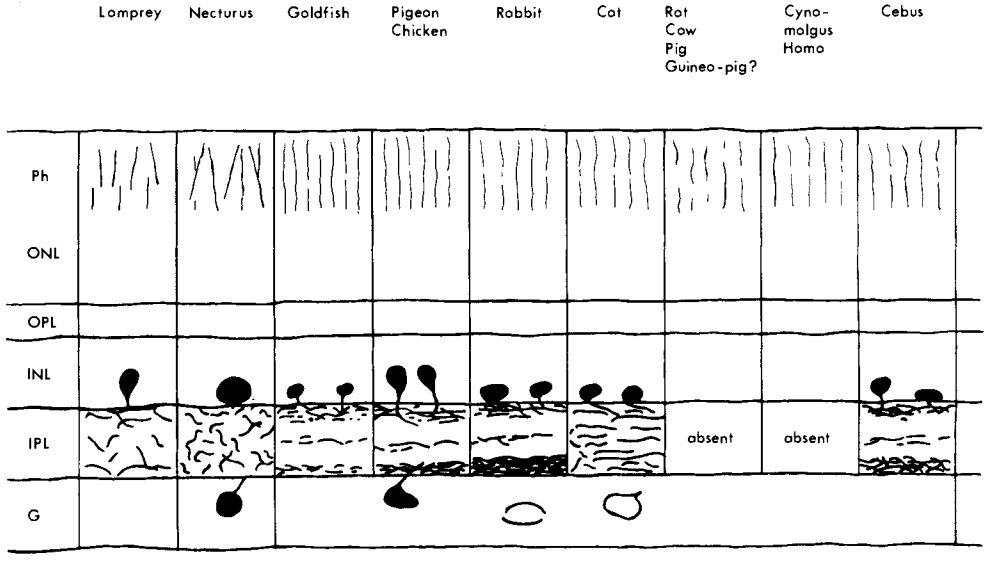

Fig. 1. Schematic drawing of the distribution of indoleamine accumulating neurons in the retina of species investigated so far. At times indoleamine accumulating cells were seen in the ganglion cell layer without much differences between some species and therefore the lines separating these species have not been extended to this layer. Ph photoreceptors; ONL outer nuclear layer; OPL outer plexiform layer; INL inner nuclear layer; IPL inner plexiform layer; G ganglion cell layer.

When incubating rabbit retina in a medium containing down to 10^{-7}M 5-hydroxytryptamine it was seen by fluorescence microscopy that at about 10^{-6}M the indoleamine

[1] 1 µg of the catecholamine on a molar basis equals 2 µg of the indoleamine.

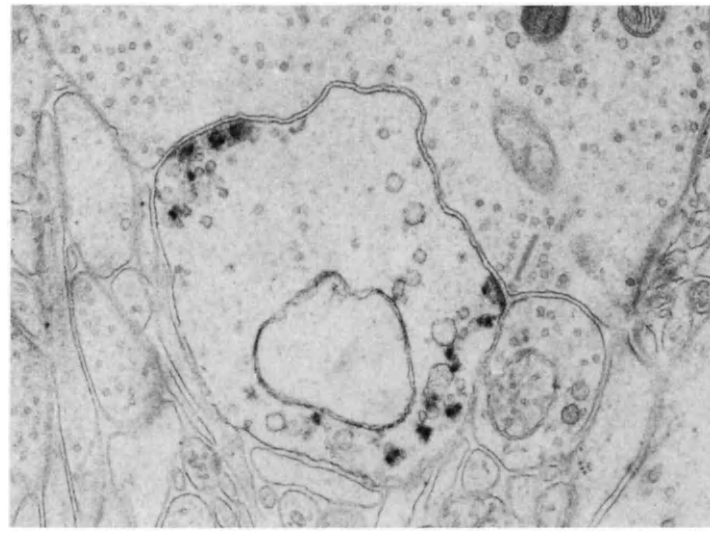

Fig. 2. A process of an indoleamine accumulating neurons (labelled with 5,6-dihydroxytryptamine so that membranes and organelles have got increased electron density) in a dyad together with an unlabelled amacrine cell process and a bipolar cell (identified by its synaptic ribbon). The indoleamine accumulating process also makes a reciprocal synapse on the bipolar cell. Rabbit retina, X 30000. (From Ehinger and Holmgren, 1979.)

accumulating neurons begin to fluoresce whereas the dopaminergic ones still show no uptake of the substance, indicating a more efficient uptake of indoleamines in the indoleamine accumulating neurons than in the dopaminergic ones. The existence of two separate, clearly distinguishable cell types with different uptake mechanisms was also demonstrated by the simultaneous intravitreal injection of a catecholamine and an indoleamine with a molar ratio 1:5 (Ehinger and Florén, 1976). Under these conditions the cell bodies and processes which normally contain a catecholamine display a greenish fluorescence whereas the cell bodies and processes of the indoleamine accumulating neurons display a yellow fluorescence. Thus, despite an indoleamine being present in a fivefold higher concentration than the catecholamine, the dopaminergic neurons preferentially accumulate the catecholamine in contrast to the indoleamine accumulating neurons which prefer the indoleamine.

The set of indoleamine accumulating neurons discovered as described above can also be identified in the electron microscope, because easily seen changes are induced in neurons which have accumulated certain hydroxylated indoleamines (Fig. 2.). The synaptic contacts of the indoleamine accumulating neurons can then be analysed. It has been found that they make other synaptic circuits than the dopaminergic neurons. The latter contact only other amacrines (Dowling and Ehinger, 1978b) whereas the indoleamine accumulating neurons receive their input from bipolar cells (Fig. 2) forming one member by many dyads (Ehinger and Holmgren, 1979). Reciprocal synapses onto the bipolars are the rule (Fig. 2) whereas the number of synapses

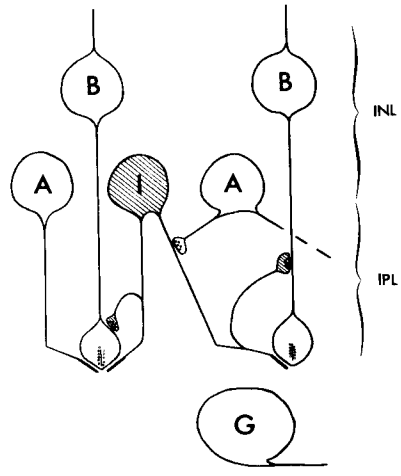

Fig. 3. Synaptic contacts of the indoleamine accumulating neurons in the inner layers of the rabbit retina. The indoleamine accumulating neuron (I) contacts bipolar cells (B) in dyads with reciprocal synapses in the innermost part of the inner plexiform layer (IPL). Unlabelled amacrines (A) make synapses onto preterminal parts of the indoleamine accumulating neurons in the outer parts of the IPL. (From Ehinger and Holmgren, 1979).

with other amacrines is very low. Thus, the indoleamine accumulating neurons form a set clearly distinguishable from the dopaminergic ones not only on light microscopical criteria, but also on ultrastructural ones, forming a different circuity (Fig. 3).

In the Cebus monkey retina indoleamine accumulating terminals appear in the inner plexiform layer in three sublayers located like in the rabbit although they are less distinct (Dowling, Ehinger and Florén, in preparation). The cell bodies were located in the innermost third of the inner nuclear layer.

In the normal cat retina the dopaminergic cell bodies send their processes to only one sublayer situated in the outermost part of the inner plexiform layer. The intravitreal injection of an indoleamine causes a 2- to 3-fold increase in the number of fluorescent cell bodies in the innermost part of the inner nuclear layer. All the cell bodies are of approximately the same size and shape. A few fluorescent cell bodies can be seen in the ganglion layer. In the inner plexiform layer there is a diffuse distribution of fluorescent fibres without discernible sublayering (Ehinger and Florén, 1976). Thus, the indoleamine accumulating neurons are also present in the cat (Fig. 1).

In the normal chicken and pigeon retina the dopaminergic terminals are distributed in three sublayers in the inner plexiform layer. The outermost sublayer is most and the innermost sublayer least pronounced. The injection of an indoleamine reveals large, bottle-shaped cell bodies situated two or three cell rows peripheral to the dopaminergic ones (Florén, 1979b). These indoleamine accumulating cell bodies are approximately twice as many as the dopaminergic ones. Occasionally fluorescent cell bodies are seen in the ganglion layer. The inner plexiform layer now displays two about equally prominent sublayers, the innermost and outermost ones. The middle sublayer is least pronounced (Fig. 1).

In the normal retina of goldfish (a teleost fish) three faint sublayers of dopaminergic terminals can at times be seen in the inner plexiform layer but more often the distribution of the processes is diffuse. Contrary to what is seen in the animals earlier discussed the innermost sublayer in the inner plexiform layer is richest in fluorescent fibres. The injection of an indoleamine increases the number of perikarya in the inner nuclear layer (Ehinger and Florén, 1976). The additional cell bodies, which are the indoleamine accumulating ones, are smaller and often situated further out than the dopaminergic cell bodies. The number of terminals in the outermost part of the inner plexiform layer is increased (Fig. 1).

In the mudpuppy (Adolph and Ehinger, in preparation), the dopaminergic neurons are confined to the border between the inner nuclear and inner plexiform layers whereas indoleamine accumulating terminals occur evenly dispersed throughout the inner nuclear layer. In the river lamprey (Ehinger, Holmberg and Öhman, 1977) the indoleamine accumulating neurons also occur throughout the inner plexiform layer whereas the dopaminergic terminals in part have a different distribution.

In the normal retina of Cynomolgus monkeys, humans, cows, pigs and rats there is one sublayer of dopaminergic terminals in the outermost part of the inner plexiform layer. The intravitreal injection of an indoleamine (Cynomolgus monkey, pig, rat) or the incubation with an indoleamine (cow, humans) did not increase the number of the distribution of the fluorescent fibres, nor was the number of fluorescent cell bodies increased (Ehinger and Florén, 1978a; Ehinger, Hansson and Tornqvist, 1979). In guinea-pigs the results were equivocal (Florén, unpublished). Injecting an indoleamine intravitreally (5-25 µg) resulted in only a small and ambiguous increase in the number of fluorescent cell bodies or terminals. Injection of both a cate-

cholamine and an indoleamine likewise resulted in nothing more than an ambiguous separation of two cell types despite the fact that several different drug proportions were tried and that similar experiments in goldfish and rabbit gave two very clearly distinguishable cell populations (Ehinger and Florén, 1976). In view of these observations the existence of a range of uptake mechanisms with different affinities for 5-hydroxytryptamine or 5,6-dihydroxytryptamine (which are the amines mainly used in this work) can be speculated on, and exemplified with the easily demonstrable ones in rabbits, goldfish, Cebus monkey, chicken and pigeon, the doubtful ones in guinea-pig and the absence of any demonstrable ones in Cynomolgus monkeys, humans, cows, pigs and rats. The neurons may nevertheless exist in all species, in homologous but not identical forms. This is suggested by the presence in Old World monkey retina of stratified amacrines which have their processes predominantly located in the innermost part of the inner plexiform layer (Ramon y Cajal , 1893/1973) and which thus could correspond to the indoleamine accumulating neurons in, e.g., the rabbit retina.

There are other examples of cells with similar morphological characteristics but different transmitters and transmitter uptake mechanisms in different species. The interplexiform cell (Dowling and Ehinger, 1975, 1978a; Boycott and co-workers, 1975; Oyster and Takahashi, 1977; Kolb and West, 1977) is an example of such a neuron in the retina. They are dopaminergic in teleost fish and New World monkeys but have a different, unknown transmitter in all other species investigated (Ehinger, 1978) and must in those cases be demonstrated with techniques other than histofluorescence.

Chemical Removal of Indoleamine Accumulating Terminals

Valuable information about the function of the indoleamine accumulating neurons can very likely be obtained by studying the effects of removing them. Intravitreal injections of 5,6-dihydroxytryptamine or 5,7-dihydroxytryptamine together with a monoamine oxidase inhibitor on two successive days were found to remove the indoleamine accumulating terminals in the rabbit and goldfish (Ehinger and Florén, 1978b). The time required for destruction of the terminals is a few days, and an adequate effect appears consequently after one week. The ordinary 4 hour exposure time to 5,6-dihydroxytryptamine or 5,7-dihydroxytryptamine used for visualization of the indoleamine accumulating neurons is too short to produce significant changes in the morphology of the neurons. The cell bodies of the indoleamine accumulating neurons appeared unaffected by the long term (1 week) exposure to the dihydroxylated indoleamines. A similar observation was made by Ehinger and Nordenfelt (1977) on the dopaminergic cell bodies when the retina was treated with a corresponding drug toxic to dopamine containing neurons, 6-hydroxydopamine. The fact that only the terminals and not the cell bodies are destroyed is presumably due to the normally higher concentration of the accumulated substance in the terminals than in the cell body and thus the acute toxic effects are likely to be most pronounced in the terminals.

Not only can the destruction of the indoleamine accumulating terminals be seen morphologically. The uptake capacity of the indoleamine accumulating neurons was, as predictable, found to be reduced (Florén, 1978): the V_{max} decreased from 9.1×10^{-8} moles/mg/min to 1.5×10^{-8} moles/mg/min (means of 4 determinations) in studies with the procedure used in other uptake studies as described below.

The b-wave of the ERG was significantly reduced in these rabbits (Florén and Nordenfelt, unpublished), which is in contrast with the previous observations with denervation of dopaminergic neurons with 6-hydroxydopamine (Ehinger and Nordenfelt, 1977). This fits with the observation that the dopaminergic neurons and the indole-

amine accumulating ones form different types of neuronal circuits (Dowling and Ehinger,1978a, b; Ehinger and Holmgren, 1979), but the b-wave is too complex a function to permit any further analysis at this stage.

5-hydroxytryptamine Content

Histochemistry. The indoleamine accumulating neurons have in all species investigated by us so far only been observed after exogenously applied indoleamines, i.e. the neurons do not fluoresce in the normal retina. This is in contrast to the dopaminergic neurons. The indoleamine accumulating neurons in the chicken retina might, however, very well correspond to the indoleamine containing cells, which Hauschild and Laties (1973) found in the normal retina of chick embryos. The position of these cells approximates that of the indoleamine accumulating neurons which are also localized three or four cell rows peripheral to the border between the inner nuclear and inner plexiform layers. The normal retina of chick embryos and newborn chicken was therefore investigated for indoleamine fluorescence but without finding any with certainty. One reason for this might be that particularly favourable conditions with higher indoleamine concentration occurred in the breed of chicken used by Hauschild and Laties. It might be noted that the indoleamine containing cells were only noticed during a short period of embryogenesis. There are several reports on fluctuation of the activity of the enzymes involved in indoleamine metabolism (Baker and Quay,1969; Smith, 1973; Suzuki, Noguchi and Yagi, 1977b) and also of the level of the formed indoleamine metabolites (Smith and Baker, 1974) in the eye of embryonic and newborn animals. Temporary increases in the amine level are therefore possible.

The reason why the indoleamine accumulating neurons can not be seen to fluoresce in the normal retina might thus be too low a concentration of their supposed transmitter, 5-hydroxytryptamine, to permit detection with the standard Falck-Hillarp method. In recent years, however, improvements of the standard method have appeared. They are mainly concerned with the fluorescence of catecholamines using perfusion with formaldehyde and/or glyoxylic acid (Hökfelt and Ljungdahl, 1972; Lindvall and co-workers,1973; Bloom and Battenberg, 1976). However, Lorén and co-workers (1976) reported on a further improved histofluorescence method based on combined formaldehyde and glyoxylic acid perfusion with high magnesium content and acid pH. In animals pretreated with L-tryptophan (the amino acid precursor of 5-hydroxytryptamine) and a monoamine oxidase inhibitor according to Aghajanian, Kuhar and Roth (1973) this glyoxylic acid method increases the fluorescence yield of 5-hydroxytryptamine compared to the standard Falck-Hillarp technique. The fluorescence of 5-hydroxytryptamine can also be improved by exposing the freeze-dried tissue to prolonged treatment with more humid formaldehyde vapour (Fuxe and Jonsson, 1967). No indoleamine fluorescence could, however, be seen in the normal retina of rabbit despite taking advantage of these improvements (Florén, 1979a). Simultaneously processed tissue pieces from the brain stem of the same animal showed good 5-hydroxytryptamine fluorescence.

Further evidence against 5-hydroxytryptamine being the transmitter of the indoleamine accumulating neurons is the failure of the neurons to respond to p-chloroamphetamine (Florén, 1979a). This substance is considered to have a neurotoxic effect on 5-hydroxytryptaminergic neurons in the brain as demonstrated by a long-term decrease in the brain levels of both 5-hydroxytryptamine and the rate limiting enzyme in its synthesis, tryptophan hydroxylase, following a single intraperitoneal dose of p-chloroamphetamine (Sanders-Bush, Bushing and Sulser, 1972; Harvey, McMaster and Yunger, 1975; Massari and co-workers, 1978). The retinal indoleamine accumulating neurons thus differ from the 5-hydroxytryptamine containing neurons of the brain in being insensitive to p-chloroamphetamine.

Chemical assays. In the earlier reports about 5-hydroxytryptamine in the retina (see Introduction) the amount of 5-hydroxytryptamine was found to be comparable to that of dopamine in the retina even though in one report (Häggendal and Malmfors, 1965) no 5-hydroxytryptamine could be detected at all. The presence of retinal dopaminergic neurons fluorescing with the Falck-Hillarp technique is well documented and therefore the absence of indoleamine fluorescence in the normal retina even in animals in which the 5-hydroxytryptamine content of the brain has been increased was in conflict with the quantitative determinations of 5-hydroxytryptamine which prompted a series of new estimates of the 5-hydroxytryptamine content (Table 1). All have in common that they show lower figures than the ones published by Levene (1962) and Welsh (1964) and they are also significantly lower than the figures for dopamine in the retina (about 200-400 ng/g, Häggendal and Malmfors, 1963, 1965; Nichols, Jacobowitz and Hottenstein, 1967; da Prada, 1977; Florén and Hansson, 1979). The lowest figures were obtained with high pressure liquid chromatography which is also the most sensitive of the methods used. In mammals, they show no more 5-hydroxytryptamine than what can be accounted for by the blood platelets, and the content can be reduced substantially by washing out the blood from the vascular tree (Ehinger, Hansson and Tornqvist, 1979). In fact, the volume of blood necessary to account for the retinal 5-hydroxytryptamine amounts to about 0.5 % of the entire rabbit retinal volume, a figure which seems quite reasonable even if the sparse vascularization of the rabbit retina is taken into account.

The low figures for 5-hydroxytryptamine in the retina thus do not favor it as a neurotransmitter because in the midbrain the concentration is at least ten times higher than the values obtained in the retina by high pressure liquid chromatography (Florén and Hansson, 1979). Noting that the number of indoleamine accumulating neurons is several times higher than the dopaminergic ones one would also expect the 5-hydroxytryptamine concentration to be in the order of one or two µg/g, but as discussed above all recent estimates are at least five to ten times less than so.

The bird retina presents a special case. Suzuki and co-workers (1977a) and Florén and Hansson (1979) agree that the figures are higher than in rabbits. As the retina of birds in contrast to that of mammals is avascular (Michaelson, 1954), the 5-hydroxytryptamine can not readily be attributed to the thrombocytes. Therefore, in the chicken, the most plausible localization of 5-hydroxytryptamine seems to be neuronal, presumably to the indoleamine accumulating neurons which seem to correspond to the indoleamine containing cell in chick embryos described by Hauschild and Laties (1973). This assumption is supported by even higher amounts of 5-hydroxytryptamine in the retina of newborn chicken (71-90 ng/g) (Florén and Hansson, 1979) than in that of older chicken (36-67 ng/g). In addition to this finding the facts that the indoleamine containing cell described by Hauschild and Laties only appeared during a short embryonic stage, and that the activity of the enzymes involved in indoleamine metabolism as well as the amount of indoleamine metabolites formed fluctuate in embryonic and newborn chicken (see above), suggest that 5-hydroxytryptamine is not necessarily the actual transmitter. 5-hydroxytryptamine might rather be an accumulated intermediate in embryos in an as yet immature indole metabolism system different from the known 5-hydroxytryptamine pathways.

Tryptophan Hydroxylase

Two enzymes, 5-hydroxytryptophan decarboxylase and monoamine oxidase, which are necessary for the metabolism of 5-hydroxytryptamine have been found in the retina (Baker and Quay, 1969; Smith, 1973; Graham, 1974). Both enzymes are, however, unspecific as they are also needed for the metabolism of dopamine. 5-hydroxytryptophan decarboxylase and dihydroxyphenylalanine (DOPA) decarboxylase are thus considered to be the same enzyme, an aromatic amino acid decarboxylase (Christensson, Dairman and Udenfried, 1972). Monoamine oxidase degrades both 5-hydroxytryptamine and dopamine although different forms of the enzyme are now being recognized. Suzuki, Noguchi and Yagi (1977b) found support in the chicken retina for the pres-

ence of type B monoamine oxidase which acts on 5-hydroxytryptamine. There are, however, no reports of the presence in the retina of the rate limiting enzyme, tryptophan hydroxylase, in the synthesis of 5-hydroxytryptamine despite several searches for it (Smith, 1973; Smith and Baker, 1974; Florén and Hansson, 1979). A direct comparison with brain tissue showed that the retinal tryptophan hydroxylase activity must be less than 1/20 of that in the midbrain (Florén and Hansson, 1979) which is difficult to reconcile with 5-hydroxytryptamine as a retinal neurotransmitter. The absence of tryptophan hydroxylase activity is in contrast to the presence of the rate limiting enzyme, tyrosine hydroxylase, in the synthesis of dopamine as shown in the chicken retina (Schwarcz and Coyle, 1976).

The level of 5-hydroxytryptamine did not increase in the rabbit retina after loading with L-tryptophan and inhibition of monoamine oxidase (Florén and Hansson,1979) although the same procedure increases the amount of 5-hidroxytryptamine several fold in the brain (Aghajanian, Kuhar and Roth, 1973). Nor did the fluorescence yield of indoleamines increase in the retina with the same procedure (Florén, 1979a). The two observations indicate indirectly the absence of tryptophan hydroxylase in the rabbit retina.

Response of Cyclic AMP to 5-hydroxytryptamine

One of the main criteria of a neurotransmitter substance is that when externally applied the substance must mimic the action of endogenous nerve activity. This is the so called criterion of identity of action. The postsynaptic response of cyclic AMP to the presynaptic release of neurotransmitters or to the external application of the substance or an analogue could in a sense be considered to be a criterion of identity of action. Several neurotransmitters including 5-hydroxytryptamine have been shown to increase cyclic AMP levels in the brain (see Daly, 1976). Enjalbert and co-workers (1978) found a close correlation between the regional distribution of the 5-hydroxytryptamine nerve terminals and the distribution of 5-hydroxytryptamine sensitive adenylate cyclase (the enzyme forming cyclic AMP from ATP). Dopamine which is an acknowledged retinal transmitter increases the level of cyclic AMP in the retina of several species (Brown and Makman, 1972; Bucher and Schorderet, 1974b; Schwarcz and Coyle, 1976; Florén and Hansson, 1979). As the rabbit retina is rich in indoleamine accumulating neurons, 5-hydroxytryptamine could be expected to increase the level of cyclic AMP if it were the transmitter of the neurons, but 5-hydroxytryptamine was found to elicit no such increase (Florén and Hansson, 1979). Similar results were reported in the rat and in the calf retina (Makman, Brown and Mishra, 1975), but as both rat and cow retina lack indoleamine accumulating neurons (see above) the result in those cases is less significant than in the rabbit retina.

Uptake of 5-hydroxytryptamine

5-hydroxytryptamine was found to be taken up in the rabbit retina by a temperature dependent saturable process as was dopamine (Ehinger and Florén, 1978). Kinetic analysis using a double reciprocal plot suggests that the uptake of 5-hydroxytryptamine consists of two components: a high affinity uptake with an apparent $K_m = 9.2 \times 10^{-7}$ M and a low affinity uptake with an apparent $K_m = 1.7 \times 10^{-5}$M (Ehinger and Florén, 1978a). The V_{max} for the high affinity uptake was 1.5×10^{-7} moles/mg/min. Thomas and Redburn (1979) also found a high affinity uptake in bovine retina, but with a V_{max} of 2.94×10^{-9} moles/mg/min. The fiftyfold difference in V_{max} is noteworthy. Suzuki, Noguchi and Yagi (1978) also found in the chicken retina a high and a low affinity uptake of 5-hydroxytryptamine with apparent K_ms of 1.0×10^{-7} M and 6,7 x

TABLE I 5-HT in retina (ng/g wet weight)

	Häggendal and Malmfors 1965 (Fluorimetry)	Suzuki and co-workers 1977 (Fluorimetry)	Thomas and Redburn 1979 (Fluorimetry)	Florén and Hansson 1979 (HPLC)	(Radioenzymatic method)	Ehinger, Hansson and Tornqvist 1979 (HPLC)
Normal rabbits	not detectable (<∿ 50 ng/g)			24 ± 5	36 ± 6	36 ± 6
Perfused rabbits						3.8 ± 0.9
Cow			100.3 ± 10.1			26.2 ± 7.2
Pig						5.9 ± 0.8
Guinea-pig						6.0 ± 2.6
Chicken		176 ± 12		34 ± 4	67 ± 17	
Chicken (newborn)				71 ± 5	90 ± 8	

10^{-6}M respectively. Shaskan and Snyder (1970) found in slices from the brain a high affinity uptake of 5-hydroxytryptamine with an apparent K_m around 1.5×10^{-7} and a low affinity uptake with an apparent $K_m = 8.0 \times 10^{-6}$M. They suggested that the high affinity system represents uptake in 5-hydroxytryptaminergic neurons and the low affinity system in catecholamine neurons. This interpretation of the data is also readily applicable to the retina as demonstrated morphologically by the preferential uptake of indoleamines in only the indoleamine accumulating neurons at low concentrations and in the dopaminergic ones first with higher concentrations (Ehinger and Florén, 1976; Florén, 1978; Ehinger and Florén, 1978a).

The specific, high affinity uptake of 5-hydroxytryptamine in the brain (Ross and Renyi; 1967; Shaskan and Snyder, 1970) is similar to the uptake also found for other neurotransmitters. The uptake seems to represent a mechanism for inactivation of the transmitter. The uptake of 5-hydroxytryptamine in the retina is comparable to that in the brain and thus per se compatible with 5-hydroxytryptamine being a transmitter in the retina as well. It should be stressed, however, that analogues of the true transmitter are also taken up by the neurons. Thus the uptake affinities for some of the dihydroxylated indoleamines are in the same range as that for 5-hydroxytryptamine in the brain (Baumgarten and co-workers, 1973). The high affinity uptake of 5-hydroxytryptamine found in the retina therefore does not rule out the possibility of a related indole as the transmitter and it is clear from Thomas and Redburn's (1979) observation of a high affinity uptake of 5-hydroxytryptamine in the bovine retina which lacks indoleamine accumulating neurons (Ehinger, Hansson and Tornqvist, 1979) that the presence of such an uptake is not by itself evidence for significant numbers of 5-hydroxytryptamine neurons. The low V_{max} in the bovine retina (Thomas and Redburn, 1979) is significant here. In a study of the rabbit retina Florén (1978) found the V_{max} to fall to similar low levels when the indoleamine accumulating neurons were removed.

Dopamine was in the retina found to have a high affinity uptake with an apparent $K_m = 5.6 \times 10^{-7}$M (Ehinger and Florén, 1978a)and a low affinity uptake with an apparent $K_m = 5.2 \times 10^{-5}$M (means of 4 determinations) (Florén, 1978). It is evident that the biphasic uptake of dopamine in the retina demonstrated by the two separate K_ms represents an uptake of dopamine into the dopaminergic neurons, as well as a less specific uptake, presumably into the indoleamine accumulating neurons. The fact that this uptake as demonstrated morphologically with higher doses of a catecholamine is into the indoleamine accumulating neurons (Ehinger and Florén, 1976; Florén, 1978) and not into the several other types of retinal cells suggests that the indoleamine accumulating neurons work with a transmitter of the amine type.

The uptake of 5-hydroxytryptamine can be clearly separated from that of dopamine as demonstrated by a different inhibitory effect of a number of drugs on the two uptake processes (Ehinger and Florén, 1976; Florén, 1979a). Special interest was taken in selective inhibitors of the uptake of 5-hydroxytryptamine. Chlomipramine has for long been known as the most selective inhibitor of the uptake of 5-hydroxytryptamine (Carlsson, 1970; Shaskan and Snyder, 1970; Ross, Renyi and Ögren, 1972) although lately other substances have been claimed to be more selective (Wong and co-workers, 1974; Claassen and co-workers, 1977; Ross and Renyi, 1977; Petersen, Olsson and Squires, 1977). FG 7051, femoxetine, norzimelidine and GEA 654 are examples of such substances. They were all found together with chlomipramine to inhibit the uptake of 5-hydroxytryptamine significantly more than that of dopamine

in the retina as well as in the brain (Florén, 1979a). The effect of the drugs in the brain was investigated to act as a comparison with the retina. For this purpose hypothalamus, rich in 5-hydroxytryptamine, and nucleus caudatus, rich in dopamine, were used. It could be concluded that the pattern of inhibition was the same in the retina and in the brain.

However, a more detailed analysis of the dose effects of FG 7051, femoxetine, norzimelidine, GEA 654 and chlomipramine on the uptake of 5-hydroxytryptamine revealed that although the dose-response curves were parallel or close to parallel for each drug (except perhaps for GEA 654) in the retina and the hypothalamus, the inhibitory effect was always less pronounced in the retina (Florén, 1979a) (Fig. 4). This difference suggests that the indoleamine uptake system of the retina is not identical with that of the hypothalamus.

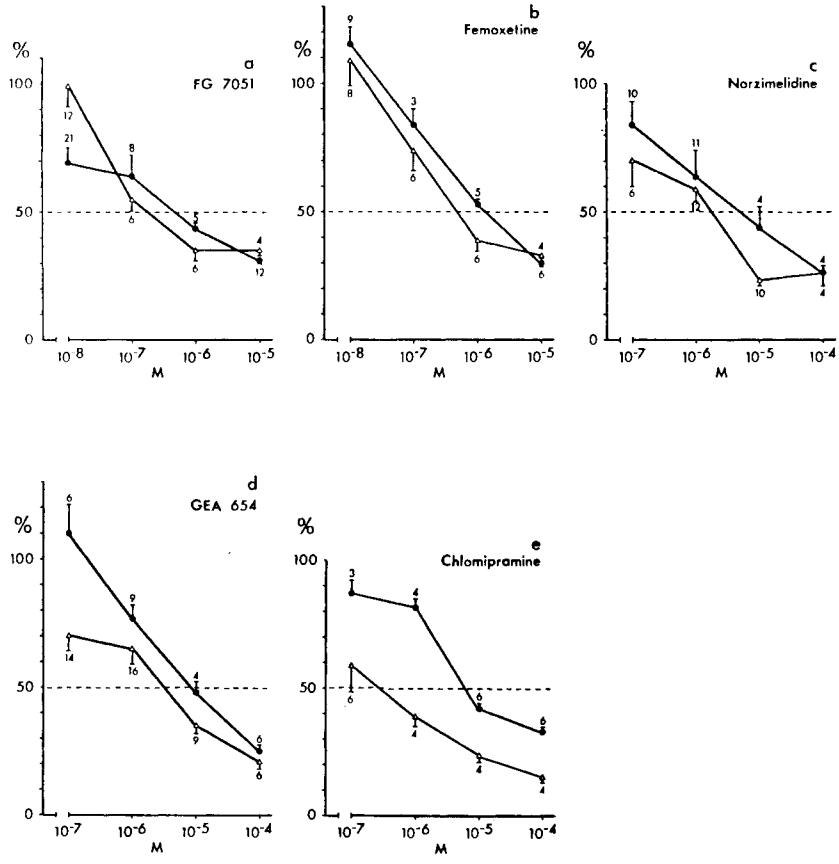

Fig. 4 a-e. Effect of varying concentrations of FG 7051, femotoxine, norzimelidine, GEA 654 and chlomipramine on the uptake of 5-hydroxytryptamine in rabbit retina and hypothalamus. The y-axis shows the relative uptake of 5-hydroxytryptamine when compared to the simultaneously run controls (control values = 100%). The x-axis shows molar concentration of the drugs. Each value is given as means ± S.E.M. and the number of observations is given above or below. ●—● retina, Δ—Δ hypothalamus.

Identity of the Transmitter of the Indoleamine Accumulating Neurons.

Because of its status as the only accepted neurotransmitter indole in the CNS 5-hydroxytryptamine has been considered as the most plausible transmitter in the retinal indoleamine accumulating neurons as well. However, as discussed above, it has not been possible to obtain positive and direct evidence for establishing 5-hydroxytryptamine as a neurotransmitter in the retina. The quantitative determinations of 5-hydroxytryptamine suggest that the indoleamine accumulating neurons operate with a different substance than 5-hydroxytryptamine buy yet sufficiently closely related to make it influence the results of e.g. the various fluorometric determinations of 5-hydroxytryptamine.

In the CNS there are reports of the presence of various indoleamines. Björklund, Falck and Stenevi (1970, 1971) suggested in microspectrofluorometric studies the presence of an indoleamine such as 5-methoxytryptamine, 6-hydroxytryptamine or N-methylserotonin in the rat brain. The neurons were, unlike ordinary 5-hydroxytryptamine containing neurons, resistant to treatment with p-chlorophenylalanine. 5-methoxytryptamine (Green, Koslow and Costa, 1973; Koslow, 1974), tryptamine (Boulton and Majer, 1972; Snodgrass and Horn, 1973; Saavedra and Axelrod, 1974) and melatonin (Koslow, 1974) have been identified in the brain using various biochemical methods. The concentrations of these indoleamines are less than that of 5-hydroxytryptamine. In rat hypothalamus where the concentration of the indoles is relatively high, the concentration of tryptamine and 5-methoxytryptamine amounts only to approximately 10-20 % of the concentration of 5-hydroxytryptamine, whereas the corresponding value for melatonin is about 40%. Melatonin is, however, unlikely as the transmitter of the indoleamine accumulating neurons as it is not taken up by the retina (Ehinger and Florén, 1978a). Bufotenine, which like melatonin is substituted on the nitrogen on the side chain, has little effect on the indoleamine accumulating system (Ehinger and Florén, 1978a) suggesting that it is not taken up in a significant amount. From this observation it can be concluded that the unknown transmitter is unlikely to be substituted on the side chain nitrogen. Such a substitution abolishes the prerequisite for the information of the fluorescing β-carbolines and would in that respect fit with a presumed nonfluorescing transmitter of the indoleamine accumulating neurons. The influence of 5-methoxytryptamine and tryptamine on the indoleamine accumulating system is greater (Ehinger and Florén, 1978a) but they are, like 6-hydroxytryptamine, 5,6-dihydroxytryptamine and 5,7-dihydroxytryptamine, unlikely as the transmitter of the indoleamine accumulating neurons as they all produce fluorophores in the histochemical procedure of Falck and Hillarp and thus should have been seen to fluoresce provided they are not present in too low a concentration. Further, 5-methoxytryptamine, tryptamine and 6-hydroxytryptamine have not been found in the rabbit retina in qualitative thin layer chromatograms according to Axelsson, Björklund and Seiler (1971) with a lower detection limit at about 150 ng/g wet retina (Florén, unpublished).

Although there is thus little doubt about the presence of a special set of cells in the retina, the indoleamine accumulating neurons, it has not been possible to ascertain their transmitter. As discussed above, we feel it should be an indoleamine, not substituted on the side chain nitrogen, but we are at present not able to define it further.

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Neurochemistry Vol. 1, pp. 231-244.
Pergamon Press Ltd. 1980. Printed in Great Britain.

THE PARTIAL PRIMARY STRUCTURE OF BOVINE RHODOPSIN AND ITS TOPOGRAPHY IN THE RETINAL ROD CELL DISC MEMBRANE

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ABSTRACT

The amino-terminal 39 amino acids of bovine rhodopsin have the sequence

CHO CHO

Ac-Met-Asn-Gly-Thr-Glu-Gly-Pro-Asn-Phe-Tyr^{10}-Val-Pro-Phe-Ser-Asn-Lys-Thr-Gly-Val-Val^{20}-Arg-Ser-Pro-Phe-Glu-Ala-Pro-Gln-Tyr-Tyr^{30}-Leu-Ala-Glu-Pro-Glx-Trp-Phe-Ser-Met^{39}

where both carbohydrate attachment sites (CHO) contain $GlcNAc_3Man_3$. This region of rhodopsin's sequence is exposed at the internal membrane surface of the rod cell disc membrane. Rhodopsin's carboxyl-terminal 40 amino acids have the sequence

Met$^{40'}$-Asn-Lys-Gln-Phe-Arg-Asn-Cys-Met-Val-Thr$^{30'}$-Thr-Leu-Cys-Cys-Gly-Lys-Asn-Pro-Leu-Gly$^{20'}$-Asp-Asp-Glu-Ala-Ser-Thr-Thr-Val-Ser-Lys$^{10'}$-Thr-Glu-Thr-Ser-Gln-Val-Ala-Pro-Ala$^{1'}$

where amino acid 1′ is the carboxyl-terminal amino acid of rhodopsin. Serines and threonines in the sequence 6′ → 15′ are phosphorylated by rhodopsin kinase in a light-dependent reaction. Trypsin can digest native rhodopsin in the disc membrane at Lys$^{10'}$↓—— Thr$^{9'}$ and thermolysin can hydrolyze bonds Thr$^{13'}$↓—— Val$^{12'}$, and Gln$^{5'}$↓—— Val$^{4'}$. Limited proteolysis by thermolysin at a site internal in the molecule has been exploited in order to prepare rhodopsin as two large fragments, F1 and F2. Cysteine$^{33'}$, is highly reactive in the dark and is modified by N-ethylmaleimide and several alkylating agents. The carboxyl-terminal region 1′-39′ reacts with the membrane-impermeable nitrene from N-(4-azido-2-nitrophenyl)-2-aminoethyl sulfonate and is therefore exposed at the external (cytoplasmic) surface of the disc membrane. 1-azidopyrene, a hydrophobic nitrene precursor, is being used to map those regions of the rhodopsin sequence which are located in a hydrophobic environment.

KEYWORDS

Rhodopsin; primary structure; topography; disc membrane; amino terminus; carboxyl terminus; phosphorylation; limited proteolysis; nitrene.

INTRODUCTION

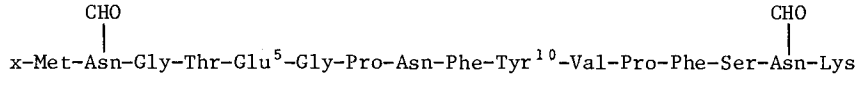
Rhodopsin is the vertebrate visual protein of rod cells which is responsible for dim light vision. Rhodopsin consists of a protein chain, opsin ($M_r \simeq 38{,}000$), to which a molecule of 11-cis retinal is bound. In the first event in vision, rhodopsin absorbs a photon causing a change in the structure or electronic state of the 11-cis retinal chromophore. This initiates a chain of events which leads to conversion of the energy of light to a neural signal--i.e., visual transduction. It is a goal of vision research to describe and understand these events at the molecular level.

We need many pieces of information in order to understand how rhodopsin functions in visual transduction. Basic to this understanding is a knowledge of rhodopsin's structure and mode of association in the disc membrane. First we need to determine rhodopsin's primary structure--the sequence of its amino acids. This task is currently underway. (It advances in parallel with developments in the state of the art of handling hydrophobic peptides from membrane proteins.) Information about rhodopsin's primary structure will aid us in learning how its polypeptide chain is folded and oriented in the membrane. Such knowledge should help us in the development of models for rhodopsin's mode(s) of action. In this paper we present our laboratory's approaches to the elucidation of the molecular structure and membrane orientation of rhodopsin.

RHODOPSIN'S AMINO-TERMINAL REGION

Rhodopsin's Tryptic Glycopeptide is its Amino-terminus

Starting points in the molecular description of a protein are the characterization of its amino- and carboxyl-terminals. Rhodopsin's amino-terminus has resisted characterization by several procedures (Albrecht, 1957; Heller, 1968). Therefore it has been concluded that the protein contains a blocked amino-terminus. Our own attempts to chemically modify rhodopsin's amino-terminal amino acid were also without result and lead us to the same conclusion (Hargrave, 1977). The first indication of what might be the amino-terminal peptide of rhodopsin came during our analysis of peptides from a tryptic digest of the protein. We found an acidic glycopeptide, designated T1, which showed no free amino terminus (Hargrave, 1977). This peptide was therefore a likely candidate for the amino-terminus of rhodopsin (although certain peptides arising from internal regions of proteins may subsequently lose their free α-amino group as a result of various treatments). We determined the sequence of peptide T1 to be

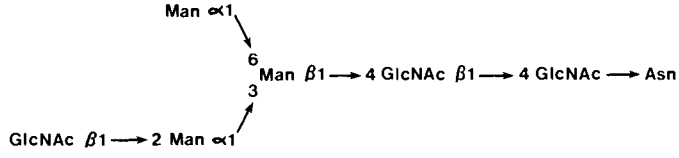


This peptide was of interest for several reasons. Amino acids 1 → 9 represent the only previously reported peptide sequence from rhodopsin (Heller and Lawrence, 1970). Heller and Lawrence isolated that nine-amino acid peptide from a peptic digest, and in their hands at least some of its amino terminus was present in unblocked form (Heller and Lawrence, 1970). In contrast we have found peptide T1 and amino-terminal peptides isolated from other enzymatic digests of rhodopsin to be blocked in all of our preparations. Rhodopsin is in fact N-acetylated (Tsunasawa, Narita, and Shichi, 1979), as are dozens of other proteins (Jornvall, 1975). The significance of this enzymatic modification of rhodopsin is unclear, but there have been several proposals made for the biological functions of protein acetylation reactions (Spencer and Carter, 1977).

We felt it was necessary to further document the amino-terminal location of peptide T1 inasmuch as there was conflicting data concerning the availability of its amino terminus. Therefore we employed a method designed to selectively prepare only the amino terminal peptide of a protein. (By application of a series of chemical and enzymatic modifications of a protein it is possible to cleave proteins at arginyl residues and render the amino-terminal peptide neutral in charge while all other peptides of the protein carry a net positive charge. Ion exchange chromatography can then uniquely separate the amino-terminal peptide from the mixture.) When this technique was applied to rhodopsin, a peptide was produced which included the glycopeptide T1 in its sequence, thus substantiating the amino terminal location of T1 (Hargrave, 1977).

All of Rhodopsin's Carbohydrate is Found at Two Sites Near its Amino-terminus

The glycopeptide T1 was also of interest due to its carbohydrate content. It has been previously reported that rhodopsin contains 3 moles of N-acetylglucosamine and 3 moles of neutral sugar (mostly mannose) per 28,000 g protein (Heller, 1968). It was also reported that the single oligosaccharide attachment site found in the nine-amino acid peptic peptide accounts for all of the carbohydrate present in rhodopsin (Heller and Lawrence, 1970). The picture became more complicated when it became clear that the molecular weight of rhodopsin was actually in the range 35,000 → 40,000 daltons (Hubbard, 1954; Daemen, deGrip, and Jansen, 1972; Lewis, Krieg, and Kirk, 1974) and when a study of the carbohydrate composition of rhodopsin yielded 9 moles of mannose and 5 moles of N-acetylglucosamine per mole of rhodopsin (Plantner and Kean, 1976). In order to obtain a coherent picture for the number of carbohydrate attachment sites, their composition, and the total carbohydrate content of rhodopsin, additional studies were performed. It now appears that rhodopsin contains approximately 6 moles each of mannose and N-acetylglucosamine per mole of protein, $M_r \simeq 39{,}000$ (Fukuda, Papermaster and Hargrave, 1979). The carbohydrate is equally distributed between two sites at Asn^2 and Asn^{15} and has the simple structure determined by Fukuda, Papermaster and Hargrave (1979) as shown below:



The same structure was determined by Liang and co-workers (1979). There is some evidence to suggest that these oligosaccharides may become further glycosylated in a small proportion of rhodopsin molecules, and that this glycosylation is important in a membrane-membrane recognition process which is postulated to occur in disc turnover (O'Brien, 1976).

Primary Structure of Rhodopsin's Amino-terminal Region

Our studies on the amino-terminal region of rhodopsin have now lead to determination of the sequence of the first 39 amino acids, representing ∿12% of the primary sequence of rhodopsin.

CHO CHO

x-Met-Asn-Gly-Thr-Glu[5]-Gly-Pro-Asn-Phe-Tyr[10]-Val-Pro-Phe-Ser-Asn-Lys-Thr-Gly-Val-Val[20]-Arg-Ser-Pro-Phe-Glu[25]-Ala-Pro-Gln-Tyr-Tyr[30]-Leu-Ala-Glu-Pro-Glx[35]-Trp-Phe-Ser-Met[39]

RHODOPSIN'S CARBOXYL-TERMINAL REGION

The Carboxyl-terminal Region of Rhodopsin: Its Localization and Partial Primary Structure

The carboxyl-terminus of rhodopsin has been reported to resist identification by both enzymatic and chemical methods (Albrecht, 1957; Heller, 1968). However, application of an improved method for hydrazinolysis revealed that the carboxyl-terminal amino acid was alanine (Hargrave and Fong, 1977). Since digestion of rhodopsin with trypsin should yield the carboxyl-terminal tryptic peptide of the protein as an acidic peptide lacking lysine or arginine, we searched for such a peptide. We located this peptide (T2) and determined its sequence to be Thr-Glu-Thr-Ser-Gln-Val-Ala-Pro-Ala (Hargrave and Fong, 1977). It contains carboxyl-terminal alanine as would be required by the hydrazinolysis results on the whole protein. Presence of the penultimate proline explains the previously reported resistance of rhodopsin to digestion by carboxypeptidase A (Ambler, 1967). Further confirmation of the carboxyl-terminal status of peptide T2 was obtained when it was isolated by a method designed to specifically isolate only carboxyl-terminal tryptic peptides from proteins (Hargrave and Fong, 1977).

More information about the carboxyl-terminal region of rhodopsin was obtained from the analysis of a peptide obtained by cleavage of rhodopsin with cyanogen bromide. (This procedure cleaves polypeptide chains at methionyl residues.) A 31-amino acid peptide (CB-3) was prepared from such a digest and its sequence was determined (Fong, 1978):

Val-Thr[30']-Thr-Leu-Cys-Cys-Gly-Lys-Asn-Pro-Leu-Gly[20']-Asp-Asp-Glu-Ala-Ser-Thr-Thr-Val-Ser-Lys[10']-Thr-Glu-Thr-Ser-Gln-Val-Ala-Pro-Ala[1'].

(Our numbering system for the carboxyl-terminal region uses "primed" numbers and starts with the carboxyl-terminal Ala as 1'.)

Rhodopsin's Fast-reactive Cysteine is Located in the Carboxyl-terminal Region

As part of a separate study on rhodopsin's cysteine reactivity we isolated a peptide which extends the sequence in the carboxyl-terminal region. Rhodopsin in rod outer segment membranes had been previously determined to react with two moles of N-ethylmaleimide (NEM) in the dark, and one of the reactive sites was found to be selectively modified with iodoacetamidosalicylate (IAS) (Sale, Towner, and Akhtar, 1977; McDowell and co-workers, 1979). We have located the IAS-reactive cysteine in an 8-amino acid cyanogen bromide peptide, CB-6 (McDowell and Griffith, 1978). Peptide CB-6 has the sequence Asn-Lys-Gln-Phe-Arg-Asn-Cys-Met. This peptide precedes the carboxyl-terminal peptide CB-3 in the sequence as shown by our isolation of a tryptic peptide whose sequence begins Asn-Cys-Met-Val-Thr-Thr. The entire 39-amino acid carboxyl-terminal portion of rhodopsin accounts for an additional 11 → 12% of the protein sequence. This portion of rhodopsin is hydrophilic and would be expected to occupy a region of the molecule exposed at an aqueous surface of the disc membrane. More information concerning its functional significance and topography in the membrane is presented in this paper.

Rhodopsin's Site of Phosphorylation is at the Carboxyl-terminus

Three laboratory groups independently discovered that when rod outer segment membranes are incubated in the presence of Mg^{++} and γ-^{32}P-ATP, rhodopsin becomes phosphorylated in a light-dependent reaction (Kühn and Dreyer, 1972; Bownds, and coworkers, 1972; Frank, Cavanagh, and Kenyon, 1973). Kühn (1974) has shown that the reaction occurs *in vivo* in frogs and that it is reversed with a time course comparable to rates observed for dark adaptation. Serine and threonine residues in bovine rhodopsin become phosphorylated and only a limited region of the protein has been reported to be involved in the phosphorylation reaction (Hargrave, Kühn, and Dreyer, 1972).

When the cyanogen bromide peptides of chromatographically purified ^{32}P-rhodopsin (or ^{32}P-ROS membranes) are analyzed by gel filtration chromatography, more than 85% of the ^{32}P is found in a peptide of $M_r \simeq 3,500$ (Fig. 1). Purification of the ^{32}P-peptide demonstrates that essentially all of the phosphate is associated with the carboxyl-terminal peptide CB-3. Peptide CB-3 contains 3 serines and 6 threonines which would be prospective candidates for phosphorylation. However, trypsin digestion of peptide CB-3 at $Lys^{24'}$ shows that all of the ^{32}P is located in the carboxyl-terminal peptide $1' \rightarrow 23'$ which contains a total of 7 serines and threonines. The extent of phosphorylation of bovine rhodopsin has steadily improved from 1.3 moles ^{32}P/mole rhodopsin (Kühn, Cook, and Dreyer, 1973) to 5 moles (Kühn and McDowell,

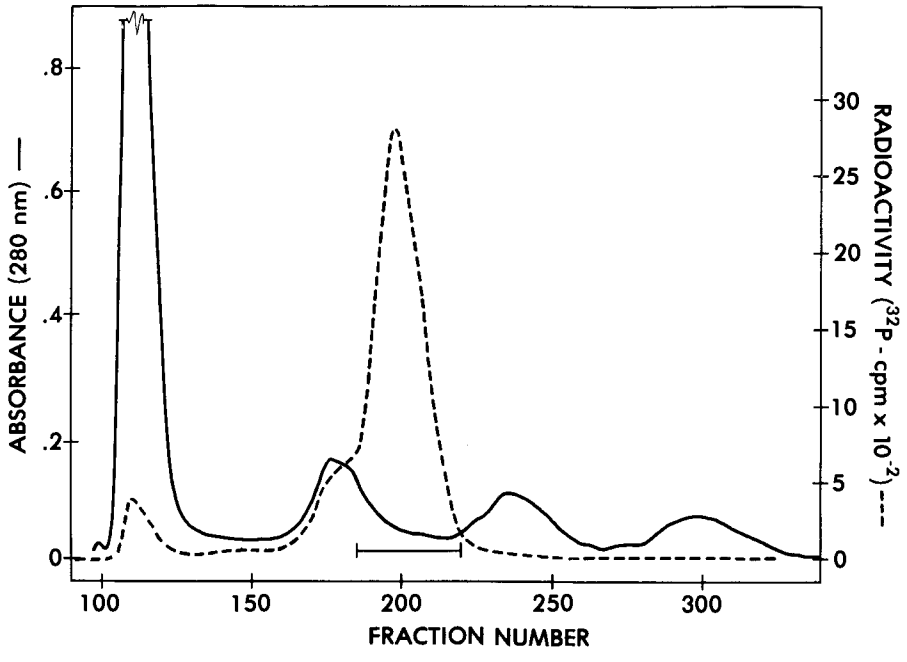


Fig. 1: Gel filtration chromatography of the cyanogen bromide peptides of ^{32}P-aminoethyl rhodopsin.

Peptides are separated on a 2.5 x 200 cm column of Sephadex G-50 equilibrated in 20% formic acid.

1977; Shichi and Somers, 1978), and recently to 6 → 7 moles ^{32}P/mole rhodopsin (H. Kühn, personal communication). Since there are a maximum of 7 serine and threonine residues in the carboxyl-terminal sequence, it would appear that each of these residues is capable of becoming phosphorylated. The phosphorylation site of rhodopsin is shown outlined in the sequence below:

$Gly^{20'}$-Asp-Asp-Glu-Ala-Ser-Thr-Thr-Val-Ser-$Lys^{10'}$-Thr-Glu-Thr-Ser-Gln-Val-Ala-Pro-$Ala^{1'}$.

It represents one of the most highly phosphorylated regions yet reported, and bears no relationship to phosphorylation sites in other proteins (Weller, 1979). The physiological importance of the reaction is not yet clear, although phosphorylation has been implicated in light adaptation (Kühn and co-workers, 1977). Phosphorylation has been reported to cause reduction of passive Ca^{++} permeability of disc vesicles upon illumination (Weller, Virmaux, and Mandel, 1975). However, important effects of phosphorylation may be mediated through rhodopsin's surface by altering its ability to interact with other proteins. It has been suggested, for example, that bleached rhodopsin activiates ROS phosphodiesterase (PDE) and that phosphorylation of rhodopsin terminates rhodopsin's ability to bind to and activate PDE (Liebman and Pugh, 1979). It is known that rhodopsin kinase and other proteins which do not normally interact with rhodopsin can selectively bind to disc membranes when rhodopsin has been freshly bleached (Kühn, 1978). It will be of interest to learn what effect phosphorylation has on the protein-binding interactions of rhodopsin.

RHODOPSIN TOPOGRAPHY IN THE DISC MEMBRANE

Limited Proteolysis as a Surface Probe

When rhodopsin is solubilized with a detergent and subjected to digestion by proteolytic enzymes it is susceptible to *extensive* digestion and ultimately yields a mixture of small peptides. But when the polypeptide chain of rhodopsin is embedded in its lipid matrix in the disc membrane, only a few regions of the folded polypeptide chain structure are susceptible to proteolytic attack. Membrane-bound rhodopsin is subject to *limited proteolysis* by a variety of proteolytic enzymes (Saari, 1974). Digestion by thermolysin cleaves rhodopsin into two membrane-bound fragments, designated F1 and F2 (Pober and Stryer, 1975). Fragment F1 contains carbohydrate and fragment F2 contains the retinyl-attachment site (Pober and Stryer, 1975).

Rhodopsin's Carboxyl-terminus is Exposed to Proteases at the Cytoplasmic Surface of the Disc Membrane

Our studies have shown that thermolysin quickly converts membrane-bound rhodopsin to an intermediate, 0′ ($M_r \simeq 30{,}500$), which is then further digested to yield F1 ($M_r \simeq 25{,}000$) and F2 ($M_r \simeq 9{,}500$) (Hargrave and Fong, 1977). Production of 0′ is accompanied by release of two small peptides from the membrane: Val-Ser-Lys-Thr-Glu-Thr-Ser-Gln, and Val-Ala-Pro-Ala. These two peptides account for the carboxyl-terminal sequence 1′ → 12′ of rhodopsin. Since these peptides are rapidly released by digestion of disc membranes of native sidedness, the carboxyl-terminal region of rhodopsin must be located on the external (or cytoplasmic) surface of the disc membrane.

Preparation of Two Thermolytic Fragments of Rhodopsin: F1 and F2

Further digestion of 0′ yields F1 and F2, although overnight digestion with ∿5% by weight thermolysin/rhodopsin is required to yield a homogeneous F2 fragment. This

demonstrates that at least one other region of rhodopsin's sequence is exposed at the disc cytoplasmic surface. Fragment F1, due to its carbohydrate content, must contain residues Asn^{2} and Asn^{15} and therefore originates from the amino-terminal region of rhodopsin. By process of elimination, and due to its retention of some ^{32}P (when phosphorylated rhodopsin is digested) fragment F2 originates from rhodopsin's carboxyl-terminus (Hargrave and Fong, 1977). We have recently prepared micromolar quantities of thermolytic fragment F2 (Fig. 2) and its structural characterization is in progress.

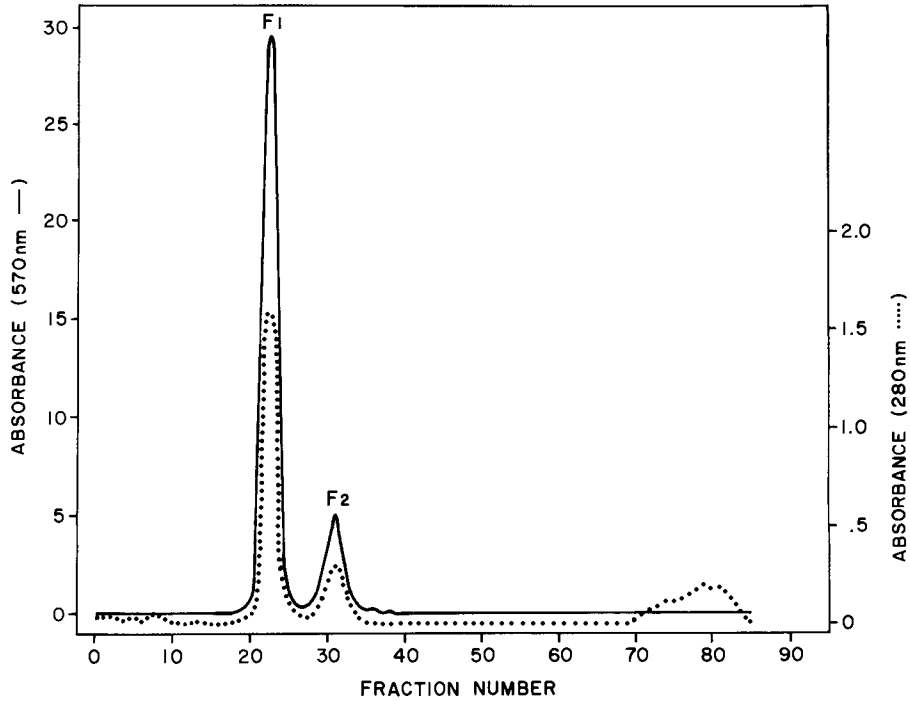


Fig. 2. Preparative separation of rhodopsin thermolytic fragments F1 and F2.

Disc membranes were digested with thermolysin. Digested membranes were solubilized in tridecyl trimethylammonium bromide and chromatographed on hydroxyapatite to purify the F1-F2 noncovalent complex. Following aminoethylation, the F1-F2 mixture was dissolved in formic acid and separated as shown above on a 1.5 x 100 cm column of Sephadex LH-60 in formic acid-ethanol (30/70, v/v). The A_{280nm} of fractions was measured directly. Aliquots were acid hydrolyzed, reacted with ninhydrin, and their A_{570nm} determined in order to locate peptides.

Although there are only two regions on rhodopsin's cytoplasmic surface which are susceptible to thermolysin digestion, the less specific proteases papain (Sale, Towner, and Akhtar, 1977) and subtilisin (Gaw, 1977) are able to cleave at one or more additional sites. Demonstration of the positions of these proteolytic cleavages in the covalent sequence of rhodopsin will eventually assist in mapping its topography in the disc membrane.

Trypsin Digests Rhodopsin's Carboxyl-terminus

It has been reported that the highly specific protease trypsin (which cleaves only at lysyl and arginyl residues) does not digest membrane-bound rhodopsin (Saari, 1974; Pober and Stryer, 1975; Trayhurn, Habgood, and Virmaux, 1975; van Breugel Daemen, and Bonting, 1975). Molday and Molday (1979) and our laboratory have observed that treatment of disc membranes with trypsin leads to apparent lowering of the molecular weight of rhodopsin by $M_r \simeq 2{,}000 \rightarrow 2{,}500$ (Fig. 3). From the

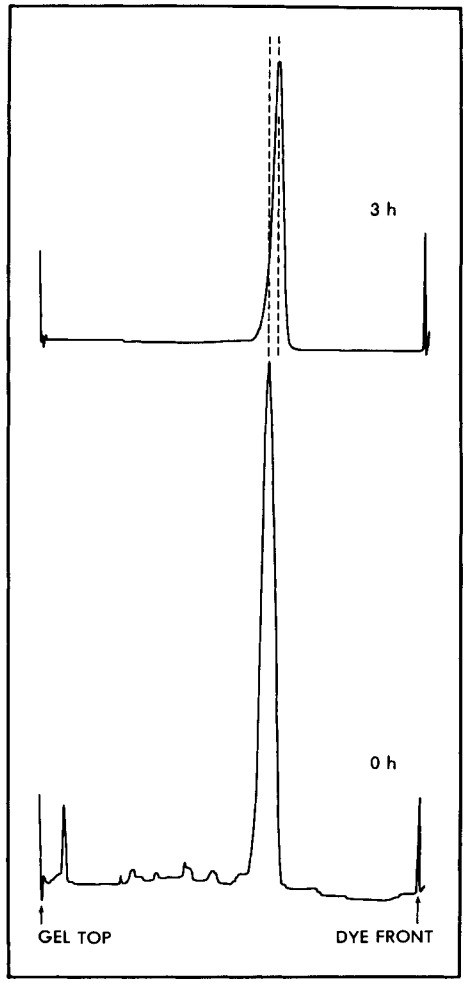


Fig. 3. Trypsin digestion of rhodopsin in disc membranes.

Disc membranes were incubated with 5% by weight of β-trypsin for 3 h at room temperature. Membranes were analyzed by $NaDodSO_4$-polyacrylamide gel electrophoresis before digestion (0 h) and following digestion (3 h). The gel scans show that all minor protein bands are eliminated by trypsin and that the molecular weight of rhodopsin is decreased. When the 0 h and 3 h samples are mixed and subjected to electrophoresis, two closely spaced bands are observed (not shown since these are not well resolved by the gel scanning technique).

digestion supernatant we have isolated the 9-amino acid tryptic peptide T2, which has M_r = 903. Analysis of the digested rhodopsin verifies that the only site of tryptic attack is at $Lys^{10'}$. Thus the actual lowering of molecular weight is less than the apparent molecular weight change as monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Rhodopsin's Carboxyl-terminus is Accessible

The above data confirm that the carboxyl-terminal region of rhodopsin is located on the external (cytoplasmic) surface of the disc membrane and is accessible to several macromolecular probes. The action of the various enzymes on the carboxyl-terminus is summarized in Fig. 4.

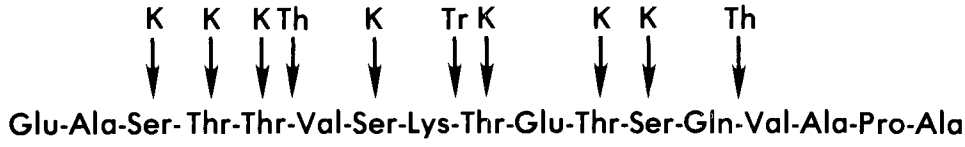


Fig. 4. Sites of enzymatic action on the carboxyl-terminal region of bovine rhodopsin.

Th = thermolysin; Tr = trypsin; K = rhodopsin kinase.

Rhodopsin's Hydrophilic Surfaces React with NAP-Taurine

We have presented evidence that portions of rhodopsin are exposed at the external surface of the disc membrane. Since rhodopsin can only be solubilized from the membrane by use of detergents, portions of the protein must be embedded in the lipid bilayer and surrounded by the fatty acid side chains of phospholipids. Finally, there is evidence that rhodopsin is a transmembrane protein (Fung and Hubbell, 1978) and that rhodopsin's carbohydrate is located at the intradiscal membrane surface (Röhlich, 1976; Adams, Tanaka, and Shichi, 1978). The complete description of rhodopsin's topography would include assignment of each part of the covalent sequence of the protein to its proper place in these three membrane environments. We are employing chemical labeling techniques as one way to help in obtaining this information.

The ideal topographic label should be restricted to a particular membrane compartment and have broad specificity for chemical modification. Certain precursors of nitrenes and carbenes are ideal candidates for such probes. We have used an anionic non-penetrating nitrene precursor, N-(4-azido-2-nitrophenyl)-2-aminoethane sulfonic acid (NAP-taurine; NAPT) as a probe for rhodopsin's external disc membrane surface (Hargrave, Mas, and Smith, 1979; Mas, Wang, and Hargrave, submitted for publication). ^{35}S-NAPT successfully labels rhodopsin in the intact disc membrane. Rhodopsin labeled with NAPT can be subsequently purified by chromatography on Concanavalin A-agarose. When CNBr peptides are prepared from the labeled protein, some peptides are found to be well labeled and some peptides contain essentially no label. Among the well-labeled peptides are peptides CB-3 and CB-6 which represent amino acids $1' \rightarrow 31'$ and $32' \rightarrow 39'$ respectively of rhodopsin's carboxyl-terminus. This result provides further evidence for the location of rhodopsin's carboxyl-terminal region at the disc membrane external surface.

In order to determine what portion of rhodopsin is located at the internal disc membrane surface we chose to label *reconstituted* membranes with NAPT. Since reconstituted membranes contain both rhodopsin molecules with normal orientation and reversed orientation, peptides labeled in reconstituted membranes but not in disc membranes should represent regions exposed at the internal disc membrane surface. We found that although rhodopsin's amino-terminal cyanogen bromide peptide CB-1 (amino acids 2 → 39) was poorly labeled in disc membranes, its labeling was much enhanced in reconstituted membranes. This result is in agreement with other evidence which places this region of the molecule at the intradiscal membrane surface.

Rhodopsin's Hydrophobic Surfaces React with 1-azidopyrene

Hydrophobic azides are designed to dissolve in the lipid bilayer and upon photolysis to form nitrenes which will react with immediately neighboring molecules, such as lipids or proteins. Rhodopsin labeling from within the lipid bilayer has been reported for the reagent 1-azidonaphthalene (Klip, Darszon, and Montal, 1976). We have synthesized another hydrophobic azide, 1-azidopyrene (AP), and used it to label membrane-bound rhodopsin (Hargrave, Mas, and Smith, 1979; Smith and co-workers manuscript in preparation). The results of such a labeling are shown in Fig. 5. Rhodopsin is modified by AP and the label is retained in the membrane-bound thermolytic fragments F1 and F2. Modification of rhodopsin appears to be restricted to regions of the protein surrounded by the lipid bilayer, since soluble proteins and peptides (lysozyme, glutathione) added to the reaction mixture do not incorporate appreciable AP. Surface regions of the protein which react well with NAPT are not targets of the hydrophobic probe AP. Characterization of sites of AP modification of rhodopsin is currently in progress.

RHODOPSIN IN ROD CELL MEMBRANES

A Schematic Model of Rhodopsin Topography

Current studies on the topography of rhodopsin from our laboratory and others allow us to construct a tentative model for the organization of rhodopsin in the membrane (Fig. 6). Some explicit parts of the model are well justified and others are quite arbitrary. The amino-terminal glycopeptide region is shown exposed at the intradiscal membrane surface. At some point beyond amino acid 35, a hydrophobic and probably α-helical region penetrates the membrane. The polypeptide chain is hypothesized to traverse the membrane an odd number of times, possibly in α-helical segments of ∿20 amino acids each. More of the rhodopsin polypeptide chain is probably exposed at the cytoplasmic face of the disc membrane than at its intradiscal surface. One of rhodopsin's two dark-reactive cysteines is located in the large amino-terminal segment, F1 (which may also contain two disulfide bridges). Rhodopsin must possess a highly exposed surface loop which is susceptible to a variety of proteolytic enzymes and also contains a glutaminyl residue modifiable by transglutaminase (Pober and co-workers, 1978). Proteolytic digestion in this loop region produces fragments F1 and F2. The remaining carboxyl-terminal ∿1/4 of the molecule, F2, contains the retinyl-binding site. Rhodopsin's carboxyl-terminal 40 amino acids are surface exposed and possess sites susceptible to proteolytic cleavage and kinase phosphorylation. Cysteine$^{33'}$, rhodopsin's fastest dark-reactive cysteine, is in this region. Shortly before amino acid 40′ the polypeptide chain must penetrate the lipid bilayer and establish the membrane-anchoring portion of F2. Additional data will be required in order to sharpen our current working hypothesis of rhodopsin's topography. Our eventual objective will be to fully describe rhodopsin's amino acid sequence and how it is arranged in the lipid bilayer. The vectorial orientation of rhodopsin and its 3-dimensional structure should assist workers in constructing a functional description of the protein. Studies on the orientation

of rhodopsin in the disc membrane also have implications for hypotheses concerning rhodopsin's interaction with other rod cell components and rhodopsin's biosynthesis. Current information on rhodopsin structure and orientation should be useful in constructing such theories.

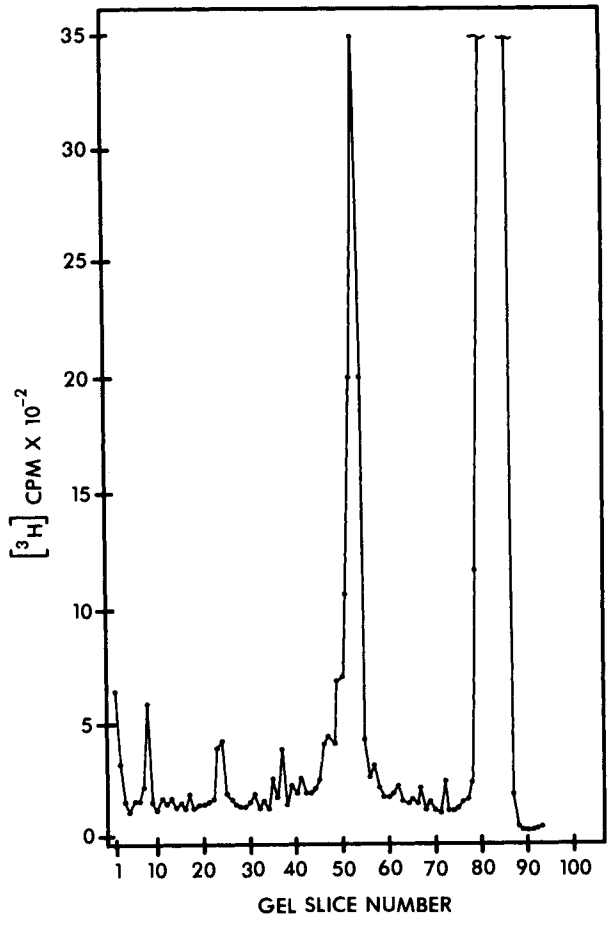


Fig. 5: Sodium dodecyl sulfate polyacrylamide gel electrophoresis of disc membranes photolyzed with [^{3}H]-1-azidopyrene.

Following electrophoresis the polyacrylamide gel was cut into 1 mm slices and their radioactivity determined. The gel top is slice 1 and the center of the rhodopsin band is gel slice 53. The tracking dye and small molecules are found at gel slices 79-88.

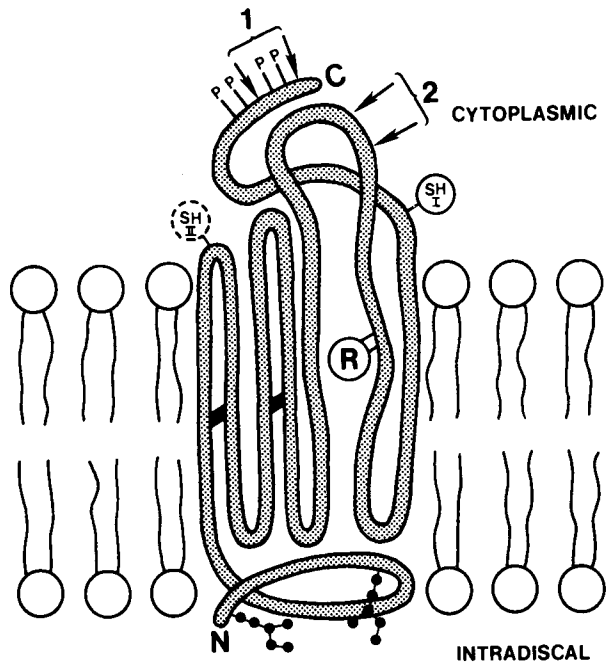


Fig. 6. Schematic Model for the Orientation of Rhodopsin in the Disc Membrane.

ACKNOWLEDGEMENT

This work was supported by U. S. Public Health Service Grants EY01275 and EY02875, and National Science Foundation Equipment Grant PCM77-17808.

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Neurochemistry Vol. 1. pp. 245-253.
Pergamon Press Ltd. 1980. Printed in Great Britain.

THE OLIGOSACCHARIDE MOIETY OF RHODOPSIN--ITS STRUCTURE AND CELLULAR LOCATION

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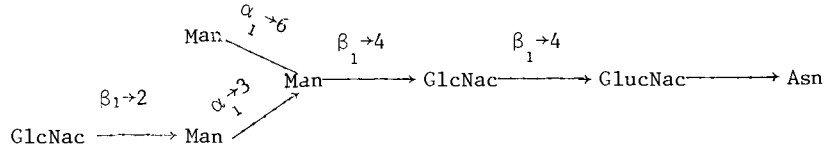
ABSTRACT

The sugar chains of bovine rhodopsin released from opsin by hydrazinolysis were reduced with $NaB(^3H)_4$ and fractionated by paper chromatography. Three oligosaccharides were obtained. The structure of the major (^3H)-oligosaccharide (ca. 60% of total) was elucidated by sequential exoglycosidase digestion, methylation analysis and endo-β-N-acetyl glucosaminidase D digestion. The structure of the sugar moiety of rhodopsin was thus identified as:



Since the terminal GlcNac serves as galactose acceptor, the location of the sugar moiety of rhodopsin in the disc membrane was studied by incorporation of (^3H)-galactose (from UDP-(^3H)-galactose) into the disk membrane. After inversion of disks by freeze-thawing, rhodopsin in the membrane incorporated one mole of (^3H)-galactose per mole purified pigment. Intact disks incorporated lower amounts of (^3H)-galactose. It was therefore concluded that the sugar moiety of rhodopsin is located on the internal surface of disk membrane. The results of lectin binding studies are consistent with the conclusion. Inverted disks, but not intact disks, bind concanavalin A (specific for α-mannosyl residue) and wheat germ lectin (specific for N-acetylglucosamine). Since intact sealed rod outer segments bind concanavalin A, the sugar moiety of rhodopsin in the plasma membrane is probably exposed on the external surface of the rod.

KEYWORDS

Bovine rhodopsin; structure of oligosaccharide moiety; cellular location; galactose incorporation; concanavalin A binding.

INTRODUCTION

The glycoprotein nature of the rod intrinsic membrane protein rhodopsin was first noted about 10 years ago (Heller, 1968; Shichi, Lewis, Irreverre and Stone, 1969). The carbohydrate composition of the oligosaccharide moiety of rhodopsin was studied by several workers (Heller and Laurence, 1970; Plantner and Kean, 1976; Fukuda, Papermaster and Hargrave, 1977). The published results unanimously show the presence of mannose and N-acetylglucosamine as its constituents but differ in the molar ratios of these two sugars. Heller and Lawrence (1970) have reported that bovine rhodopsin contains 3 moles each of the two sugars per mole, while Plantner and Kean (1976) have found 7 to 9 moles of mannose and 4.5 to 6 moles of N-acetylglucosamine per mole of pigment. A glycopeptide of 16 amino acid residues containing two asparagine-linked sugar chains was isolated from tryptic digests of bovine rhodopsin (Hargrave, 1977). Although this confirmed the presence of two oligosaccharide moieties per rhodopsin molecule, the chemical structure of the chains remains yet to be elucidated. We have therefore attempted to determine the complete structure of the sugar moieties of rhodopsin. We have then used the structural information for studies of the spatial orientation of rhodopsin in rod membranes.

MATERIALS AND METHODS

Bovine rhodopsin was purified on a column of calcium phosphate-Celite by the method described previously (Shichi and co-workers, 1969). The sugar chains were liberated from opsin by hydrazinolysis and reduced with $NaB(^3H)_4$ after N-acetylation. A detailed account of the procedure as well as of paper chromatography, exoglycosidase digestion, methylation analysis and endo-β-N-acetylglucosaminidase D digestion will be found elsewhere (Liang and co-workers, 1979). To prepare inverted disks, sealed outer segments were prepared by centrifugation in a continuous metrizamide gradient (Adams, Tanaka and Shichi, 1978) and disks were released in 5% Ficoll by the method of Smith, Stubbs and Litman (1978). Disks were then inverted by freeze-thawing and separated from intact (uninverted) disks by affinity chromatography on concanavalin A (Adams and co-workers, 1978). Glycosylation of disks was carried out with bovine milk galactosyltransferase (Sigma) and (^{3}H)-UDP-galactose (Adams, Somers and Shichi, 1979). For lectin binding studies, fluorescent isothiocyanate (FITC)-labeled lectins (Miles Labs.) were used. Disks were incubated with either FITC-concanavalin A, FITC-wheat germ agglutinin or FITC-Ricinus Communis agglutinin 60 for 20 minutes at 20°C and centrifuged at 58,000 xg for 20 minutes. The fluorescence of the supernatant was determined with an Aminco-Bowman spectrophotofluorometer. Disks were also incubated in a similar manner in the presence of appropriate inhibitor (α-methylmannoside for concanavalin A, N-acetylglucosamine for wheat germ agglutinin, and galactose for Ricinus lectin).

RESULTS AND DISCUSSION

It would be helpful to have a quick look at the proposed structures of rhodopsin sugar moieties (Fig. 2) before evidence for the structures is discussed. The structures possess one N-acetylglucosamine (at the reducing end) which is linked to an asparagine residue and another N-acetylglucosamine (at the non-reducing end) which is linked to the mannose cluster.

Acid hydrolysis followed by N-acetylation of the reduced oligosaccharides yielded N-acetylglucosaminitol as the only radioactive sugar. Therefore, the reducing termini of the oligosaccharides must be N-acetylglucosamine. Paper chromatography of the $NaB(^3H)_4$-reduced oligosaccharides gave rise to three fractions, A, B, and C in

a molar ratio of 55:12:33 (Fig. 1). Carbohydrate compositions of the three oligosaccharides are shown in Table 1. Since the N-acetylglucosamine at the reducing termini of the samples was already reduced with $NaBH_4$, glucosamine values in Table 1 are 1 mole less than the actual numbers. The corrected numbers are shown in parentheses.

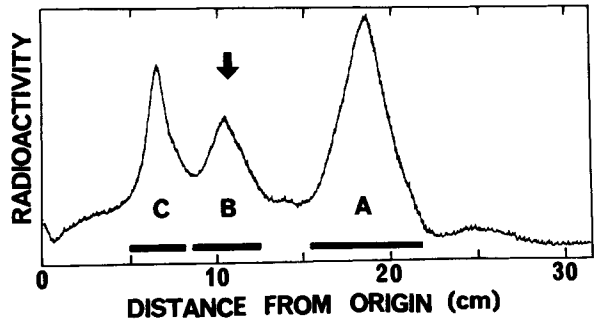


Fig. 1. Paper chromatography of radioactive oligosaccharides liberated from bovine rhodopsin by hydrazinolysis and subsequent reduction with $NaB(^3H)_4$.

TABLE 1 Monosaccharide Composition of Oligosaccharides A, B and C

Oligosaccharides	Molar ratio	
	Mannose	N-acetylglucosamine
A	3.15	2.00 (3.00)*
B	4.10	2.00 (3.00)*
C	4.92	2.00 (3.00)*

*Numbers in parentheses are corrected integers obtained by adding 1 mole of N-acetylglucosamine located at the reducing termini.

After removal of one N-acetylglucosamine at the non-reducing end by digestion with β-N-acetylhexosaminidase, oligosaccharides A, B, C became susceptible to α-mannosidase and released 2, 3 and 4 mannose residues, respectively. The remaining triitols were identical with Manβ1→ 4GlcNAcβ1→ 4GlcolNac. This was further confirmed by sequential digestion with β-mannosidase and N-acetylhexosaminidase. These results indicate that the structure of oligosaccharides A, B, and C can be written as GlcNAcβ $(Man\alpha)_n$.Manβ1→ 4GlcNAcβ1→ 4GlcNAc, where n=2 for A, n=3 for B, and n=4 for C.

In order to determine the location of each glycosidic linkage, oligosaccharides A, B, and C were subjected to methylation analysis (Table 2).

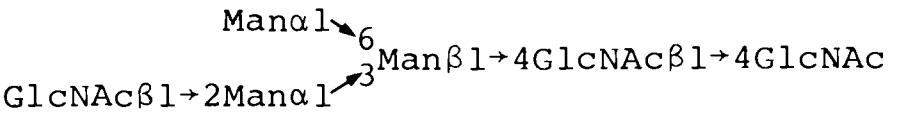
The data in Table 2 confirmed that oligosaccharides A and B contain one 3,6 disubstituted mannose and C contains two of it. When de-N-acetylglucosaminyl oligosaccharides obtained by β-N-acetylhexosaminidase treatment of oligosaccharides A, B and C were subjected to methylation analysis, 3, 4, 6-tri-O-methylmannitol acetate

TABLE 2 Molar Ratio of Alditol Acetates Obtained from Hydrolysates of Permethylated Oligosaccharides

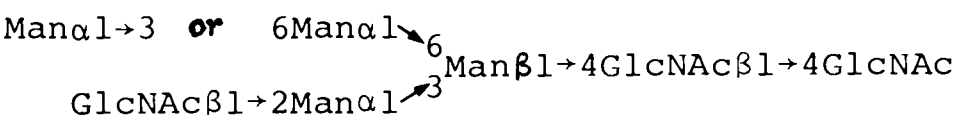
	Molar ratio[a]					
	Oligosaccharide A		Oligosaccharide B		Oligosaccharide C	
	Intact	-GlcNAc	Intact	-GlcNAc	Intact	-GlcNAc
Mannitol						
2,3,4,6-Tetra-O-methyl-(1,5-di-O-acetyl)	1.0	2.1	1.1	1.8	1.9	2.8
2,4,6-Tri-O-methyl-(1,3,5,-tri-O-acetyl)	0	0	0.2	0.2	0	0
3,4,6-Tri-O-methyl-(1,2,5-tri-O-acetyl)	1.1	0	1.0	0	0.9	0
2,3,4-Tri-O-methyl-(1,5,6-tri-O-acetyl)	0	0	0.8	0.8	0	0
2,4-Di-O-methyl-(1,3,5,6-tetra-O-acetyl)	1.0	1.0	1.0	1.0	2.0	2.0
2-N-Methylacetamido-2-deoxyglucitol						
1,3,5,6-Tetra-O-methyl-(4-mono-O-acetyl)	0.8	0.9	0.9	0.9	0.9	0.8
3,4,6-Tri-O-methyl-(1,5-di-O-acetyl)	0.9	0	0.9	0	1.0	0
3,6-Di-O-methyl-(1,4,5-tri-O-acetyl)	1.0	0.9	0.8	0.9	0.9	1.0

(a) Numbers were calculated by making the values of 2,4-di-O-methylmannitol as either 1.0 or 2.0

Oligosaccharide A



Oligosaccharide B



Oligosaccharide C

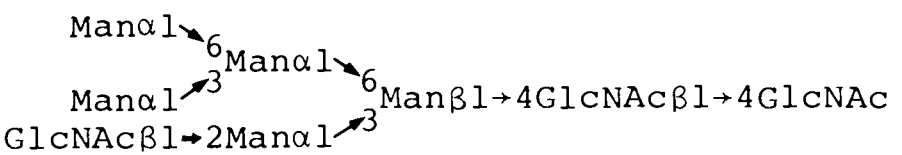


Fig. 2. Proposed structures of oligosaccharides A, B and C.

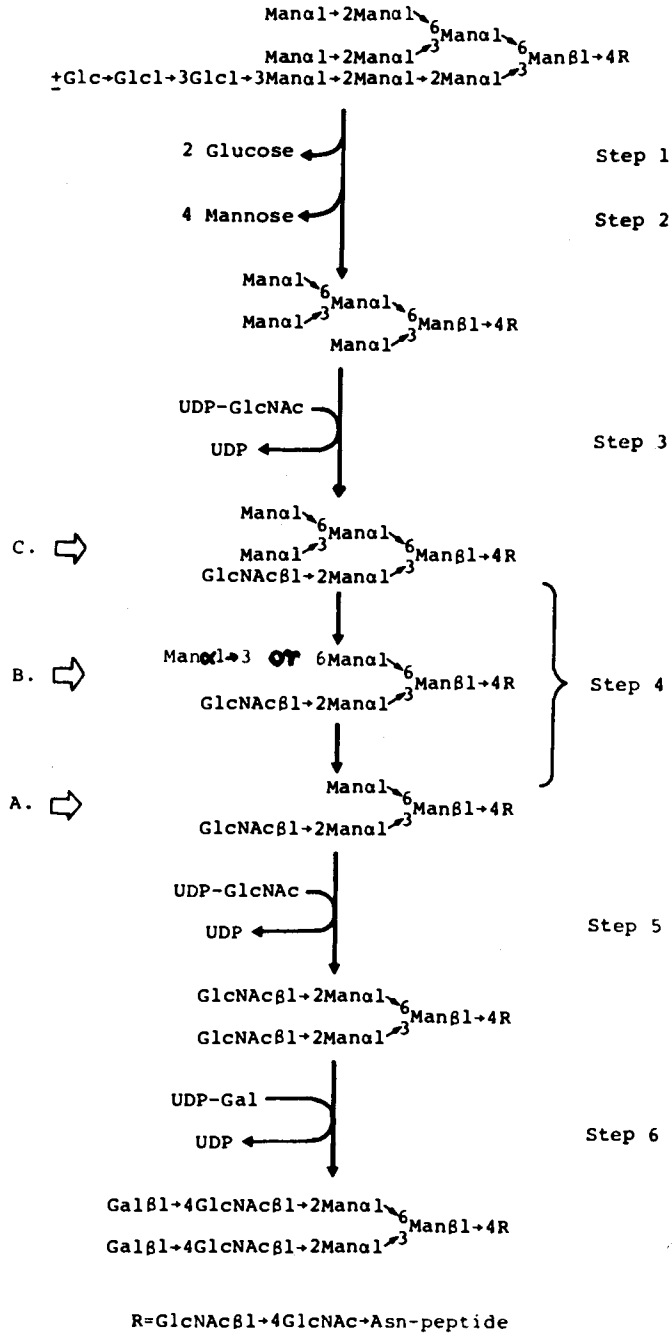


Fig. 3. Processing in the biosynthetic pathway of complex type asparagine-linked sugar chains of glycoprotein.

disappeared with the concomitant appearance of 1 mole of 2,3,4,6-tetra-O-methyl-mannitol acetate in all three oligosaccharides. Therefore, the non-reducing terminal N-acetylglucosamine residues in all three oligosaccharides should occur as GlcNAcβ1→2Man grouping. These results, taken together, lead us to conclude that oligosaccharides A, B, C have chemical structures shown in Fig. 2.

A biosynthetic pathway of complex type asparagine-linked sugar chains of glycoprotein has recently been proposed by Kornfeld, Li and Tabas (1978) (Fig. 3). The proposed pathway indicates that the mannose-rich oligosaccharide moiety is de-mannosylated in a stepwise fashion and then glycosylated (i.e., galactosylated, sialylated, and fucosylated). If the sugar moiety of rhodopsin is processed by the proposed pathway, oligosaccharide C would be de-mannosylated one at a time in two steps to oligosaccharide A. Since oligosaccharide A is the major fraction produced by hydrazinolysis of opsin, the structure is considered to be the final form of rhodopsin sugar moiety. This suggests that the inner segment of bovine rod, where processing of the sugar moiety occurs, lacks glysosyltransferases such as galactosyl transferase and sialyltransferase which are involved in further modification of oligosaccharides.

The structure of oligosaccharide A containing mannoseα1→3 and mannoseα1→6 linkages is consistent with the previous finding (Adams and co-workers, 1978) that the glycoprotein rhodopsin is capable of binding concanavalin A. The N-acetylglucosamine at the non-reducing end of the oligosaccharide moiety of rhodopsin should be able to serve as a galactose acceptor for UDP-galactose: glycoprotein galactosyl transferase (EC 2.4.1.22). We took advantage of the structural feature to study the spatial arrangement of rhodopsin in the disk membrane. After inverted disks were incubated with UDP(^{3}H)-galactose and galactosyltransferase, rhodopsin was extracted and purified on an ECTEOLA-cellulose column. The first peak from the column is non-phosphorylated rhodopsin and the second peak is phosphorylated rhodopsin (Shichi and Somers, 1978).(^{3}H)-Galactose was incorporated into both rhodopsin fractions. The stoichiometry of incorporation was 0.92 galactose per rhodopsin. If rhodopsin has two carbohydrate moieties (Hargrave, 1977), only one of them might be available as galactose acceptor. Intact (uninverted)disks, when similarly treated, incorporated 0.6 galactose per rhodopsin. This low significant level of incorporation may be attributed to vesiculated disks which took up UDP-galactose and transferase during membrane resealing without inversion. Shaper and Stryer (1977) demonstrated incorporation of galactose into the carbohydrate moiety of rhodopsin but did not answer the question on the localization of sugar moiety in the membrane. In our work, galactose-labeled inverted disk vesicles were first separated, rhodopsin extracted, and then the presence of galactose in purified rhodopsin was demonstrated. Lectin binding studies (Table 3), show that inverted disks but not intact disks bind not only concanavalin A but also wheat germ agglutinin which are specific for α-mannosyl residue and N-acetylglucosamine, respectively (Lis and Sharon, 1973). In contrast, Ricinus lectin that is specific for galactose (Lis and Sharon, 1973) did not bind to disks both intact and inverted. The results are consistent with the lectin binding property of rhodopsin that the chemical structure of the carbohydrate moiety would predict. We therefore conclude that the carbohydrate moiety of the pigment is exposed on the internal (intradiscal) surface of the disk. We have previously shown that intact sealed rod outer segments bind concanavalin A (Adams and co-workers, 1978). If the lectin binding is attributed to the sugar moiety of rhodopsin associated with the plasma membrane (Jan and Revel, 1974; Basinger, Bok and Hall, 1976), the results support the conclusion that the disks are formed by infolding of the rod plasma membrane; the membrane is inverted during the infolding process.

TABLE 3 Lectin Binding to Disks

Lectin	Sample	Fluorescence of supernatant*
FITC-concanavalin A	Fresh disk - inhibitor	5.9
	Fresh disk + inhibitor	6.6
	Frozen disk - inhibitor	1.7
	Frozen disk + inhibitor	5.0
FITC-wheat germ agglutinin	Fresh disk - inhibitor	4.1
	Fresh disk + inhibitor	6.5
	Frozen disk - inhibitor	1.9
	Frozen disk + inhibitor	4.6
FITC-Ricinus lectin	Fresh disk - inhibitor	6.0
	Fresh disk + inhibitor	6.1
	Frozen disk - inhibitor	4.0
	Frozen disk + inhibitor	4.3

*Fluorescence is in arbitrary units.

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Neurochemistry Vol. 1, pp. 255-267.
Pergamon Press Ltd. 1980. Printed in Great Britain.

LECTINS AS PROBES OF GLYCOPROTEIN AND GLYCOLIPID OLIGOSACCHARIDES IN RODS AND CONES

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ABSTRACT

The oligosaccharides of rod and cone membranes were investigated with the aid of fluorescence and ^{125}I-labeled lectins. Additionally, the ability of lectins to cause agglutination in ROS suspensions was used as an index for the presence of the corresponding lectin receptors. The specificities of lectin-ligand interactions were determined from studies of inhibition by various haptene sugars. The membranes of both rods and cones have receptors for Con A, PNA, RCA-120, RCA-60, SBA and WGA. The affinity of PNA for accessory cones is much higher than for the principal cones. There do not appear to be receptors for UeA and LTA on rods or cones. Additionally, receptors for HPA and DBA were identified on ROS. These results suggest the existance of the following sugar residues: (GlcNAc $\xrightarrow{\beta}$) Man $\xrightarrow{\alpha}$ Man; Gal $\xrightarrow{\beta}$ GalNAc; Gal $\xrightarrow{\alpha,\beta}$; GalNAc $\xrightarrow{\alpha,\beta}$; ($\longrightarrow$) GlcNAc $\xrightarrow{\beta}$ GlcNAc ($\longrightarrow$). There is no evidence for Fuc $\xrightarrow{\alpha}$. The binding of Con A and WGA to ROS membrane proteins electrophoresed on SDS-polyacrylamide gels was also investigated. In addition to rhodopsin, these lectins also bind to the 291,000-dalton protein, indicating that it is a glycoprotein containing mannose and GlcNAc.

KEYWORDS

Lectins, glycoproteins, glycolipids, plasma membrane, rods, cones, oligosaccharides, agglutination, rhodopsin.

INTRODUCTION

Glycolipids and glycoproteins are anchored in the plasma membranes of all cells, oriented so that their hydrophilic carbohydrate chains extend outwards into the extracellular space (Hughes, 1976; Nicolson and Singer, 1974). These carbohydrates are believed to be involved in many normal and pathologic cell functions, including cell recognition, development, contact inhibition, immunological phenomena, synaptic function, cell proliferation in cancer, and reactions to external agents such as toxins and hormones (Horowitz and Pigman, 1977).

The visual system consists of many different neuronal and associated cells, many closely juxtaposed and making specific contacts with each other. It is believed that glycoprotein and glycolipid oligosaccharides may play a pivotal role in determining the way in which these contacts are established and maintained (Lilien and Balsamo, 1978).

Lectins are proteins that display marked specificities for certain haptene sugars (Dulaney, 1978; Goldstein and Hayes, 1978; Lis and Sharon, 1978), and consequently may be used as probes for the oligosaccharides of cell surfaces. Various techniques have been used in the present work to investigate the lectin receptors on visual cells: these have included binding of fluorescence-labeled lectins and the binding of ^{125}I-labeled lectins to the membrane glycoproteins of ROS separated by SDS-PAGE. Since most lectins are multivalent, they can cause agglutination by forming cross-bridges between receptors on neighboring cell plasma membranes (Nicolson, 1974). Agglutination in suspensions of ROS has been used in the present work as an index of lectin reactivity with the ROS plasma membrane and for investigating the effects of various haptene sugars.

MATERIALS AND METHODS

Lectins and FITC-lectins were obtained from a variety of sources: Miles-Yeda (Con A, LTA, WGA, SBA)[1], L'Industrie Biologique Francaise (UeA, PNA, WGA, HPA), Sigma (DBA, RCA-120, RCA-60, WGA), P. L. Biochemicals (RCA-120, SBA, WGA), Pharmacia (HPA). Haptene sugar inhibitors used were as follows: methyl-α-D-mannopyranoside (200 mM in Ringer; Calbiochem); chitobiose (10 mM, L'IBF); D-galactose (100 mM, L'IBF); N-acetyl D-galactosamine (100 mM, L'IBF); D-glucose (100 mM, Fisher); lactose (50 mM, L'IBF); α-D- and α-L-fucose (50 mM, Sigma); N-acetyl D-glucosamine (100 mM, L'IBF); methyl-α-D-galactopyranoside (50 mM, Sigma); methyl-β-D-galactopyranoside (50 mM, Sigma).

Except where indicated in the Results, freshly dissected retinas were used. Frogs (*R. pipiens*) were usually dark-adapted for 3-12 hours, and the retinas separated in dim red light under modified Ringer's solution (see Bridges, 1976). Cattle eyes were placed fresh on ice in the slaughter house and the retinas dissected within 2-3 hours. For fluorescence studies, either the whole retina was incubated in the FITC-lectin in Ringer (0.5 to 612 μg lectin per ml), or it was quickly dipped in and out of a drop of Ringer placed on a glass slide. In that event, the resulting suspension of photoreceptor cells was mixed with the FITC-lectin and coverslipped. Alternatively, the lectin was run in from the side. The latter technique avoided much of the agglutination that interfered with the first approach (cf. Bridges, 1978). After incubating at room temperature for 5-15 minutes, the surplus lectin was rinsed out by leaving the coverslip in place and gently irrigating with fresh Ringer until no background fluorescence was visible. Thorough rinsing prior to incubation with lectin was sometimes carried out, without affecting the results.

When required, haptene sugars were added with the lectin, or to the rinsing solution.

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[1]Abbreviations used: Con A (concanavalin A), LTA (agglutinin of *Lotus tetragonolobus*), WGA (wheat germ agglutinin), SBA (soybean agglutinin), UeA (*Ulex* agglutinin I), PNA (agglutinin from *Arachis hypogaea*, peanut), HPA (*Helix pomatia* agglutinin), DBA (*Dolichos biflorus* agglutinin, horse gram), RCA-120 and RCA-60 (agglutinins from *Ricinus communis*, castor bean).

Fluorescence was observed with a Zeiss Universal microscope equipped for epi-fluorescence with an FITC dichroic mirror/filter combination. Illumination was provided by a xenon or mercury lamp. Black and white photography was carried out with Kodak Tri-X film pushed to an effective exposure index of 1600 by developing in Diafine (Acufine, Inc., Chicago, Illinois). Exposure times ranged from 5 to 45 seconds, averaging about 15-25 seconds. In general, little improvement was obtained beyond 30 seconds, owing to fading of the FITC chromophore. Further, fresh rods were observed to disintegrate with prolonged exposure to the irradiating light (440-490 nm).

For studies of agglutination, retinas from which adhering pigment epithelium had been cut away were placed in a conical tube containing Ringer's solution and gently swirled by hand. The suspension was pipetted into a clean tube, diluted with more Ringer and centrifuged at a few hundred r.p.m. for about one minute. The comparatively clear upper layers were removed, and the more concentrated lower suspension was diluted with fresh Ringer and centrifuged a second time. In this method, the yield of ROS was sacrificed in favor of integrity. The ROS from 2-4 retinas generally produced ½ ml of suspension. In order to observe agglutination, 0.1 ml of suspension was mixed in a conical glass tube with 0.1 ml of lectin solution in Ringer and 0.1 ml of Ringer with or without the haptene sugar. After 10 minutes incubation at room temperature a drop of this suspension was gently withdrawn and examined in dark-field. Occasionally agglutination was subjectively scored on a scale of 1 to 4, but it proved simpler for the observer to note whether agglutination had occurred or not. Agglutination, as opposed to the occasional random cluster, could be checked by gentle agitation of the slide. In truly agglutinated samples, the ROS were clearly seen to be adhering to one another. Equivalent results were obtained if the solutions were mixed on silicone-coated slides and incubated in a moist chamber. To test for reversibility of agglutination, ROS were incubated for 20 minutes with the minimum effective concentration of lectin, examined, then half of the agglutinated suspension mixed with a solution of the appropriate haptene sugar. Complete or almost complete reversal could be obtained with some of the lectins tested (e.g., HPA and RCA-120), but with Concanavalin A the agglutination score was reduced from four to between one and two with 100 mM α-methyl mannopyranoside, some slight residual agglutination still being observed. The reason for this probably lies in the extended binding site of Concanavalin A, which recognizes a complex oligosaccharide sequence with much higher affinity than the simple monosaccharide used here (Goldstein and co-workers, 1973; Kornfeld and Ferris, 1975; Krusius, Finne and Fauvala, 1976; Ogata, Muramatsu, and Kobata, 1975).

ROS membranes were prepared from the retinas of dark-adapted *R. pipiens* (northern variety) by a procedure modified from Papermaster and Dreyer (1974) and Basinger, Bok and Hall (1976). Twelve retinas were placed in 17 ml cellulose nitrate centrifuge tubes and homogenized with a Teflon pestle in 4 ml of a 1.17 gm/ml solution of sucrose in 67 mM pH 7 phosphate buffer. The homogenate was then successively layered over with 4 ml volumes of 1.15, 1.13 and 1.11 gm/ml sucrose in phosphate buffer. After centrifuging in a SW27 rotor at 27,000 r.p.m. for 60 minutes, the 1.11/1.13 and 1.13/1.15 interface material was harvested, diluted with 2 volumes of 67 mM pH 7.0 phosphate buffer and sedimented for 30 minutes at 12,000 r.p.m. in a Sorvall SS34 rotor. The pellet was resuspended in water.

Con A was bound to Sephadex and iodinated by the lactoperoxidase method (Phillips and Furmanski, 1976). WGA was iodinated using Iodogen (chloroamide 1,3,4,6 tetrachloro-3a-6a-diphenyl glycoluril : Pierce Chemical Co., Rockford, Illinois) as described by Fraker and Speck (1978) and Markwell and Fox (1978).

SDS-PAGE was carried out on 150 mm slabs (Laemmli, 1970). Ten µl of membranes suspended in water (2 mg protein/ml) were incubated for 15-30 minutes at room temperature with an equal volume of a solution containing 2.5% sodium dodecyl sulfate, 2.5% DTT, 0.5 mM EDTA, 5% sucrose, 5 µg/ml Pyronin Y and 10 mM pH 8.0 Tris-HCl. Electrophoresis was carried out in a Bio Rad Model 220 slab gel equipment, initially at 12.5 mA (stacking), then 25 mA (separating). Gels were stained overnight with 0.1% Coomassie Blue in 20% isopropanol and 7% acetic acid, then destained in 10% acetic acid containing a small amount of Dowex-1X4 resin.

Localization of lectin-binding glycoproteins was carried out as follows. The stained gel was washed in several changes of buffer (50 mM pH 7.5 Tris-HCl, 140 mM NaCl, 0.1% sodium azide; 1 mM $MnCl_2$ and 1 mM $CaCl_2$ were added if Con A was being used: the last wash contained 0.1% bovine serum albumin). The gel was then incubated for 18 hours at 4° with 0.5 - 2.0 x 10^7 cpm/ml of ^{125}I-lectin in a small, sealed plastic bag. After washing with several buffer changes at room temperature for at least 48 hours, the gel was protected by sealing in a plastic bag. Autoradiography was carried out by placing the bag in contact with Kodak NS-5T X-Ray film. As controls, parallel gels were washed and incubated in media containing 200 mM α-methyl mannopyranoside (Con A) or 200 mM N-acetyl glucosamine (WGA). These sugars block the corresponding binding sites and prevent the lectins from interacting with their receptors.

RESULTS

Interactions of Lectins with Rods

Figure 1 is a surface view of a fresh frog retina that has been incubated with FITC-Con A. The fluorescent label is distributed as a bright line defining the surface of the rod outer segments, indicating that the lectin has bound to the plasma membrane. No labeling is observed if 100 mM α-methyl mannopyranoside is added to the incubating medium. In ROS exposed to hypotonic media, the plasma membrane becomes ruptured and the discs are exposed to the surrounding medium. No labeling of the discs is observable if this medium contains FITC-Con A.

Figure 2 is a fluorescence micrograph of a fresh ROS incubated with FITC-Con A. As seen in the whole retina, labeling of the plasma membrane is clearly visible. The rod is completely sealed at the distal end (left), but failure to label the disc surfaces gives the appearance of a hollow tube open at the broken, proximal end (right).

In addition to labeling the surfaces of ROS, concanavalin A had another action. At physiological pH, the molecule is a tetramer consisting of four subunits each possessing one sugar binding site. Because of this multivalence, it is possible to form cross-bridges between neighboring Con A receptors. If these receptors are on different ROS, then the two organelles will be linked together. This effect is manifested by agglutination of ROS suspensions in the presence of Con A, even at concentrations as low as 0.05-0.5 µg/ml. Agglutination was abolished by 3-33 mM α-methyl mannopyranoside and to a certain extent by D-glucose. Lactose, D-galactose, α-D-fucose, α-L-fucose, N-acetylglucosamine, chitobiose and N-acetylgalactosamine had no effect.

A total of ten lectins with various sugar specificities were examined. These are listed in Table 1. Only LTA and UeA failed to cause agglutination or exhibit fluorescent labeling of the ROS surface when used as the FITC conjugate.

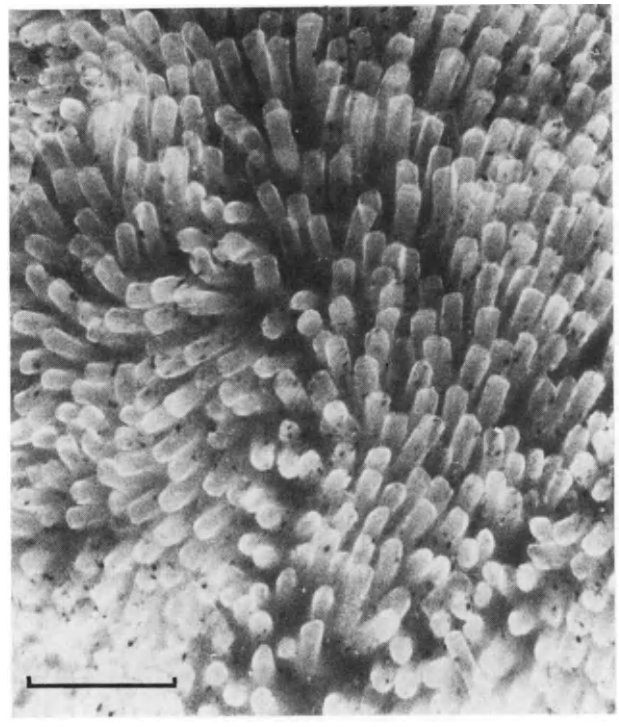

Fig. 1. Fluorescence photograph of the surface of a fresh frog (*R. pipiens*) retina after incubation with FITC-Con A (500 µg per ml, 3 minutes, room temperature). Bar - 50 µm.

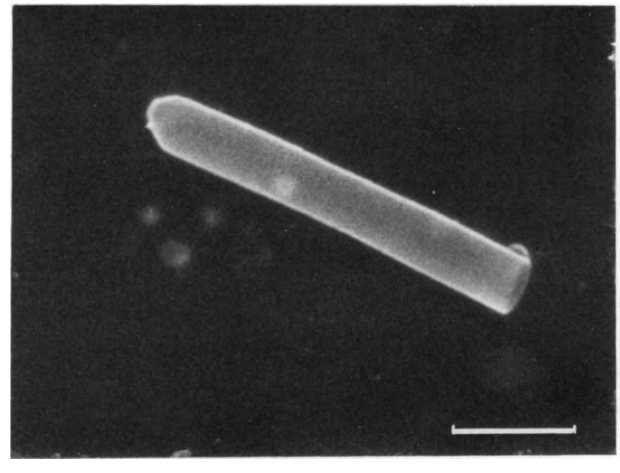

Fig. 2. Isolated fresh frog ROS incubated with FITC-Con A (100 µg per ml, 10 minutes, room temperature). Bar - 13 µm.

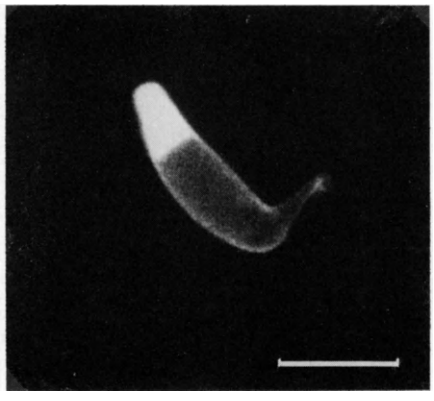

Fig. 3. Isolated fresh frog cone incubated with FITC-Con A (150 µg per ml, 10 minutes, room temperature). Bar - 10 µm.

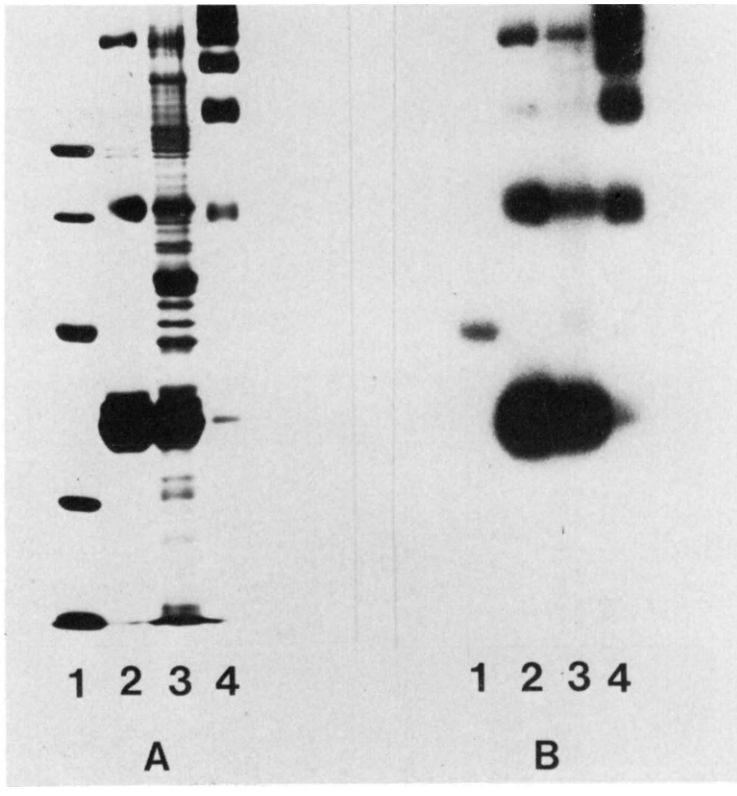

Fig. 4. SDS-PAGE of ROS membrane proteins on slabs subsequently stained and incubated with ^{125}I-concanavalin A.

A - Coomassie blue-stained gel. B - Autoradiogram. Track 1: protein standards (phosphorylase b, 94,000 daltons; BSA, 68,000 daltons; egg albumin, 43,000 daltons; carbonic anhydrase, 29,000 daltons; soybean trypsin inhibitor, 21,000 daltons; lysozyme, 14,300 daltons); track 2: 1.11/1.13 density interface (mainly ROS); track 3: 1.13/1.15 density interface; track 4: 1.11/1.13 membranes heated to 100^{0} in the SDS dissociating buffer (this procedure almost eliminates rhodopsin monomer, and generates multimers that also bind Con A.

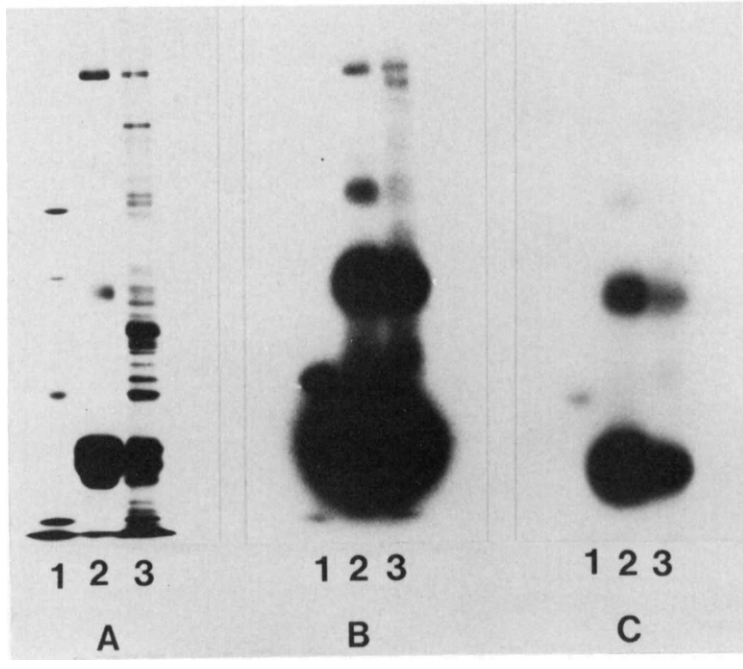

Fig. 5. SDS-PAGE of ROS membrane proteins on slabs subsequently incubated with ^{125}I-WGA.

A - Coomassie blue-stained gel. B - Autoradiogram (4 hours exposure). C - Autoradiogram (2 hours exposure). Tracks 1, 2 and 3 as in Fig. 4.

TABLE 1 Interactions of Lectins with Rod and Cone Outer Segments

Lectin	Agglutination of ROS		FITC-label	
	Min conc. (μg/ml)	Most potent inhibitory sugar tested	ROS	COS
Con A	0.05-0.5	α-methyl mannopyranoside	+++++	++++
PNA	2.0-19.5	D-galactose	+++	+++++
RCA-120	25.0	D-galactose	+++	++
RCA-60	10.0	D-galactose	+++	++
HPA	not known	N-acetyl galactosamine	n.t.	n.t.
DBA	250	N-acetyl galactosamine	n.t.	n.t.
SBA	62.5	N-acetyl galactosamine	+	++
WGA	250	di (N-acetylglucosamine)	++	++
LTA	none	—	none	none
UeA	none	—	none	?

Binding of fluorescence-labeled lectins to cones

Figure 3 illustrates an isolated frog cone cell incubated with FITC-Con A. The inner segment clearly bears a surface label, but the outer segment is uniformly fluorescent, unlike ROS. This arises from penetration of the lectin into the intra-disc space, which is confluent with the extracellular medium.

Results obtained with other lectins are summarized in Table 1. In addition to Con A, cone outer segments bind PNA, RCA-120, RCA-60, SBA and WGA. However, where as both accessory and principal cones are labeled by Con A, WGA and SBA, only the accessory cone is labeled appreciably by PNA.

ROS Glycoproteins--Identification of Con A and WGA Receptors on Polyacrylamide Gels

SDS-PAGE of ROS membranes was carried out, and the resulting slab gels are illustrated in Figs. 4 and 5. After each gel was stained with Coomassie Blue it was incubated with ^{125}I-concanavalin A (Fig. 4) or ^{125}I-WGA (Fig. 5). Of the standard proteins in tracks #1, only the glycoprotein ovalbumin binds Con A and WGA. Tracks #2 and #3 are membranes collected respectively from the 1.11/1.13 and 1.13/1.15 interfaces of the discontinuous sucrose gradient. The former material is the purer ROS preparation, as evidenced by the paucity of protein bands. Three bands bind ^{125}I-Con A. From the bottom, they are the rhodopsin complex, rhodopsin dimer and the high molecular weight protein described by Dreyer, Papermaster and Kuhn (1972) and Bownds and co-workers (1974). A similar pattern is observed with ^{125}I-WGA, which binds to rhodopsin and its multimers as well as to the high molecular weight protein. WGA binds relatively less intensely to the latter than Con A, and the exposure times required to visualize it adequately on the X-ray film result in heavy over-exposure to the rhodopsin label (Fig. 5B). These findings show that the high MWt protein is a glycoprotein similar to rhodopsin. The characteristic doublet visible in track #2 at the approximate level of phosphorylase B (MWt 94,000) does not bind Con A or WGA, and may correspond to the cyclic nucleotide phosphodiesterase described by Miki and co-workers (1975). The more complex pattern seen in track #3 does not show any prominent extra bands on the autoradiogram, with the exception of one just below the high MWt protein in the WGA-incubated gel. Track #4 in Fig. 4 was obtained from a 1.11/1.13 interface sample that had been treated with the SDS-dissociating

mixture for 5 minutes at 100°, compared with the customary 15-30 minutes at room temperature. In this case, most of the rhodopsin was converted into Con A-binding multimers. As recently noted by Molday and Molday (1979), we find that the frog rhodopsin monomer region is complex in these high-resolution gels, and consists of three distinct bands separated by a few thousand daltons. This will be the topic of a future publication.

DISCUSSION

Receptors for a wide variety of lectins clearly exist on the surfaces of rods and cones. A major glycoprotein in ROS is rhodopsin, which has a high affinity for Con A and WGA (see Figs. 4 and 5; also Steinemann and Stryer, 1973), consistent with its oligosaccharide structure GlcNAc β 1 → 2 Man α 1 → 3 (Man α 1 → 6) Man β 1 → 4 GlcNAc β 1 → 4 GlcNAc → Asn (Liang and co-workers, 1979; Papermaster, Fukuda and Hargrave, 1979). Rhodopsin is a likely constituent of the ROS palsma membrane (Basinger, Bok and Hall, 1976; Jan and Revel, 1974) and hence may represent the majority of Con A and WGA surface receptors (see also Fong and Bridges, 1979; Kalish and Branton, 1978). As shown in Figs. 4 and 5, the protein of 291,000 daltons MWt (Bownds and co-workers, 1974; Dreyer, Papermaster and Kuhn, 1972) also binds Con A and WGA, but is probably only a minor membrane-bound component of the ROS (Papermaster and co-workers, 1978).

The identity of the other ROS lectin receptors has not been established as yet. Lectin-ligand interactions can be extremely complex, and do not always involve a simple haptene sugar. For example, Con A may recognize a mannobiosyl chain with terminal β(1→2) linked GlcNAc (Kornfeld and Ferris, 1975); PNA has an affinity for the disaccharide Gal β 1→3 GalNAc (Lotan and co-workers, 1975); WGA binds to β 1→4 linked multimers of GlcNAc, and possibly other sequences involving Man and NeuNAc residues (e.g., Bhavanandan and Katlic, 1979). Lectins that bind to single terminal sugar residues include RCA-120 and RCA-60 (Gal; Etzler, 1974); HPA (GalNAc; Hammarström, 1973); DBA (GalNAc; Carter and Etzler, 1975); possibly SBA (GalNAc and Gal; Lotan and co-workers, 1974). Consequently, our present observations also suggest the presence of GalNAc (HPA, DBA, SBA and PNA) and Gal (RCA-120, RCA-60 and PNA) on ROS plasma membranes as summarized in Table 2. While some rhodopsin molecules may carry galactose (see O'Brien, 1978), this sugar may also be present on another unidentified glycoprotein or even a glycolipid. GalNAc, hitherto unsuspected, may also be attached to plasma membrane glycolipids.

As Table 1 shows, lectins that bind to rod outer segments also bind to cones, although there may be differences in intensity. For example, PNA binds strongly to the accessory cones *in situ*, but scarcely at all to the principal cones. WGA, on the other hand, binds almost equally to both members of the pair. These differences should be interpreted circumspectly, since they may entail differences in the ability of these molecules to penetrate the intra-discal space.

Unfortunately, our knowledge of cone biochemistry is very sketchy. Iodopsin is a glycoprotein like rhodopsin, although it appears to have a different affinity for Con A (Fager and Fager, 1979). Consequently, cone pigments may be responsible for the Con A binding to frog COS (e.g., in Fig. 3). Fucose may have a role, as suggested by Bunt (1978), but its presence on cone outer segment membranes is not supported by our observations with UeA and LTA. The failure of these fucose-specific lectins to bind to cone outer segments (as well as ROS) indicates that these residues are not present, although interference with lectin-ligand interactions by steric factors (e.g., neighboring residues) cannot be ruled out.

TABLE 2 Lectin Receptors on ROS

Lectin	Residue suggested
Con A	(GlcNAc $\xrightarrow{\beta}$) Man $\xrightarrow{\alpha}$ Man
PNA	Gal $\xrightarrow{\beta}$ GalNAc
RCA-120 RCA-60 (PNA) (SBA)	Gal $\xrightarrow{\alpha,\beta}$
HPA DBA SBA (RCA-60)	GalNAc $\xrightarrow{\alpha,\beta}$
WGA	(⟶) GlcNAc $\xrightarrow{\beta}$ GlcNAc (⟶)
UeA LTA	No evidence for Fuc $\xrightarrow{\alpha}$

ACKNOWLEDGEMENT

This work was supported by grants from the Retina Research Foundation of Houston, the National Institutes of Health (ROl EY 02489; P30 EY 02520) and Fight-for-Sight, Inc., New York City.

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Neurochemistry Vol. 1, pp. 269-285.
Pergamon Press Ltd. 1980. Printed in Great Britain.

LIGHT-INDUCED, REVERSIBLE BINDING OF PROTEINS TO BOVINE PHOTORECEPTOR MEMBRANES. INFLUENCE OF NUCLEOTIDES

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ABSTRACT

At least four polypeptides are extractable, using centrifugation in aqueous buffers, from dark adapted bovine photoreceptor outer segments but become membrane-bound upon illumination of rhodopsin. Their approximate molecular weights are 68,000, 48,000, 37,000, and 35,000 daltons, as estimated by gel electrophoresis. The 68 K polypeptide was previously identified to carry rhodopsin kinase activity. Both the 37 K and 35 K polypeptides together are shown to carry GTPase activity. The GTPase in its soluble form is inactive but is reactivated if washed rod outer segment membranes are added to it in the light.
In the dark after bleaching, the bound polypeptides (enzymes) slowly become soluble again. Nucleotides strongly interact with the light-induced binding of all four of the polypeptides. GTP, for instance, prevents and reverses the light-induced binding of the GTPase. The binding of the GTPase to bleached membranes and its subsequent specific elution with GTP was used to purify the GTPase to homogeneity. It is postulated that these light-induced changes in interaction of proteins with the photoreceptor membrane reflect part of the mechanism by which the enzymes are light-activated via bleaching of rhodopsin.

KEYWORDS

Rhodopsin, GTPase, kinase, light-activated enzymes, peripheral membrane proteins, enzyme purification.

ABBREVIATIONS

ROS, rod outer segments; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; cGMP, cyclic guanosine 3', 5'-monophosphate; K, molecular weight of polypeptides in thousands (e.g., 37 K = 37,000 daltons); EDTA, ethylenediamine tetraacetic acid; DTT, dithiothreitol.

INTRODUCTION

A number of enzymatic reactions in vertebrate rod outer segments (ROS) are activated by light, including GTPase, cGMP phosphodiesterase, and phosphorylation of rhodopsin

by a kinase (for a recent review see Pober and Bitensky, 1979). These light-induced reactions are thought to be part of the mechanism by which light absorption is transduced into excitation of the rod cell, although their precise function in excitation or adaptation is unknown. The enzymes are distinct entities which can be separated from rhodopsin.

Nothing is known about the mechanism of their activation by light except that the action spectrum of their light activation in all cases matches the absorption spectrum of rhodopsin (see review by Pober and Bitensky, 1979). This implies that bleaching of rhodopsin is the primary event of enzyme activation, and that the enzymes must somehow interact with bleached rhodopsin in order to get activated.

I have found that light induces, in fact, profound changes in the interaction of several proteins with the photoreceptor membrane. These light-induced changes are reversible in the dark, and are strongly influenced by GTP and a few other nucleotides. A previous report (Kühn, 1978) demonstrated that two proteins (68 K, 48 K), which are soluble at moderate ionic strength (e.g., 70 mM phosphate buffer) in the dark, become membrane-bound for a limited time after illumination. One of them (67 - 69 K) has been shown to carry rhodopsin kinase activity. An additional polypeptide (37 K) was mentioned also to be extractable in large amounts from dark-kept but not from bleached membranes if extraction was performed at very low ionic strength (Kühn, 1978). In the present report these findings have been extended using a variety of different ionic conditions, and the influence of nucleotides on the binding has been studied. It is demonstrated that at least four polypeptides undergo light-induced changes in their interaction with the disc membrane. Two of them (37 K, 35 K) together carry GTPase activity, and the GTPase was purified by light-induced adsorption to the membranes followed by specific elution with GTP.

METHODS

Bovine ROS were purified from freshly dissected retinas as described (Kühn, 1978). Their absorption ratio A_{280} / A_{500} was 2.0 - 2.4. Amounts of rhodopsin were determined from its absorption difference at 500 nm before and after bleaching. The molar extinction coefficient was assumed to be 41,000, and the molecular weight 38,000 daltons.

Extraction of soluble proteins in darkness and light was performed as described (Kühn, 1978), with some modifications. Orange light ($\lambda >$ 540 nm) was used to bleach the ROS in most experiments, except those shown in Figures 2 and 6 where white light was used. During the 2 - 3 min period of illumination with orange light, about 50 - 80 % of the rhodopsin was bleached. Bleaching was carried out at 20° if not stated otherwise. Infrared radiation of the bleaching light was filtered off by a water bath. The ROS were centrifuged for 10 - 15 min at 50,000 g, and the supernatants were again centrifuged for 30 - 60 min at the same speed to remove any residual membraneous material. Normally, the first supernatant was already clear and free of rhodopsin but the second centrifugation step was always added. The final supernatants were used for GTPase and kinase assays and for SDS-PAGE.

The buffers used for extraction were either Tris-HCl (pH 7.4) at 5 mM or 100 mM concentration; or sodium phosphate (pH 7.0) at 5 mM or 70 mM; or 1 M NH_4Cl. All buffers, except 1 M NH_4Cl, contained 1 mM DTT and 0.05 mM phenylmethylsulfonyl fluoride. $MgCl_2$ or EDTA were sometimes added as stated in RESULTS.

Kinase activity of extracts was assayed as described (Kühn, 1978), using kinase-depleted, alum-treated ROS membranes as a substrate in the presence of light.

GTPase activity was assayed by a modification of the methods described by Neufeld and Levy (1969) and by Wheeler and Bitensky (1977). The assay mixture contained 2 μM γ - ^{32}P-GTP (specific activity 500 - 3,000 cpm / pmol); 20 mM Tris-HCl (pH 7.4); 5 mM $MgCl_2$; 1 mM DTT; and the appropriate amounts of ROS and extracts, in a final volume of 200 μl. For the assay of extracted GTPase, alum-treated ROS membranes (Kühn, 1978), which were free of intrinsic GTPase activity, were added for activation of the GTPase (4.7 μg rhodopsin per sample). If extracts containing solubilized GTPase were assayed in the absence of ROS membranes, their apparent GTPase activity was only 1 - 2 % of that measured in the presence of added, alum-treated ROS membranes. The measured GTPase activity was approximately linear with the amount of extract but was not strongly influenced by the amount of alum-treated ROS if more than 3 μg rhodopsin were added. Incubation was started by adding previously dark-kept ROS to the pre-warmed samples in white light. After 2 - 8 min at 30°, the reaction was terminated by addition of 200 μl of ice-cold, 25 % trichloroacetic acid / 40 μM P_i, and the $^{32}P_i$ formed during hydrolysis was determined as described by Neufeld and Levy (1969). GTPase activity is expressed as pmol P_i liberated per min per amount of protein shown on the gels of Fig. 1. The values are averages of 3 - 4 determinations which did normally not differ by more than 5 %.

Gel electrophoresis in the presence of 0.1 % dodecyl sulfate (SDS-PAGE) was performed in two systems: The discontinuous system described by Laemmli (1970), and the continuous system of Weber and Osborn (1969) with the modifications described (Kühn, 1978). For molecular weight estimation, the continuous system was preferred, using fluorescein-labeled chymotrypsinogen as an internal R_f marker. The discontinuous (Laemmli, 1970) gels yielded better separation and sharper bands but had some peculiarities which did not seem to favour molecular weight estimations: They never gave straight logarithmic calibration lines with standard proteins and, reproducibly, the order of sequence of certain polypeptide bands on the discontinuous gels was reversed as compared to continuous gels. For example: On continuous gels, rhodopsin (38 K) migrates on top of the two GTPase polypeptides (37 K and 35 K) whereas on discontinuous gels, it migrates below them (see Fig. 1). Similarly, the order of the light-dependent 48 K polypeptide and its light-independent neighbour is reversed on the two types of gels.

Staining for protein using Coomassie Brilliant Blue (Fig. 1) and for glycoprotein using PAS was performed according to Fairbanks, Steck and Wallach (1971). The amount of protein in the bands of stained gels was measured by comparison of gel densitograms with standard gels containing known amounts of aldolase (Sigma). The staining intensity of the aldolase standard was nearly the same (only slightly higher)as that of known amounts of opsin.

The nucleotides and nucleotide analogues were purchased from Boehringer and Sigma.

RESULTS

Polypeptide composition of rod outer segments and of dark extracts.

Although rhodopsin is the predominant protein in purified ROS, the presence of a number of additional polypeptides can be demonstrated by separation on SDS-PAGE (Fig. 1; in agreement with previous reports such as Godchaux and Zimmerman, 1979 a; Kühn, Cook and Dreyer, 1973; Kühn, 1978). Most of these additional polypeptides can be eluted from dark-kept ROS with aqueous buffers (Fig. 1 d, g, k, q), and little protein besides rhodopsin (opsin) remains with thoroughly washed membranes (Fig. 1 c). Many proteins can already be extracted at moderate, "near-physiological" ionic strength (e. g., 100 mM Tris or 70 mM phosphate buffer; Fig. 1 k, q), provided the ROS plasma membrane is mechanically disrupted which can be easily

achieved by freezing / thawing or by homogenization (Kühn, 1978). This suggests that those proteins are soluble in the ROS cytoplasm or only very weakly membrane-bound.

Other polypeptides, namely the prominent doublet band at 37 K / 35 K and the doublet at 95 K, are nearly insoluble at moderate ionic strength but are soluble, in the dark, at low ionic strength (e.g., 5 mM Tris buffer, Fig. 1 d), suggesting that they are peripherally bound membrane proteins which need extremes of ionic strength to be solubilized. The 37 K / 35 K doublet is also solubilized, in the dark, at very high ionic strength (1 M NH_4Cl, Fig. 1 g).

Staining for carbohydrate using PAS stain revealed only two glycoproteins to be present in ROS, namely opsin and a high-molecular weight polypeptide (> 200 K); both polypeptides remain membrane-bound even after extensive washing. None of the extractable proteins was stained by PAS, not even when large amounts of extract, containing up to 40 - 45 µg of each of the polypeptides 37 K and 35 K, were applied to a gel. For comparison, 0.5 µg of rhodopsin already yielded a distinct pink band if stained with PAS under the same conditions.

Light-induced binding. Illumination of the ROS suspensions prior to centrifugation causes certain polypeptides to disappear almost completely from the soluble supernatant. They sediment with the bleached membranes and are found in the pellet (not shown in Fig. 1) from which they can be eluted by subsequent treatments (see, e.g., Fig. 1 n). Four polypeptides were found, reproducibly with many ROS preparations, to undergo this light-induced binding: The polypeptides 68 K, 48 K, 37 K, and 35 K. However, different buffers are needed for the different polypeptides to demonstrate their differential extractability in light and dark. The 68 K and 48 K polypeptides are soluble in the dark at both moderate and low ionic strength, and therefore, their light-induced binding is obvious under both conditions (Fig. 1 e, l, r).

The light-dependent 37 K and 35 K polypeptides, on the other hand, are nearly insoluble at moderate ionic strength in the dark as well as in light (Fig. 1 k, l, m); therefore, very low or very high ionic strength is necessary to show that the interaction of these two peripheral membrane proteins with the membrane is also changed upon illumination (Fig. 1 d, e, and g, h, respectively). 5 mM Tris buffer (and, to a lesser extent, 1 M NH_4Cl) provides sufficient ionic stress to disrupt their binding to the membrane in the dark but is not sufficient to disrupt their light-induced binding which is obviously much stronger. For the 48 K polypeptide, 1 M NH_4Cl is too strong an extractant to keep its light-induced binding intact; therefore, this polypeptide is extracted independently of light in 1 M NH_4Cl whereas the 68 K, 37 K, and 35 K polypeptides undergo the light-induced binding (Fig. 1 h).

The interpretation of the polypeptide patterns in the 30 K - 40 K range is somewhat complicated by the fact that at least four different polypeptides are present in this range only two of which are light-dependent.[1] The light-dependent binding of the 35 K polypeptide, for instance, is masked to some extent by the presence of another 35 K polypeptide which is extracted independently of light and which, in

[1] Since 5 mM Tris buffer is more effective an extractant than 100 mM Tris or 1 M NH_4Cl, different amounts of extract were applied to gels in order to give approximately equal staining intensity. The amounts of extract applied to the gels correspond to the following amounts of rhodopsin present in the original ROS suspension before extraction: 35 µg rhodopsin for each of the samples applied to gels (d) - (f); 80 µg rhodopsin for each (g) - (i); and 120 µg rhodopsin for each (k) - (m).

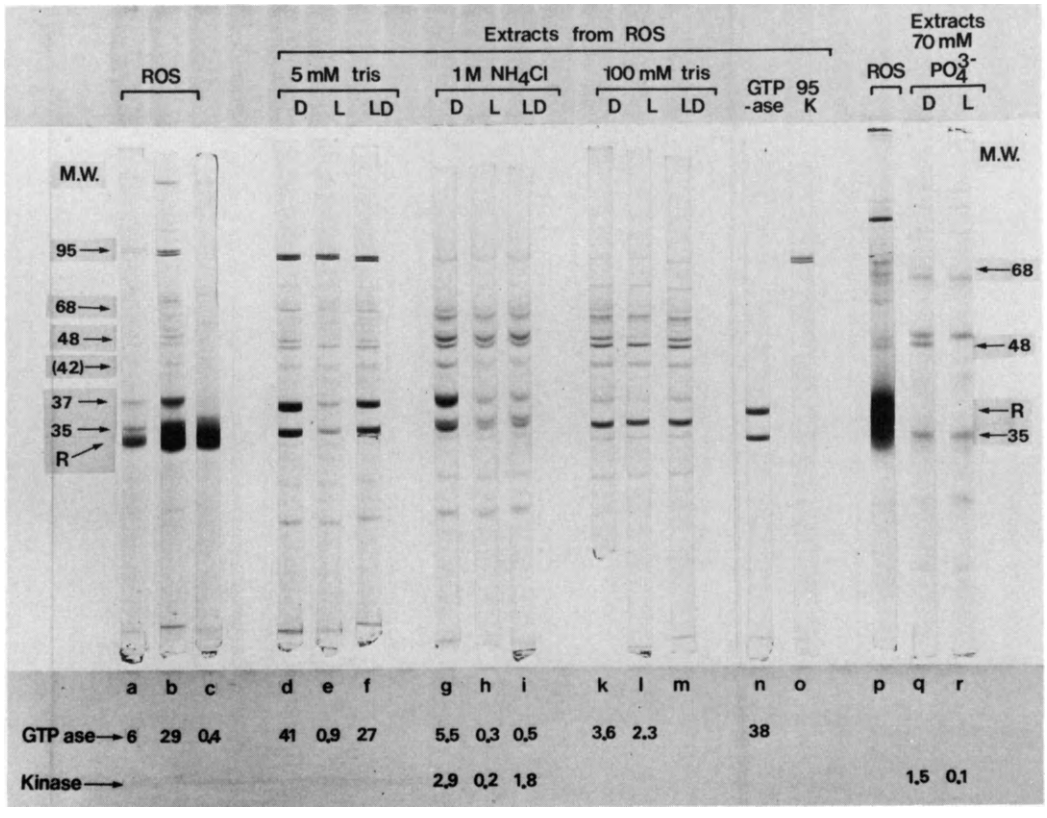

Fig. 1. Polypeptide composition and enzyme activities of ROS and of extracts obtained from ROS in various buffers.

contrast to its light-dependent counterpart, is soluble already at moderate ionic strength (see Fig. 1 k, l, m). The two 35 K polypeptides could sometimes be separated on modified Laemmli gels (which is demonstrated, to some extent, in Fig. 1 g, h, i). Because of this masking, the differences in enzyme activity (GTPase) of dark versus light extracts are always greater than the differences in the amount of polypeptides.

In addition to the four light-dependent polypeptides described above, two other polypeptides have been found often but not always to be present in larger amounts in dark extracts than in corresponding light extracts. One of them (42 K) exhibits light-dark differences only if 1 M NH_4Cl is the extractant (faintly visible in Fig. 1 g, h). The other one migrates near the kinase (in the range of 60 K - 80 K) and is preferentially seen if light / dark extraction is performed at low ionic strength in the presence of EDTA. They have not been further investigated.

Reversibility of light-induced binding.

If ROS membranes are bleached but are then further incubated for 70 min at 20° before centrifugation, the initially bound polypeptides (68 K, 48 K, 37 K, 35 K) are found to be released again into the soluble supernatant. This is reproducibly observed both at moderate ionic strength for the 68 K and 48 K polypeptides (see also Kühn, 1978), and at low ionic strength for all four of the polypeptides (Fig. 1 m, f).[2]

The polypeptides were separated by SDS-PAGE both in a discontinuous (gels a - o) and a continuous (p - r) electrophoretic system. The numbers (M. W.) on either side indicate molecular weights in thousands. The position of the rhodopsin (opsin) band, as identified by PAS staining of separate gels, is marked by "R". Samples (a), (b), and (p) represent whole ROS containing 2.4 µg, 12 µg, and 22 µg of rhodopsin, respectively. (c), ROS membranes after extensive hypotonic extraction; 12 µg rhodopsin. All other samples represent extracts of soluble proteins obtained in the buffers indicated on top of the gels, under the illumination conditions as denoted: (D), dark extracts from unbleached ROS; (L), extracts from bleached ROS centrifuged immediately after illumination; (LD), extracts from bleached ROS centrifuged after a 70 min dark incubation period at 20° following illumination. Extracts (d) - (f) are in 5 mM Tris-HCl / 0.5 mM $MgCl_2$; extracts (g) - (i) are in 1 M NH_4Cl; extracts (k) - (m) are in 100 mM Tris-HCl / 0.5 mM $MgCl_2$. Sample (n) represents purified GTPase (2.8 µg protein), and sample (o) purified 95 K polypeptides (see section about purification of ROS proteins).
GTPase and rhodopsin kinase assays were performed in each case with the same amount of extract or ROS, respectively, which was also applied to the corresponding gel. For details see METHODS. GTPase activity is expressed as pmol P_i liberated per min, and kinase activity as mol phosphate incorporated per mol rhodopsin (of alum-treated ROS) per 30 min at 30°.

In 1 M NH_4Cl, only the 68 K polypeptide (rhodopsin kinase) is released from its light-induced binding, whereas the three polypeptides 37 K, 35 K, and 42 K appear to be bound irreversibly (Fig. 1 i). The reason for this irreversible binding may be that 1 M NH_4Cl too strongly disorders the system. Another reason for the apparent irreversibility may be the absence of the protecting agent, DTT, from NH_4Cl solutions (see METHODS).

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[2]The release of the bound polypeptides in the dark can also be demonstrated if ROS are centrifuged immediately after bleaching and if the bleached pellet is then resuspended at 20° in the dark with buffer for an hour followed by a second centrifugation step (not shown in Fig. 1).

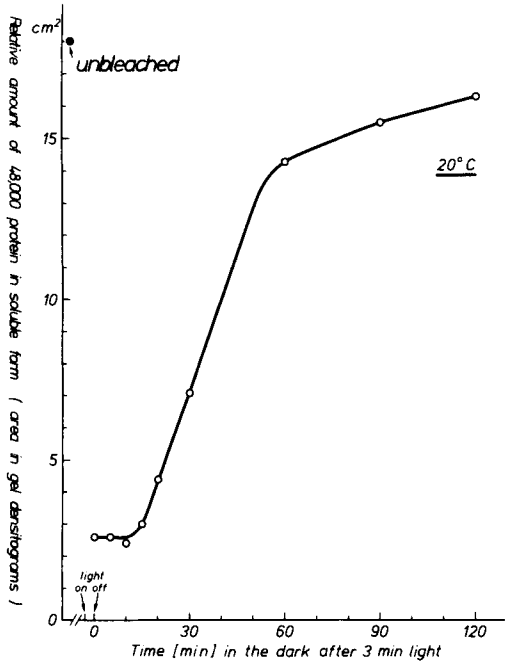

Fig. 2. Extractability of the 48 K polypeptide as a function of the time elapsed between bleaching and centrifugation of ROS suspensions.

For experimental conditions see METHODS, and see Kühn (1978). The buffer was 70 mM phosphate / 2 mM DTT / 1 mM $MgCl_2$ / 0.1 mM EDTA. Aliquots of the soluble supernatants were applied to gels.

The rate at which the 48 K polypeptide is released from its light-induced binding is shown in Fig. 2. The release appears to be a fairly slow reaction, taking about one hour at 20°. The release of the kinase (Kühn, 1978) and of the 37 K and 35 K polypeptides (unpublished) occurs in the same time range, although the rate of release is not necessarily the same for all of the proteins.

Light-induced binding of kinase and GTPase activities.

Some of the preparations shown in Fig. 1 were assayed for kinase and for GTPase activities, and the results are shown at the bottom of the gels in Fig. 1.

Rhodopsin kinase activity was high in dark extracts and low in corresponding light extracts (Fig. 1 g, h, and q, r). The light-induced binding of the kinase was essentially reversible at both high and moderate ionic strength (Fig. 1 i; and Kühn, 1978). The presence of kinase activity always paralleled the presence of the 68 K polypeptide. This was observed comparing kinase activities and polypeptide patterns of many extracts obtained under a wide variety of conditions, indicating that the 68 K polypeptide carries the kinase activity. This confirms and extends previously published findings (Kühn, 1978).

GTPase activities of whole ROS, (Fig. 1 a, b) were found to be of the same order of magnitude as those reported for frog ROS by Wheeler and Bitensky (1977). Thoroughly washed ROS membranes, on the other hand, showed negligible GTPase activity (Fig. 1 c) which indicates, also in agreement with these authors, that the GTPase can be eluted from the membranes. No GTPase activity could be measured in any of the extracts if assayed in the absence of rhodopsin (see METHODS). However, if washed ROS membranes, free of intrinsic GTPase, were added with illumination to the GTPase assays of extracts, high GTPase activity was found in dark extracts (Fig. 1 d, g) but not in light extracts (Fig. 1 e, h). The activity in dark extracts was found to be up to 50 times higher than in corresponding light extracts. Obviously, the

GTPase is extracted from dark-kept membranes in an inactive form which can be reactivated by adding bleached ROS membranes, in agreement with Wheeler and Bitensky's (1977) and Godchaux and Zimmerman's (1979 b) observations. It can, however, not be eluted from bleached membranes shortly after bleaching because it undergoes the light-induced binding to the membranes. This binding is reversible in 5 mM Tris (Fig. 1 f) but irreversible in 1 M NH_4Cl (Fig. 1 i). At moderate ionic strength, most of the GTPase remains membrane-bound both in darkness and light (Fig. 1 k, l).

Comparing GTPase activities and polypeptide patterns of many extracts obtained under different conditions, the presence of GTPase activity always and quantitatively paralleled the presence of the two light-dependent polypeptides 37 K and 35 K. Both of these polypeptides seem to be necessary for GTPase activity (unpublished experiments with partial chromatographic separation of the two polypeptides). It is therefore assumed that both of them represent subunits of the GTPase. This is in agreement with recent findings by Godchaux and Zimmerman (1979 b) who also assign GTPase activity to the same polypeptide doublet.

Temperature dependence of binding and release

It would be of great interest to know how fast the light-induced change in interaction between the soluble proteins and the membrane occurs. It may be considered to be a physiologically relevant mechanism of light-induced enzyme activation only if it takes less than a second to occur at physiological conditions (i. e., 37°, and high concentrations of all biological material). Unfortunately, the centrifugation method used to separate soluble from membrane-bound proteins, has a poor time resolution (about 10 min). Therefore, it was investigated whether any effect of temperature on the light-induced binding can be demonstrated within this time resolution.

Fig. 3 (middle section) shows that between 0° and 29°, the two GTPase polypeptides (37 K, 35 K) undergo the light-induced binding faster than can be resolved by the time resolution of the method. Even at 0°, the binding occurs to the same extent as at higher temperatures. This leaves open the possibility that it may be, at physiological temperature, a fairly fast reaction.

An ROS suspension in 5 mM phosphate buffer was divided into 18 equal portions. All samples were warmed in the dark for 5 - 10 min to the temperatures indicated in the abscissa. Six of them were centrifuged without bleaching (left section); six were bleached for 2 min shortly before centrifugation (middle section); and six were bleached but then further incubated in the dark for 88 min at the indicated temperatures before centrifugation (right section). All samples were cooled in an ice water bath in the dark for 2 - 5 min before centrifugation was started. Aliquots of the clear supernatants were applied to discontinuous gels; the amount of 37 K and 35 K polypeptides was estimated from densitograms of stained gels (see METHODS) and is shown in the ordinate.

The extent to which the bound polypeptides are released in the dark in an 88 min incubation period, on the other hand, highly depends on the temperature (Fig. 3, right section). This is in agreement with the observation that the release is a slow reaction at 20° (see Fig. 2 for the 48 K polypeptide, and Kühn, 1978, for the kinase). At 16°, only half of the bound GTPase polypeptides is released within 88 min in the dark, and at 0°, no release at all seems to occur. This lack of release at 0° is helpful, from an experimental point of view, in handling several samples at the same time: After they have been bleached at 20°, subsequent cooling to 0° in the dark greatly reduces the risk of release of bound polypeptides during processing.

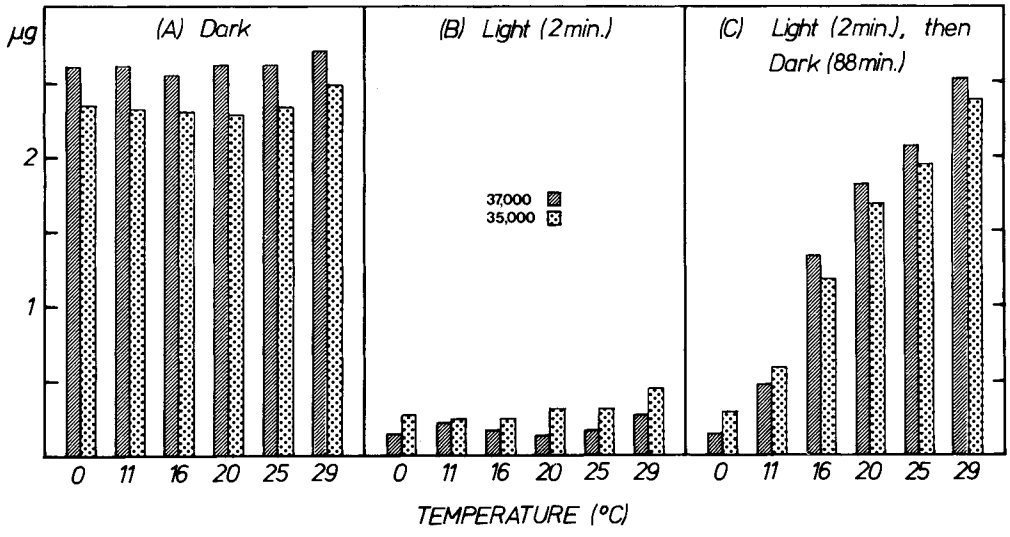

Fig. 3. Temperature dependence of binding and release of GTPase polypeptides.

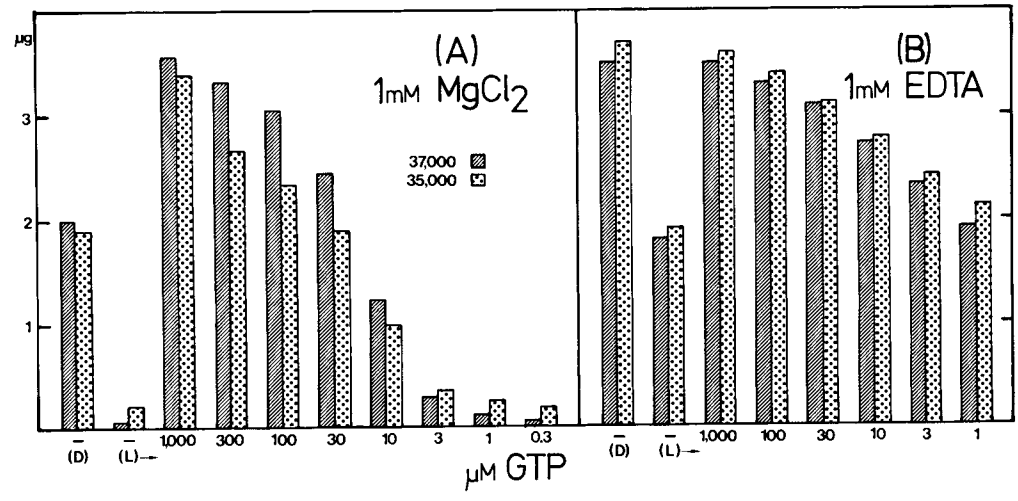

Fig. 4. Solubilization of 37 K / 35 K polypeptides from bleached ROS by various concentrations of GTP.

The extractability from unbleached membranes was found to be independent of temperature (Fig. 3, left section).

Influence of Mg^{++} ions

$MgCl_2$ (1 mM) significantly enhances the binding of GTPase to the membranes in both darkness and light. In the presence of 1 mM EDTA (Fig. 4 B, (D) and (L) controls), higher amounts of the 37 K / 35 K polypeptides are eluted from dark-kept (D) as well as from bleached (L) ROS than in the presence of 1 mM $MgCl_2$ (Fig. 4 A, (D) and (L) controls). The ratio of binding in dark versus light is, therefore, greater in the presence of $MgCl_2$ than in its absence. On the other hand, the difference in the amounts of extracted polypeptides (dark minus light) is almost the same in Fig. 4 A and B. This effect of $MgCl_2$ was observed with 4 different ROS preparations suspended in 5 mM Tris buffer (see also Fig. 5). The binding of the kinase and of the 48 K protein, on the other hand, appears to be much less influenced by $MgCl_2$ (Kühn, 1978).

All of the 18 samples are aliquots from the same ROS suspension in 5 mM Tris / 2 mM DTT. $MgCl_2$ or EDTA, respectively, was added before bleaching; GTP in the final concentrations indicated in the abscissa was added shortly after bleaching. For details see text. Similar results were obtained in 2 separate experiments. The amount of polypeptides in the soluble supernatants is shown in the ordinate.

Influence of GTP on the binding of GTPase

The light-induced binding of the 37 K / 35 K polypeptides (GTPase) is completely inhibited if GTP (0.2 - 1 mM) is present during illumination (not shown), and is quickly reversed if GTP is added in the dark after illumination (Fig. 4). All samples, except the dark controls (D), were bleached at 20° in the absence of GTP in order to induce the binding of the GTPase to the membranes. GTP was then added in the dark to the final concentrations indicated in the abscissa. Immediately after mixing, the suspensions were cooled in an ice water bath and then centrifuged. The amount of 37 K and 35 K polypeptides in the clear supernatants was determined from densitograms of stained gels.

Obviously, the solubilizing effect of GTP is independent of $MgCl_2$ since it works in 1 mM EDTA (Fig. 4 B) as well as in 1 mM $MgCl_2$ (Fig. 4 A). The main difference is only that in the absence of $MgCl_2$, the background value of unbound GTPase is higher (Fig. 4 B, (L)-control) than in the presence of $MgCl_2$ (Fig. 4 A, (L)-control). It should be noted that, in contrast to the solubilizing action of GTP which is independent of $MgCl_2$, the enzymatic activity of the GTPase requires $MgCl_2$ and is abolished by EDTA (unpublished experiments).

The solubilizing effect of GTP on the GTPase polypeptides is exhibited over a wide range of GTP concentrations. It is near saturation at 0.3 - 1 mM GTP and becomes smaller at lower GTP concentrations, but as low as 1 µM GTP still had a small but significant effect in the presence of both $MgCl_2$ and EDTA (compare with the corresponding (L)-controls in Fig. 4 A and B).

This solubilizing effect of GTP was observed both at high (1 M NH_4Cl) and at low ionic strength (5 mM Tris or 5 mM phosphate buffer) but not at moderate ionic strength. In 100 mM Tris buffer, addition of GTP to ROS membranes in light or darkness did not significantly increase the solubility of the GTPase.

Nucleotide specificity

The effect to reverse the light-induced binding of the GTPase is relatively, but not quite, specific for GTP (Fig. 5). GDP is almost as effective as GTP, and the γ-blocked GTP analogue guanylyl imidodiphosphate (GMP-PNP) is partially effective. Another γ-blocked analogue, ß,γ-methylene GTP, is nearly ineffective. The following nucleotides or derivatives were found to be completely ineffective in reversing the light-induced binding of the 37 K / 35 K polypeptides: 5'-GMP, cGMP (cG), guanosine, adenosine, adenylyl imidodiphosphate (AMP-PNP), ß,γ-methylene-ATP (AMP-PCP), γ-P-fluorinated GTP (GTPF, kind gift by Dr. Eckstein, Göttingen) (the latter five not shown in Fig. 5).

Those guanosine nucleotides which are effective in the presence of $MgCl_2$ are also effective in the presence of EDTA (compare with the (L)-control values in Fig. 5 A and B, respectively). This does not apply, however, to the adenosine nucleotides: ATP (0.2 mM) is partially effective in the presence of $MgCl_2$ but ineffective in EDTA. ATP at even 10 times higher concentration (2 mM), which is more than sufficient to fully solubilize the GTPase in the presence of $MgCl_2$, is completely ineffective in the presence of EDTA. This interesting phenomenon needs further studies which may provide insights into the mechanism of the action of nucleotides.

In any event, these specificity studies in the presence and absence of $MgCl_2$ indicate that hydrolysis of the γ-phosphate group of GTP is not a necessary step in its action to reverse the light-induced binding of the GTPase.

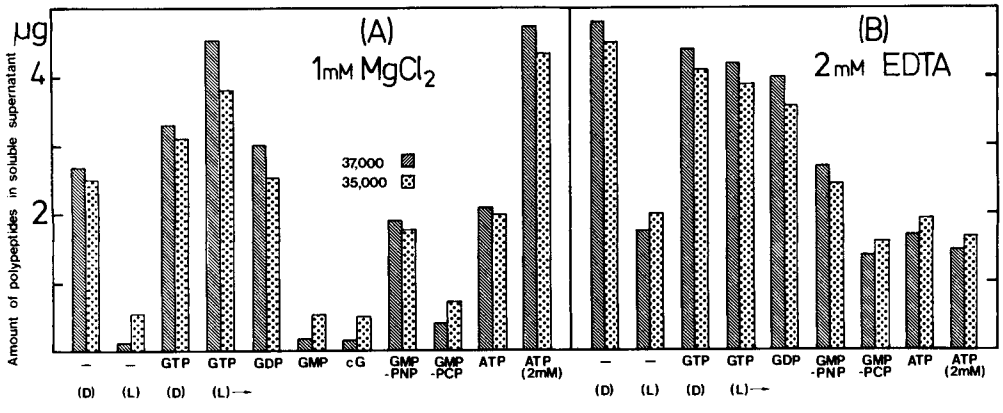

Fig. 5. Efficiency of various nucleotides in reversing the light-induced binding of the 37 K / 35 K polypeptides.

All of the ROS suspensions were bleached except those labeled with (D). The buffer was 5 mM Tris / 2 mM DTT, with the additions indicated in each case. The final concentration of all nucleotides was 0.2 mM, except for ATP which was both 0.2 mM and 2 mM (as indicated). Shortly after addition of the nucleotides at 0^{o}, the suspensions were warmed to 20^{o} in the dark for 3 min, bleached for 3 min, cooled in the dark and centrifuged. Similar results were obtained in 3 separate experiments.

Effect of nucleotides on the release of kinase in the dark

Fig. 6 shows that ATP also influences the release of rhodopsin kinase from its light-induced binding to the membranes. ATP has little influence on the extractability of the kinase in the dark before bleaching (filled symbols in Fig. 6), nor does it prevent the light-induced binding (Fig. 6, the two open symbols in parentheses, where ROS suspensions were bleached in presence of nucleotides). ATP and its γ-blocked analogue, adenylyl imidodiphosphate (AMP-PNP) do, however, significantly increase the rate of release of kinase in the dark after bleaching (open symbols in Fig. 6). This enhancement of kinase solubilization by ATP occurs, however, on a much slower time scale than the solubilization of the GTPase by GTP or ATP which appeared to occur "immediately" after addition of the nucleotide to bleached ROS.

Effect of nucleotides on the binding of the 48 K polypeptide

Both ATP and GTP also influence the light-induced binding of the 48 K polypeptide, but in an opposite direction as compared to the GTPase and kinase: The binding of this polypeptide becomes stronger and apparently irreversible if ATP or GTP are present during or after illumination (not shown in the Figures of this report). Even in 1 M NH_4Cl, where the 48 K polypeptide normally does not undergo the light-induced binding (Fig. 1 h), it becomes bound to bleached disc membranes if ATP is present.

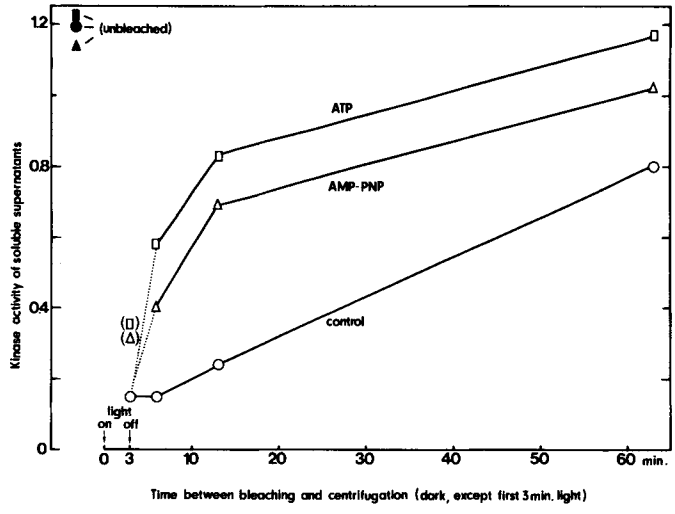

Fig. 6. Influence of nucleotides on the light-induced binding and dark release of rhodopsin kinase.

ROS were suspended in 1 M NH_4Cl containing 1.3 mM $MgCl_2$. All suspensions, except those indicated by the filled symbols, were bleached with white light for 3 min at 20°. Open symbols in parentheses: 2 mM ATP (□) or 2 mM AMP-PNP (△), respectively, were present during illumination, and samples were centrifuged immediately after illumination. Other open symbols: samples were bleached in the absence of nucleotides, and 2 mM ATP or 2 mM AMP-PNP, respectively, were added immediately after

bleaching, followed by an incubation period in the dark at 20° for the times indicated on the abscissa. After centrifugation, aliquots of the clear supernatants were assayed for kinase activity as described (Kühn, 1978). Similar results were obtained in 3 separate experiments.

Purification of ROS proteins using light-induced binding

The phenomenon of light-induced binding and substrate-induced release of certain ROS proteins can be used to purify them simply by washing bleached ROS membranes several times successively in the appropriate buffers using centrifugation.

The GTPase was purified in the following way: ROS membranes were bleached at 20° in 5 mM Tris / 1 mM $MgCl_2$ / 1 mM DTT, cooled to 0°, and centrifuged. The pellet containing the bound GTPase was washed in the dark first with 5 mM Tris buffer at 0° to remove residual soluble proteins, and was then treated several times for 30 - 60 min at 20° with 100 mM Tris buffer to elute the light-induced bound polypeptides 68 K and 48 K. The GTPase remains membrane-bound at this ionic strength. The GTPase was finally eluted from the pellet using 40 µM GTP in 5 mM Tris / 2 mM DTT. The resulting preparation contained high GTPase activity and only the two polypeptides 37 K and 35 K (Fig. 1 n). Even if 10 times more protein was applied to gels than shown in Fig. 1, no polypeptide besides these two could be detected. The yield of purified GTPase was 60 - 70 µg of each polypeptide per mg of rhodopsin, indicating that no more than 15 rhodopsin molecules are present in ROS per molecule of each of the two GTPase polypeptides.

The 95 K polypeptide doublet which does not undergo the light-induced binding was purified in the following way: Dark-kept ROS membranes were washed several times with 100 mM Tris / 2 mM $MgCl_2$ / 1 mM DTT to remove soluble proteins. (Both the 95 K polypeptides and the GTPase remain largely membrane-bound at this ionic strength.) The ROS were then bleached in 5 mM Tris / 1 mM $MgCl_2$ / 1 mM DTT and centrifuged; the membraneous pellet contained the GTPase, and the supernatant contained the 95 K doublet in nearly pure form. (Fig. 1 o).

The purification of the 48 K protein to 95 % purity, based on a similar method, has been reported earlier (Kühn, 1978).

DISCUSSION

The present report demonstrates that both light and certain nucleotides are involved in regulating the binding of several proteins to the photoreceptor membrane. Among these proteins are a GTPase (consisting of 37 K and 35 K polypeptides), rhodopsin kinase (68 K), and a 48 K protein of as yet unknown function. Light increases the binding affinity of the membrane for all of the proteins investigated, whereas nucleotides have differential effects on the different proteins: The light-induced binding of the GTPase is inhibited and quickly reversed by GTP and a few other nucleotides (Figures 4 and 5); the light-induced binding of the kinase is also reversed by ATP, but on a much slower time scale (Fig. 6); and the light-induced binding of the 48 K protein, in contrast, is strengthened by ATP.

The solubilizing effects of GTP and ATP on the GTPase (fast) and on the kinase (slow) are not mediated via phosphorylation of rhodopsin since they are displayed even under conditions where the phosphorylation reaction is totally inhibited (i.e., in the presence of EDTA, and with γ-blocked nucleotides, respectively). The nucleotides seem to interfere mainly with the light-induced type of binding of the proteins to the membranes. Not much influence of nucleotides on the binding to

dark-kept membranes was seen (except for the compensating effect of higher concentrations of nucleotides on the binding which is caused by Mg^{++} ions, see Fig. 4 A and 5 A).

The light-induced binding of all four of the polypeptides is reversible in the dark even in the absence of nucleotides (Fig. 1) if the appropriate conditions are used (temperature, presence of DTT, type of buffer).

Different buffer systems were used in this study to reveal different aspects of the light-induced binding. All of them are more or less artificial, 100 mM Tris and 70 mM phosphate ("moderate ionic strength") buffers probably being the closest to physiological ionic strength conditions.

For the two proteins (68 K, 48 K) which are extractable at moderate ionic strength, the light-induced binding can be demonstrated to take place using moderate as well as low ionic strength, since under both conditions they are soluble in the dark and membrane-bound in the light. The GTPase, on the other hand, is largely membrane-bound at moderate ionic strength in both light and darkness. Therefore, extreme ionic conditions must be applied in order to reveal light-dark differences in its binding affinity to the membrane. Low ionic strength (and, to a lesser extent, very high ionic strength) is capable of disrupting the binding of the GTPase to dark-adapted membranes but not to freshly bleached membranes. Separate experiments (to be published elsewhere) have shown indirectly that the light-induced increase in binding affinity also occurs at moderate ionic strength: After bleaching ROS in 100 mM Tris buffer, the GTPase is bound so tightly that it cannot be extracted by subsequent treatment in the dark with 5 mM Tris at 0^{o}, whereas it is easily eluted from unbleached membranes by changing the buffer from 100 mM to 5 mM Tris.

It should be noted that all of the soluble proteins are colourless and cannot directly react with the bleaching light used ($\lambda > 540$ nm). The only protein in ROS known to absorb the orange light used is rhodopsin, the major constituent of the disc membrane. Any light-induced change concerning the soluble proteins must therefore occur, directly or indirectly, via interaction with bleached rhodopsin. Similarly, it is suggested by the action spectra of the light-activated ROS enzymes (λ_{max} 500 nm, see review by Pober and Bitensky, 1979), that these enzymes are also activated via bleaching of rhodopsin. It seems likely that the observed light-induced change in the interaction with the photoreceptor membrane of GTPase, kinase, 48 K protein, and perhaps other proteins, reflects a general mechanism by which enzymatic reactions in ROS are activated by light. For the kinase, it has been shown earlier (Kühn, 1978) that its light-induced binding decays in the dark at the same rate as the light-induced capacity of rhodopsin to be phosphorylated decays, suggesting that the light- and time-dependent binding of kinase serves to regulate the activity of the phosphorylation reaction. The light activation of the GTPase seems to involve complicated interactions between the enzyme, GTP, and the membrane which need further studies. The function of the 48 K protein, although it has been obtained in high purity via light-induced binding, is still unknown; it may be a light-regulated enzyme, or an activator or inhibitor of an enzyme.

The reversible interconversion of enzymes (e.g., glycolytic enzymes) between soluble and membrane-bound forms has been described in a number of recent reports and is generally thought to serve as a regulatory mechanism of enzyme activity (reviewed by Wilson, 1978). The two forms are reported to differ in their kinetic properties, and the membrane-binding is specifically influenced by substrates or allosteric effectors of the enzymes. With regard to the findings of the present report, a report by Margoliash and others (1976) is particularly interesting, showing that the binding of cytochrome c to cytochrome c oxidase in the mitochon-

drial membrane is inhibited by ATP. The authors discuss the possibility that this effect of ATP on the binding may "provide the basis of a mechanism for mitochondrial respiratory control".

In the present report, the differential effects of nucleotides and of light on the binding of the different proteins, and the physiological role of these agents, are not understood in detail. It is, for instance, plausible that light-induced phosphorylation of rhodopsin by ATP involves light-induced binding of the kinase; however, it is then difficult to understand why the presence of ATP weakens the binding of the kinase (Fig. 6). Some additional control mechanism seems, therefore, to be involved. Similarly, it is not known which reaction may be regulated by the light-induced binding of the 48 K protein, and by the ATP-induced enhancement of the light-induced binding of this protein.

In conclusion:
There is, at the present time, no detailed explanation for all of the phenomena observed. They all probably reflect, under different conditions, various aspects of the same event: A profound change in the arrangement and interaction of proteins in the disc membrane, a change which is induced by light, is reversible in the dark, and is strongly influenced by certain nucleotides. These changes in molecular interaction may well reflect the mechanism by which enzymes are light-activated via bleaching of rhodopsin.

ACKNOWLEDGEMENT

I thank Ms. O. Mommertz for expert technical assistance and Dr. P. A. Hargrave for reading of the manuscript. The work was supported by SFB 160 of Deutsche Forschungsgemeinschaft.

Note added.

Recent observations have shown that, in addition to the light-dependent polypeptides described, a peptide is present in dark extracts and absent in light extracts. It migrates with the tracking dye upon electrophoresis on discontinuous gels (Laemmli, 1970) and is eluted from the continuous gels (Weber and Osborn, 1969) under the conditions used in this report (15 hr electrophoresis time); therefore, it has not been detected earlier. If analyzed by a gel electrophoretic system designed to resolve peptides in the molecular weight range of 2,000 - 20,000 daltons (Swank, R. T., and K. D. Munkres (1971), Anal. Biochem. 39, 462-477), this peptide migrates with an apparent molecular weight of about 5,000 - 6,000 daltons.

Most of the extracts represented in the figures of this report were again subjected to electrophoresis under these conditions, leading to the following results: The 5 K peptide is present in all of the extracts which contain the 37 K / 35 K polypeptides (GTPase). It is soluble in the dark in 5 mM Tris but insoluble in 100 mM Tris buffer, like the GTPase. It becomes reversibly membrane-bound upon illumination, and its light-induced binding is reversed by the same nucleotides which also reverse the light-induced binding of the 37 K / 35 K polypeptides (Fig. 5). It is also present in the purified GTPase (Fig. 1 n) and may, therefore, be tentatively regarded as a subunit of the GTPase in addition to the 37 K and 35 K polypeptides.

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Neurochemistry Vol. 1, pp. 287-297.
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RHODOPSIN PHOSPHORYLATION IN THE FROG RETINA: ANALYSIS BY AUTORADIOGRAPHY

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ABSTRACT

Light microscopic autoradiography was used to localize phosphorylated rhodopsin and to examine rate and extent of light activated rhodopsin phosphorylation in frog rod outer segments and in the isolated frog retina. The even distribution of label in totally bleached rod outer segments and partial labeling of rod outer segments after local illumination indicates that the light induced transfer of ^{32}P from exogenous (γ-^{32}P) ATP to rhodopsin is not restricted to particular parts of the outer segment. Light activated phosphorylation of rhodopsin in retinas incubated in $^{32}P_i$ starts with the formation of a gradient of labeled rhodopsin suggesting that either light affects the formation of ^{32}P labeled ATP or that outer segments contain a gradient of a diffusable metabolite involved in the phosphorylation of rhodopsin.

Evaluation of autoradiograms by microdensitometry shows that rate and extent of phosphate incorporation in individual rod outer segments significantly differ from the mean value of phosphate incorporation into opsin. Phosphorylation of rhodopsin in single rod outer segments may occur at a rate of 2 moles of phosphate incorporated per mol rhodopsin per minute with a half-time of less than 30 seconds.

KEYWORDS

Vision; retina; light activated phosphorylation; rhodopsin.

INTRODUCTION

Various studies now indicate that protein phosphorylation is of central importance in the control of diverse biological processes such as hormone and neurotransmitter action. Accordingly the light activated phosphorylation of rhodopsin may have a function in the control of the photoreceptor response. Establishing the physiological relevance of this reaction requires information about the localization of phosphorylated rhodopsin and the kinetics of rhodopsin phosphorylation in the photoreceptor cell. Although phosphorylation of rhodopsin has been already studied in isolated retinas (Kühn and Bader, 1976; Kühn and others, 1977; Miller and Paulsen, 1975) and living frogs (Kühn, 1974) our information on these points remains incomplete. Therefore, we have recently started to examine light activated phosphorylation of rhodopsin by light microscopic autoradiography (Paulsen and Schürhoff, 1979)

Autoradiograms, prepared from isolated frog rod outer segments after the light triggered transfer of phosphate groups from (γ-^{32}P)-ATP to rhodopsin, showed that the phosphate incorporation was apparently not restricted to a particular part of the rod outer segment. However, since we could not exclude that the even distribution of radioactive label over the entire length of an outer segment may have resulted preferentially from phosphate incorporation into newly synthesized rhodopsin, which then diffuses laterally in the outer segment plasma membrane, we have prepared autoradiograms from locally illuminated rod outer segments. Furthermore, we have examined the distribution of phosphorylated rhodopsin after incubating isolated retinas in organic ^{32}P-phosphate, so that the phosphate transfer occurred from energy-rich phosphates endogenous to the photoreceptor cell.

Striking differences in the concentration of label associated with single rod outer segments further indicated that the extent and kinetics of rhodopsin phosphorylation are strongly affected by the differing permeability of the plasma membrane of isolated outer segments for ATP. By measuring simultaneously the light activated phosphate incorporation into rhodopsin and, by microdensitometry of autoradiograms, the light induced labeling of rod outer segments we are now able to estimate rate and extent of rhodopsin phosphorylation in single rod outer segments.

MATERIAL AND METHODS

Preparation of Retinas and Rod Outer Segments

Frogs (Rana esculenta) were kept for 3 hours in darkness and then killed by decapitation. Eye cups were prepared from excised eyes with a razor blade and were placed into an isotonic salt solution buffered to pH 7.0 (Hepes Ringer) which contained 115 mM NaCl, 2 mM $MgCl_2$, 2 mM KCl, and 10 mM Hepes (N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid) (Miller and Paulsen, 1975). The retina was removed from the eye cup and was gently separated from the pigment epithelium. It was then stored, receptor side-up, in Hepes Ringer until it was transferred into the incubation medium. Suspensions of rod outer segments were prepared by agitating a retina slowly in a small volume of Hepes Ringer. All the above manipulations were carried out at room temperature (20 to 22° C) by use of an infra-red converter.

Phosphorylation of Rhodopsin in Isolated Retinas and Suspensions of Rod Outer Segments

For phosphorylation of isolated rod outer segments the Hepes Ringer (pH 0.7) was supplemented by 5 mM (γ-^{32}P) ATP (spec. activity about 20 mCi/mmol). Rhodopsin phosphorylation was assayed as described by Miller and Paulsen (1975). Isolated retinas or small pieces of retina (2 mm in diameter), punched out from a larger retina segment, were incubated at 22°C in Hepes Ringer, pH 7.0 (0.1 to 0.2 ml per retina), containing 5 mM glucose and 0.1-0.2 mCi carrier-free orthophosphoric acid ($^{32}P_i$) (Amersham/Buchler). O_2/CO_2 was bubbled through the incubation medium throughout the experiment.

Phosphorylation in suspensions of rod outer segments and in the isolated retina was initiated by illumination (1 min) with white light which bleached about 80% of the rhodopsin present. For local illumination about 50 rod outer segments were suspended in 0.1 ml Hepes Ringer containing (γ-^{32}P) ATP. The outer segments were allowed to sediment to the surface of a cover glass which formed the bottom of a small incubation chamber. This chamber was placed under a microscope. Local illumination was performed by focussing a spot of white, unpolarized light (about 7 µm in diameter) by the condensor onto a part of the outer segment while viewing the area around the specimen under red light ($\lambda > 665$ nm). Light controls for phosphorylation of to-

tally bleached outer segments were obtained by illuminating a larger area of the incubation chamber.

Incubation times prior and after illumination are given in the results. Phosphate incorporation into rod outer segments and retinas used for microautoradiography was stopped by fixation with a solution consisting of 1 % formaldehyde, 5 % glutaraldehyde, and 0.1 M sodium phosphate buffer, pH 7.4. In order to measure rhodopsin phosphorylation in the isolated retina, rod outer segments were, at the end of the incubation time, shaken from the pieces of retina into ice-cold Hepes Ringer. The outer segments were collected by a 30 seconds, low speed centrifugation and dissolved in a denaturing medium containing 10 % sodium dodecyl sulfate·(SDS) and 2 % 2-mercaptoethanol. Aliquots of this solution were subjected to SDS gel electrophoresis according to the method of Laemmli (1970). Coomassie stained gels were scanned by a gel scanner equipped with an integrator unit in order to be able to correct for small differences in the opsin content of the samples. Radioactivity in the opsin band was counted as described by Miller and Paulsen (1975).

Microautoradiography and Microdensitometry of Autoradiograms

Fixatives and soluble radioactive compounds were removed from fixed outer segments and fixed retinas by washing the preparation eight times with 1.0 ml of 0.1 M phosphate buffer and afterwards five times with distilled water. Aldehyde-fixed pieces of retina used for the preparation of autoradiograms of 0.5 µm sections were fixed additionally in phosphate buffered 3.6 % osmium tetroxide, dehydrated and embedded in Epon. Autoradiography of rhodopsin phosphorylation in the isolated retina was performed on rod outer segments which were broken loose by a fine forceps from fixed retinas. Rod outer segments or Epon sections were placed on a microscope slide and covered with a Kodak AR-10 stripping film. Then, autoradiography was performed as described by Paulsen and Schürhoff (1979). Exposure times varied between 3 and 7 days. The illuminated specimen and the corresponding dark controls were placed side by side on the same microscope slide and were therefore treated identically.

Photometric measurements of the absorbance produced by the silver grains in the film above a single outer segment were carried out with the microspectrophotometer described by Schlecht, Hamdorf and Langer (1978) at a fixed wavelength (540 nm). The absorbance was measured in an area 50 µm in diameter around single rod outer segments. Data aquisition, computation, and storage were done by an on-line PDP 11/10 computer.

Histograms which illustrate the differing degree of phosphate incorporation were obtained by measuring the blackening of the stripping film above 150 to 170 single rod outer segments from illuminated samples and above 100 outer segments from the corresponding dark controls. The mean absorbance of the background in areas free of rod outer segments has been subtracted from all other values. The absorbance classes used to construct histograms were based on the absorbance of dark controls immediately before illumination. In order to establish the relationship between film blackening and ^{32}P incorporation we computed the ratio of the mean absorbance of outer segments at different times after illumination to the mean absorbance of the corresponding dark controls and compared this ratio with the light-dark ratio of the ^{32}P incorporation into rod outer segments and rhodopsin. The ratios agreed within the limits of error which suggests a linear relationship (e.g. for the histograms of Fig. 2 and Fig. 6) between absorbance and ^{32}P incorporation.

RESULTS

Distribution of Label after Phosphorylation of Rhodopsin in Isolated Rod Outer Segments

In the previous paper (Paulsen and Schürhoff, 1979) we demonstrated that the light induced incorporation of ^{32}P from (γ-^{32}P) ATP into isolated rod outer segments can be visualized by light microscopic autoradiography. The conclusion that the light induced labeling of rod outer segments reflects the phosphorylation of rhodopsin is further supported by recent experiments in which we dissolved rod outer segments in ionic detergents. We then separated rhodopsin from lipids and other proteins by chromatography on concanavalin A-sepharose and subsequent gel electrophoresis. The ^{32}P content of opsin from illuminated rod outer segments corresponded to the amount of radioactive phosphate which was incorporated after illumination into the rod outer segments. None of the other fractions showed a significant light induced binding of ^{32}P. Accordingly, differences in the distribution of label, such as shown in the autoradiograms of Fig. 1, should indicate a different location of phosphorylated rhodopsin.

Figure 1 shows autoradiograms from a dark control (Fig. 1a), a preparation in which the outer segments were totally bleached (80 % of the rhodopsin present) (Fig. 1b), and from outer segments which were locally bleached by a spot of light (Fig. 1c). Although the dark adapted outer segments had been incubated for 60 minutes in 5 mM (γ-^{32}P)ATP, they remained unlabeled. After illumination which bleached the whole outer segment, about 80 % of the outer segments became labeled. Typically, the label was evenly distributed over the entire length of an outer segment (Fig. 1b). A heterogenous distribution of label was observed only in about 1 % of the labeled outer segments. The important result of this experiment is that after local illumination of outer segments from the same preparation about 60 % of the labeled outer segments became heterogenously labeled. The autoradiograms (Fig. 1c) show that the labeled region of the outer segment is larger than the diameter (7 µm) of the light spot used to illuminate a specific area of the outer segment.

This may in part result from bleaching of rhodopsin by stray light and, further, from the low resolution of autoradiography which is achieved with ^{32}P. In addition, we observed in this and other experiments that, after local illumination of an outer segment, only about 20 % of the illuminated rod outer segments were labeled. Thus, under the conditions used here, the probability that an outer segment becomes labeled increased with the area exposed to light. Furthermore, local illumination appeared to induce phosphorylation of rhodopsin primarily in broken rod outer segments. Most of the labeled outer segments were noticeably shorter than the average rod outer segment length of about 50 µm.

Rate and Extent of Light Activated Labeling of Isolated Rod Outer Segments

The autoradiograms prepared from strongly bleached rod outer segments consistently revealed differences in the concentration of label associated with illuminated rod outer segments. The different extent to which rod outer segments are labeled is illustrated by Fig. 2. During the light activated phosphorylation of rod outer segments, indicated by the time course in Fig. 2a, samples were removed from the suspension and examined by autoradiography. The absorbance histograms obtained by microdensitometric measurements of the silver grain absorbance above single rod outer segments (Fig. 2b) show that throughout the experiment a relatively high percentage of rod outer segments fell into the class of the unlabeled outer segments while others were labeled at a different rate and to a different extent. Rod outer segments falling into the class of maximally labeled outer segments were present

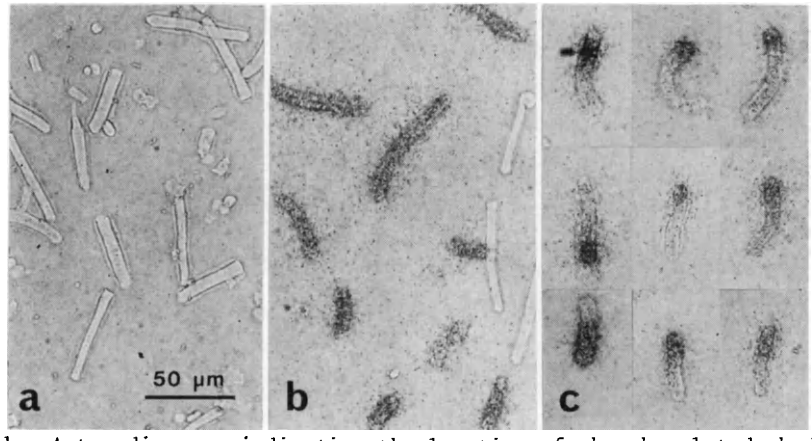

Fig. 1. Autoradiograms indicating the location of phosphorylated rhodopsin in isolated frog rod outer segments. Rod outer segments were incubated for 40 min at 20°C in Hepes Ringer containing 5 mM (γ-^{32}P) ATP (20 mCi/mmol). - (a) Rod outer segments incubated in the dark. - (b) Rod outer segments which were totally bleached at the beginning of incubation by a 1 min exposure to white light. - (c) Single rod outer segments which were, during incubation in (γ-^{32}P)ATP, locally illuminated for 1 min by a spot of white light (diameter 7µm).

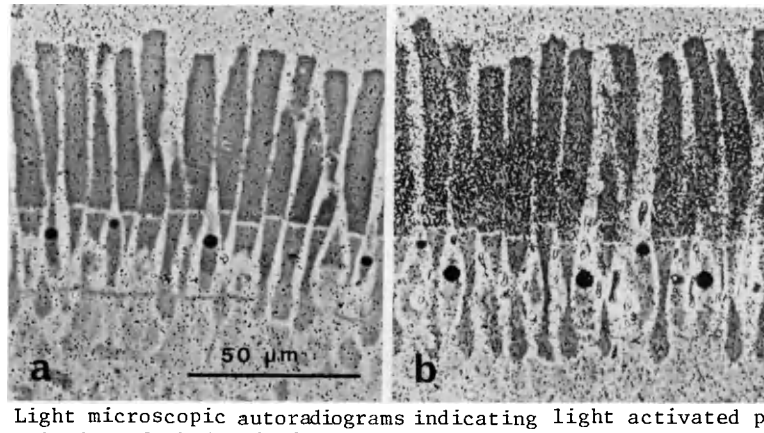

Fig. 3. Light microscopic autoradiograms indicating light activated phosphorylation of rhodopsin in the isolated frog retina. 0.5 µm sections, phase contrast. Retinas were incubated at 20°C in 0.2 ml Hepes Ringer containing 360 µCi carrier-less $^{32}P_i$. - (a) Retina incubated for 70 min in the dark. - (b) Retina incubated for 60 min in the dark, then for 1 min in white light and for another 10 min in the dark. Light induced labeling is restricted to the outer segment layer of rods.

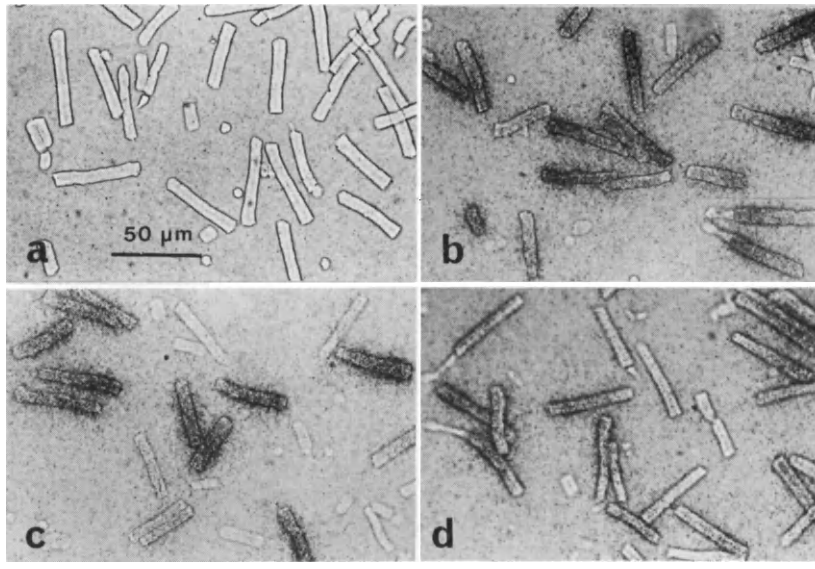

Fig. 4. Autoradiograms showing light activated phosphorylation of rhodopsin in rod outer segments from the isolated frog retina. For autoradiography rod outer segments were prepared from retinas which were fixed after incubation at 20°C in Hepes Ringer containing $^{32}P_i$. Experimental conditions were: - (a) Incubation for 18 min in the dark with $^{32}P_i$ present; - (b) incubation for 8 min in the dark, followed by 1 min in the light (80 % bleach) and 9 min in the dark, with $^{32}P_i$ present all the time; - (c) incubation for 8 min in the dark without $^{32}P_i$ and, after addition of $^{32}P_i$, for 1 min in the light and 9 min in the dark; - (d) incubation for 8 min in the dark with $^{32}P_i$ present and, after removal of $^{32}P_i$, for 1 min in the light and 9 min in the dark.

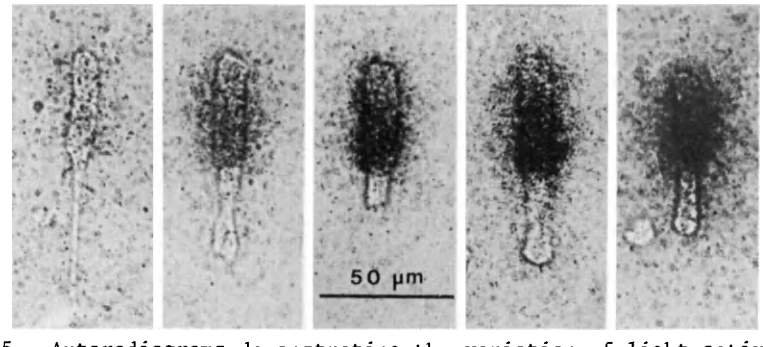

Fig. 5. Autoradiograms demonstrating the variation of light activated ^{32}P incorporation into rod outer segments of the same frog retina. The retina was incubated in 0.1 ml Hepes Ringer, containing 130 µCi $^{32}P_i$, for 60 min in the dark, then illuminated for 1 min and fixed after another 7 min in the dark. Rod outer segments from the dark control were not labeled.

for the first time between two and four minutes after the onset of illumination. The absorbance measured above these outer segments was five times higher than the mean absorbance of illuminated rod outer segments at four minutes after the onset of illumination.

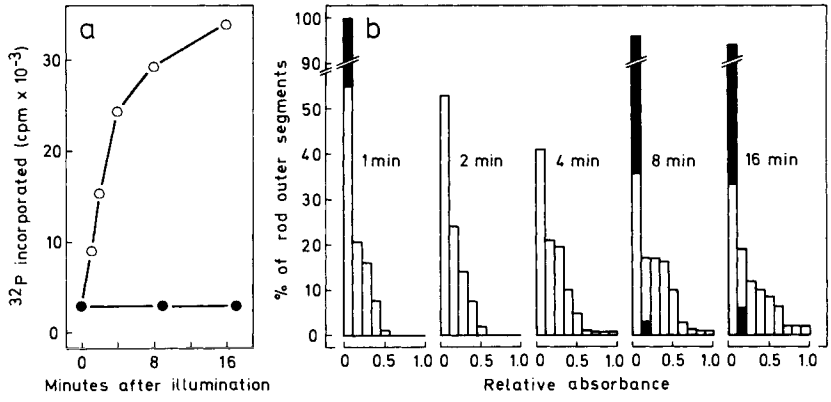

Fig. 2 Frequency distribution of labeled rod outer segments during light activated phosphorylation of rhodopsin. Isolated rod outer segments were incubated at 20°C in Hepes Ringer, pH 7.0, containing 5 mM (γ-^{32}P) ATP (20 mCi/mmol). - (a) Time course of $^{32}P_i$ incorporation into rod outer segment membranes. (●), dark controls; (○), rod outer segments which were illuminated for 1 min with white light. - (b) Histograms obtained by measuring the absorbance of label from single rod outer segments. The indicated times are minutes from the onset of illumination at which rod outer segments were removed from the suspension and fixed for autoradiography. (■), dark controls; (□) illuminated rod outer segments.

The initial rate of light activated phosphate incorporation for the whole outer segment population calculated from the time course shown in Fig. 2a, was 0.43 moles phosphate per mol rhodopsin per minute. Therefore, phosphorylation in rod outer segments which were maximally labeled at four minutes from the onset of illumination, must have occurred at a rate of about 2 moles phosphate per mol rhodopsin per minute with a half-time of less than 30 seconds.

Distribution of Label after Phosphorylation of Rhodopsin in the Isolated Retina

Autoradiograms prepared from semi-thin sections of retinas incubated in $^{32}P_i$ show a light induced ^{32}P incorporation particularly in the outer segment region of the photoreceptor layer (Fig. 3). In agreement with earlier reports (Kühn, 1974; Miller and Paulsen, 1975) we find that the main site of ^{32}P binding is again the protein moiety of rhodopsin. As the light induced labeling can be inhibited by adding 2mM dinitrophenol or 5 mM potassium cyanide to the incubation medium the phosphorylation reaction apparently requires mitochondrial synthesis of energy-rich phosphates. Rod outer segments removed from unbleached frog retinas incubated with $^{32}P_i$ also contain phosphorylated proteins. These proteins, with molecular weights of about 13,000 and 30,000 were, however, dephosphorylated in the light and may therefore be identical with proteins isolated from bullfrog retinas (Polans and others, 1978) and bovine rod outer segments (Lolley, Brown and Farber, 1977). ^{32}P incorporation into phospholipids or rod outer segments membranes was comparatively low, in particular, after short incubation times, and did not increase significantly in the light.

In order to visualize the distribution of label and to be able to measure the absorbance of silver grains above single rod outer segments we removed rod outer segments from fixed retinas and spread them over a larger area on the microscope slide. Figure 4 shows autoradiograms prepared from those outer segments. In the particular experiment illustrated by Fig. 4, retinas were incubated in $^{32}P_i$ for 19 minutes in the dark (Fig. 4a), for 8 minutes before and 10 minutes after illumination (Fig. 4b), for 9 minutes after illumination (Fig. 4c), and for 8 minutes before illumination (Fig. 4d). In the majority of the photoreceptors, illumination induced a phosphorylation of rhodopsin.

The most interesting finding is the distinct gradient of label indicating that binding of ^{32}P has primarily occurred at the base of the outer segment. This gradient of ^{32}P-phosphorylated rhodopsin is most apparent in retinas in which $^{32}P_i$ has been present during and after the illumination (Fig. 4b and c). Extending the incubation in $^{32}P_i$ prior to illumination to 1 - 4 hours has the effect that, after illumination, the extent to which rod outer segments are labeled shows a greater variation. The differing extent to which outer segments from rods of one and the same retina may be labeled is indicated by the autoradiograms in Fig. 5. During prolonged incubation of retinas in $^{32}P_i$ after an illumination, the gradient of phosphorylated rhodopsin along the outer segment changes into a more even distribution, similar to that observed after incubation in (γ-^{32}P) ATP. Interpretation of this observation is however difficult, as during this time, concomitantly with the phosphorylation, rhodopsin also becomes dephosphorylated.

Even after being incubated for several hours in $^{32}P_i$ some illuminated photoreceptors do not incorporate a detectable amount of ^{32}P. So far our attempts to induce a visible light induced labeling in all photoreceptors have failed. Addition of ouabain which should inhibit consumption of endogenous ATP by sodium-potassium pumps increases the average number of radioactive phosphates bound to rhodopsin but does not lead to a light induced labeling of all outer segments. Control experiments carried out with other Ringer solutions showed that the different extent of labeling does not specifically result from the use of Hepes Ringer.

Rate and Extent of Light Activated Labeling of Rod Outer Segments in the Isolated Retina

In frog rod outer segments incubated in (γ-^{32}P)ATP dephosphorylation of rhodopsin is observed only when particular experimental conditions are met (Miller and Paulsen, 1975; Miller, Paulsen and Bownds, 1977). In illuminated retinas incubated in $^{32}P_i$ however, rhodopsin usually undergoes, like in living frogs, a complete phosphorylation-dephosphorylation cycle (Kühn, 1974; Kühn and others, 1977). Our previous experiments had indicated (for example, see Fig. 5) that even after incubating a retina several hours in $^{32}P_i$ light did not induce phosphorylation in all photoreceptors to the same extent. As this will have consequences for the evaluation for example of the rate of rhodopsin phosphorylation and dephosphorylation we compared the light activated incorporation of ^{32}P into rhodopsin with the density of label above single rod outer segments from the same retina.

Figure 6 illustrates such an experiment in which we measured phosphorylation and dephosphorylation of rhodopsin in the retina and, in parallel, monitored incorporation of ^{32}P into rod outer segments by autoradiography. Clearly the histograms presented in Fig. 6b reflect the different ^{32}P binding to rhodopsin during the phosphorylation-dephosphorylation time course shown in Fig. 6a. It is obvious that the value obtained for the phosphorylation of rhodopsin by measuring the phosphate con-

tent of opsin does represent only the mean of a ^{32}P incorporation which may differ at the receptor level by a factor of 10. Accordingly, the rates of phosphorylation and dephosphorylation may also be different. Whether this proves to be true can only be evaluated if one succeeds in labeling the endogenous pool of energy-rich phosphates in all photoreceptors to the same extent.

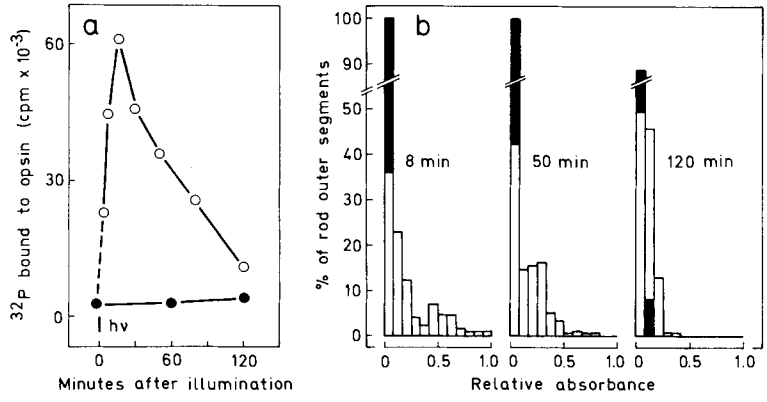

Fig. 6. Frequency distribution of labeled rod outer segments during light activated phosphorylation in the isolated frog retina. Retina pieces were incubated before illumination at 20°C for 60 min in the dark in Hepes Ringer containing 100 µCi $^{32}P_i$ per 0.1 ml. - (a) Time course of ^{32}P incorporation into opsin; (●) opsin from dark controls; (○) opsin from pieces of retina which were exposed for 1 min to white light. Triplicate determinations which agreed within 10%. - (b) Histograms obtained by measuring the absorbance of label above single rod outer segments. The indicated times are minutes from the onset of illumination at which pieces of retina were fixed for autoradiography. (■) Dark controls; (□) rod outer segments from illuminated pieces of retina.

Finally the histograms of Fig. 6b reveal that labeling of rod outer segments from the dark control increases during two hours of incubation in the dark. Thus, the residual binding of ^{32}P observed in illuminated rods after 120 minutes may not only result from phosphorylation of rhodopsin but also from the labeling of other proteins or phospholipids.

DISCUSSION

The analysis of autoradiograms prepared from single rod outer segments shows that the rhodopsin phosphorylation in outer segments from one and the same suspension may greatly differ in rate and extent. In a preceeding paper (Paulsen and Schürhoff, 1979) we discussed a differing permeability of the outer segment plasma membrane for ATP as one possible reason for the differing labeling of rod outer segments in the presence of exogenous (γ-^{32}P)ATP. In the meantime we have attempted to increase the leakiness of isolated rod outer segments for ATP without perturbing at the same time light activated rhodopsin phosphorylation. Shocking outer segments hypo-osmotically or ultrasonication in the presence of (γ-^{32}P)ATP did not increase reproducibly the rate of rhodopsin phosphorylation. Only partial lipolysis of outer segment phospholipids by phospholipase A_2 increased the initial

rate of phosphorylation about 3 - 4 fold. However, the maximum phosphate incorporation decreased by about 70 %.

It appears that the process of rhodopsin phosphorylation is sensitive to experimentally induced changes in the structural and functional integrity of the rod outer segment. This is further supported by the observation that dephosphorylation of rhodopsin, which is normally observed in the isolated retina of Rana esculenta, does not occur or is at least greatly reduced in isolated rod outer segments, and that maximum phosphate incorporation in preparations of outer segment membranes is usually lower than in "intact" outer segments. The lability of the rhodopsin phosphorylating system may however be only one reason for the differing degree to which outer segments become labeled. It does not explain why sometimes more than 30% of the illuminated photoreceptors of a retina did not incorporate $^{32}P_i$ into rhodopsin.

Shichi and Somers (1978) who examined phosphorylation in isolated rod outer segments containing ^{3}H-labeled rhodopsin reported that newly synthesized rhodopsin became preferentially phosphorylated. Accordingly, light activated phosphorylation of rhodopsin should be restricted to the basal region and the plasma membrane of a rod outer segment. However, from the autoradiograms, prepared from isolated rod outer segments after rhodopsin phosphorylation in presence of exogenous (γ-^{32}P)ATP, emerges a different picture. After illuminating the whole outer segment the label is distributed evenly along a rod outer segment. This even distribution cannot primarily be caused by phosphorylation of rhodopsin diffusing in the plasma membrane: Firstly, the amount of phosphate transferred to rhodopsin in intensely labeled rod outer segments is far too high to be explained by the phosphorylation only of rhodopsin located in the plasma membrane (Paulsen and Schürhoff, 1979). Secondly, after local illumination labeling remains confined to a part of an outer segment. This is to expect if the phosphorylated rhodopsin is located within disc membranes separated from the plasma membrane. The even distribution of label in totally illuminated rod outer segments then indicates that, in isolated rod outer segments and with (γ-^{32}P)ATP present in excess, rhodopsin of newly synthesized and old disc membranes is phosphorylated to about the same extent.

Therefore, the formation of a distinct gradient of phosphorylated rhodopsin in photoreceptors from retinas incubated in $^{32}P_i$ is most likely not based on differing properties of rhodopsin. As the gradient is most apparent in strongly bleached outer segments when exogenous $^{32}P_i$ is present during and after illumination, light appears to have an effect apart from activating rhodopsin phosphorylation. Illumination may, for example, increase the influx of $^{32}P_i$ into the receptor cell, increase the rate of ATP formation in the inner segment and reduce ATP consumption by other processes. This would increase the specific activity of ^{32}P labeled γ-groups in ATP. γ-groups of the ATP, when it diffuses into the outer segment, would then be transferred to the bleached rhodopsin located at the base of the outer segment.

Another possibility would be that the gradient of phosphorylated rhodopsin is the result of differences in the ATP concentration within the outer segment or less likely, of gradients of factors such as divalent cations which participate in the light activated phosphorylation of rhodopsin. The existence of a gradient of a diffusable metabolite has been recently discussed by Baylor, Lamb and Yau (1979) as one possible reason for the different response kinetics measured at the tip and the base of single toad rod outer segments.

The evaluation of autoradiograms from single rod outer segments finally shows that kinetics measured in suspensions of isolated rod outer segments or isolated retinas represent only an average value for the ^{32}P incorporation into rhodopsin which may proceed in individual outer segments with a significantly differing rate. Thus,

phosphorylation from intracellular energy-rich phosphate could be faster than indicated by the time courses of phosphate transfer from exogenous ^{32}P sources and may be more rapid at the base of an outer segment than at its tip. By extrapolation from an initial rate of 2 moles of phosphate bound per mol rhodopsin per minute one can estimate that within a few milliseconds from the onset of illumination each disc should contain several hundred phosphorylated rhodopsins. This might be a sufficiently high number to alter disc membrane properties or to regulate enzyme activities, for example to inhibit light dependent phosphodiesterase activities. However, when considering possible functions of rhodopsin phosphorylation on the basis of the results which we have obtained by autoradiography, it has to be taken into account that, in order to incorporate high amounts of ^{32}P into an outer segment, we generally bleached about 80% of the rhodopsin present. Experiments with isolated bullfrog rod outer segments (Miller, Paulsen and Bownds, 1977) suggest that rate and maximum phosphate incorporation are different when, in order to initiate phosphorylation of rhodopsin, more physiological light stimuli are used.

ACKNOWLEDGEMENT

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 114). We thank Mrs. G. Kuschewski for valuable technical assistance and Dr. W. S. Jagger for a critical reading of the manuscript.

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Neurochemistry Vol. 1, pp. 299-309.
Pergamon Press Ltd. 1980. Printed in Great Britain.

SEARCH FOR A PHYSIOLOGICAL ROLE OF CYCLIC GMP METABOLISM IN THE PHOTORECEPTORS

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ABSTRACT

Data on the time-course of the light-activation of the cyclic GMP phosphodiesterase and of the GTPase, and results on the influence of cyclic GMP on the disc membrane permeability are presented. On the basis of the kinetic data, it is not possible to separate the light-activation of these two enzymes from the early steps of photoreceptor transduction. In addition, the cyclic GMP increases the permeability of the disc membranes, indicating that a decrease of the endogenous cyclic GMP concentration, consequent to the light-activation of the phosphodiesterase, can decrease the membrane permeability shortly after illumination.

KEYWORDS

GTPase; cyclic GMP; cyclic GMP phosphodiesterase; membrane permeability; rod outer segments.

INTRODUCTION

In this communication we make a brief review of the work done in our laboratory in the last few years. Our attention was focused on the physiological significance of two light-activated enzymes of the ROS, the cyclic GMP phosphodiesterase (cG-PdE) and the GTPase.

The time-course of the light-activation of these two enzymes was studied in order to see whether it was comparable with the electrical events.

Furthermore, to understand whether the light-induced activities are responsible for the light-induced permeability changes of the photoreceptor membranes, we have been studying the influence of cyclic GMP (cGMP) and GTP on these membranes.

RESULTS AND DISCUSSION

Light-Activation of the cG-PdE and of the GTPase

It has been possible to study the time-course of the light-activation of the cG-PdE

and of the GTPase in the broken ROS with a true resolution of 3 s, by means of a fast perfusion system (Caretta and co-workers , 1979a).

Briefly, one drop of perfusate was collected every 1.5 s from a preparation of broken ROS perfused with a solution containing a suitable amount of radioactive nucleotide, and, during the perfusion, a flash of light was presented to the broken ROS. Subsequently, by means of thin layer chromatography separation of the nucleotides produced by the hydrolysis, and by means of radioisotope counting, the rate of the hydrolysis was determined, Fig. 1. The data were somewhat distorted by the dynamic characteristics of the perfusion apparatus, but it was possible to correct for them and obtain a description of the activation time-course with a nominal resolution of less than 3 s.

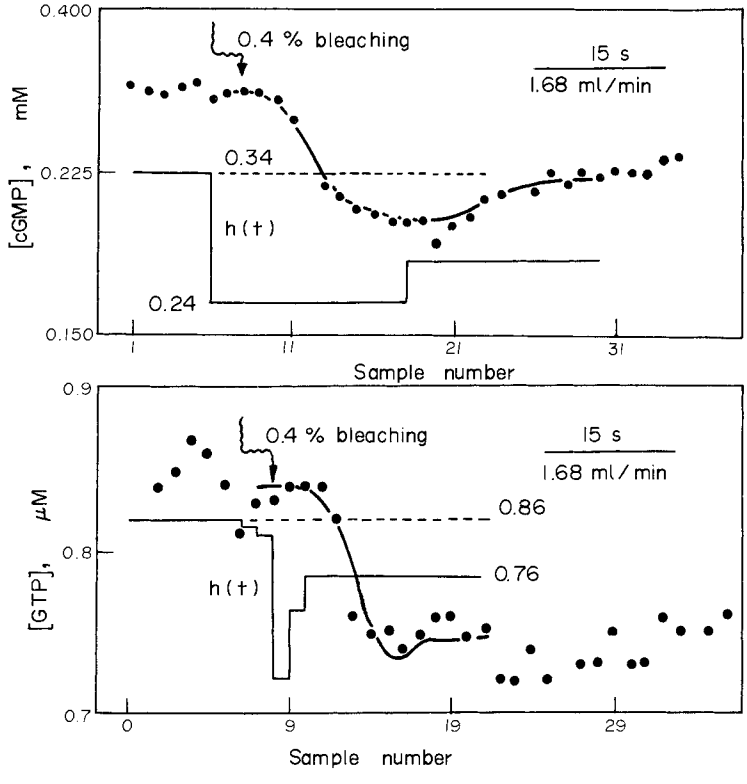

Fig. 1. Time-course of the activation by light. A. Phosphodiesterase. 0.4×10^6 broken ROS; 0.5 mM cGMP, 0.5 mM GTP; samples of 1 drop every 1.5 s. The continuous curve is the convolution of a suitable function of activation, h(t), with the characteristic function of the perfusion. B. GTPase. 1 μM GTP; the rest as in A. (from Caretta and co-workers, 1979a).

The onset of the activation of the cG-PdE was without apparent delay, whereas that of the GTPase seemed somewhat delayed.

At the same time, also other laboratories were obtaining data on the activation kinetics of the cG-PdE. Yee and Liebman (1978) were studying the proton rather than the nucleotide products of the cG-PdE hydrolysis in the broken ROS and their data are in agreement with ours. Bownds and collaborators (Woodruff and others, 1977; Woodruff and Bownds, 1979) measured the endogenous cGMP content in isolated ROS after illumination with a better time resolution and their data suggest that the activation of the cG-PdE by light occurs in less than 0.1 s. Discussing the pioneering study on the light-activated cG-PdE (Miki and others, 1973), Bitensky and collaborators were inclined to rule out the possibility that the activation of the cG-PdE might represent an early event in the chain leading to the photoreceptor

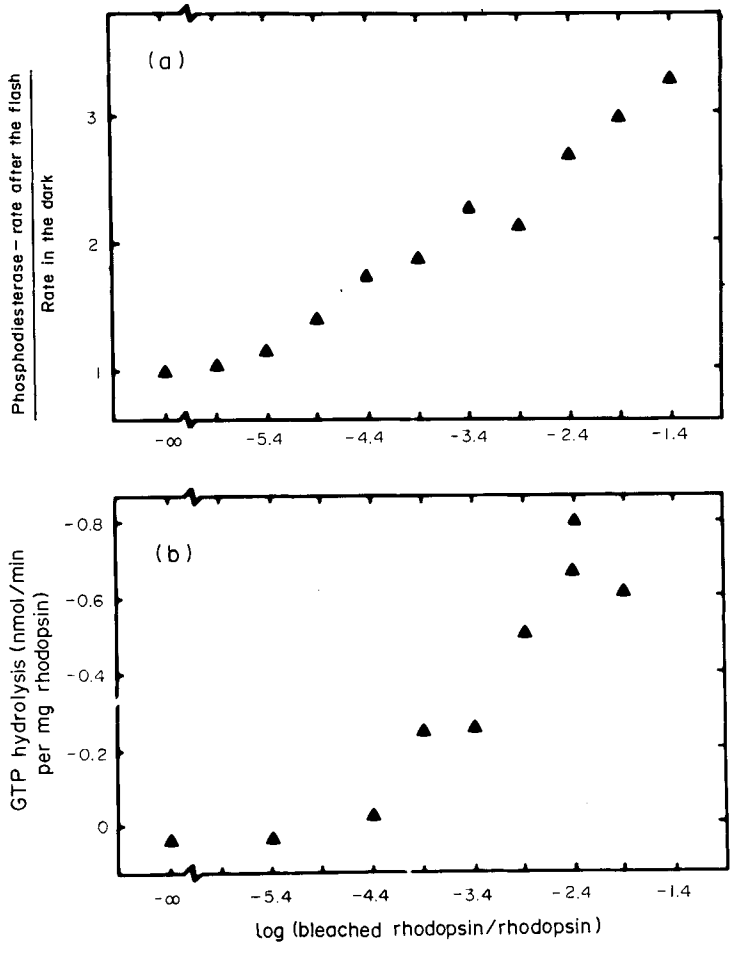

Fig 2. Effect vs. intensity relationship. A. cG-PdE. 0.4x 10^6 broken ROS; 1 mM cGMP, 0.5 mM GTP. B. GTPase. 0.4x10^6 broken ROS; 1 μM GTP. (from Caretta and others, 1979a).

excitation, mainly on the basis of kinetic data. (Bitensky and others, 1978). With these new kinetic informations, however, we could not reject any more the idea that the light-induced modulation of these two enzymes intervenes early in the process of photoreception.

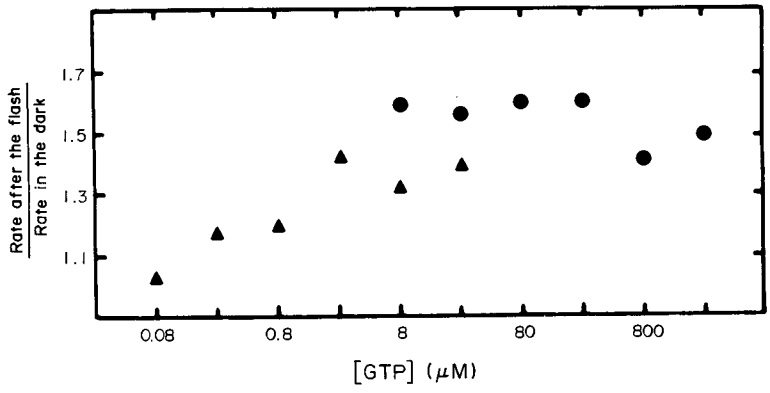

Fig. 3. Light-effect on cG-PdE vs. GTP concentration. 0.4×10^6 broken ROS; 0.5 mM cGMP; flashes bleaching 0.014 % of the rhodopsin; the tests on two preparations are shown with different symbols.

The relationship between the size of the activation peak and the intensity of the stimulus was also investigated. Since in our experimental set-up it was not possible to stimulate the broken ROS uniformly, because they were embedded in a small opaque cellulose column, only the average bleaching was estimated. The size of the effect of light on the two enzymes was determined by the ratio of the hydrolysis in the first sample after the flash to the hydrolysis in the dark, with different light intensities. The light intensity that gave the half-maximal effect was about the same for both enzymes, one bleached rhodopsin over 2,500, and the lowest intensity sufficient to sizeably activate the cG-PdE was one bleached rhodopsin over 2.5×10^6, that is 10^2 times less than necessary to activate the GTPase, Fig. 2. We do not know whether this difference is due to a true different sensitivity of the two enzymes, or to an apparent difference due to a slower time-course of activation for the GTPase at low light intensities, or, finally, to a different sensitivity of the assays for the hydrolysis products.

Following a peak of activation after the flash, the two activities diminished with time, but, in our experimental conditions, they never reached the previous dark value. Moreover, since a second flash bleaching 4% of the rhodopsin, presented to the ROS up to 2 min after the first one of the same intensity, did not reactivate the enzymes, this inactivation does not correspond to a recovery of the sensitivity.

Previously, the light-activation of the cG-PdE has been reported to depend on the presence of mM concentrations of ATP (Miki and others, 1973). Surprisingly, in our conditions, the cG-PdE was activated by flashes bleaching 10% of the rhodopsin also without ATP (Bignetti and others, 1978). Later on it was possible to prove that the effect of the light on the cG-PdE was dependent on μM concentrations of GTP in the perfusing medium, Fig. 3. In fact, without GTP the light-activation was

slower and 10^4 times more intense lights were needed to elicit an effect, Fig. 4. GMP-

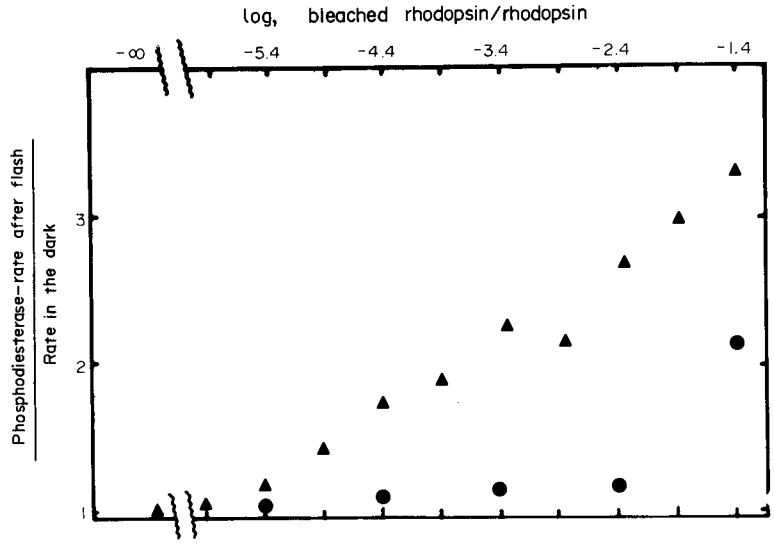

Fig. 4. Effect vs. intensity relationship in cG-PdE. y-axis: rate of hydrolysis measured at the 5th (▲) or at the 16th sample (●) divided by the rate before the flash; 0.4×10^6 broken ROS. Triangles: 1 mM cGMP, 0.5 mM GTP. Points: 1 mM purified cGMP, no GTP. (from Caretta and others, 1979a).

PNP, a GTP analogue that is reported to be resistant to the GTPase hydrolysis, was a good substitute for GTP. Similar conclusions were obtained in overseas laboratories and the apparent discrepancies were thus resolved (Bitensky and others, 1978; Wheeler and Bitensky, 1977; Yee and Liebman, 1978).

When the broken ROS were washed with low ionic solutions, most of the cG-PdE activity was found in the supernatant, but we have not been able to detect any GTPase activity in the ROS membranes or in the supernatant, suggesting that the washed fraction was necessary to the GTPase activity. Other authors have been able to solubilize this enzyme with different methods, but the biochemical definition of the GTPase is still an open problem (Godchaux and Zimmerman, 1979; Somers and Shichi, 1979; Wheeler and others, 1977).

Effect of cGMP on the Ionic Permeability of the Disc Membranes.

The physiological relevance of the activation of the cG-PdE by light rests on two assumptions: I- that the intracellular cGMP concentration is modified shortly after the illumination, II- that the intracellular cGMP modifies the membrane permeability. The first assumption has been elegantly proved and the second was first suggested by the data on the swelling of the isolated rods in Bownds laboratory (Brodie and Bownds, 1976; Woodruff and others, 1977).

To test more directly the effect of cGMP on the membrane permeability, we added cGMP to the liquid perfusing the broken ROS that had been previously loaded with ^{22}Na

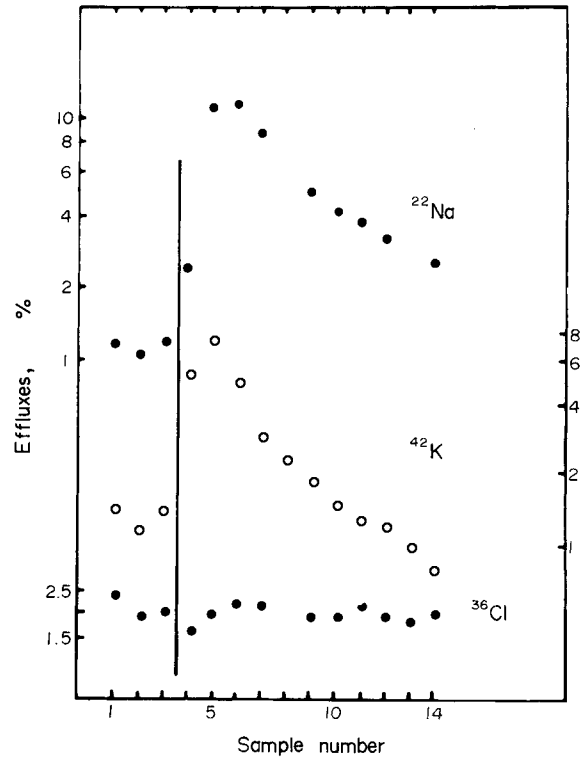

Fig. 5. Effect of cGMP on ^{22}Na, ^{42}K and ^{36}Cl effluxes. 1mM cGMP and 0.1 mM GTP were introduced with the test perfusion (vertical bar); 3 perfusions with the same preparation; one sample every 17 s. (from Caretta and others, 1979b).

(Caretta and others, 1979b). It was seen that cGMP increases the sodium efflux, Fig. 5. The efflux of potassium was also increased by the addition of cGMP, whereas that of chlorine was not affected. Although at 0.1 - 1 mM cGMP concentrations there was no difference between the effect on sodium and on rubidium (a cheaper substitute for potassium) efflux, at lower concentration cGMP still increased the sodium, but not the rubidium efflux, Fig. 6. Since broken ROS, incubated with cGMP, also loaded more ^{22}Na than those incubated without cGMP, the effect on the effluxes was due to changes of permeability and not to other properties of the ROS. cAMP was able to increase the sodium efflux to some extent, even though less than cGMP, whereas guanosine and GMP were ineffective, Fig. 7. Guanosine and GMP are respectively more and less hydrophobic than cGMP, a fact that excludes the polarity of the molecule as the cause of the effect. GTP was ineffective in eliciting permeability changes in dark-adapted, washed broken ROS.

The effect of cGMP on the membrane permeability was independent on the ionic strength of the perfusing medium. Partially depleting the membranes of cG-PdE by

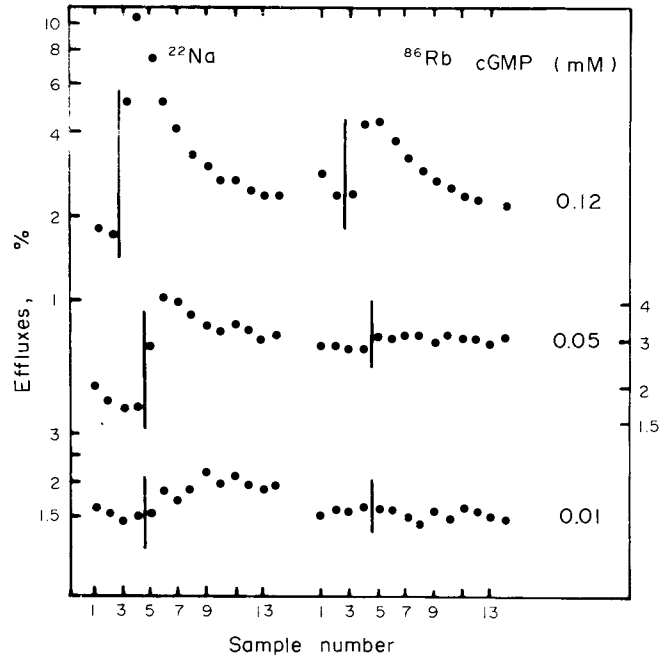

Fig. 6. Effect of different concentrations of cGMP on ^{22}Na (on the right) and on ^{86}Rb effluxes (on the left). From above: 0.12, 0.05 and 0.01 mM cGMP; the two upper traces are two perfusions with the same preparation and the four lower traces are with another preparation; one sample every 12 s. (from Caretta and others, 1979b).

washing with low ionic solutions was not harmful and the experiments with low cGMP concentrations were made with membranes partially depleted of cG-PdE. Neither the presence or the absence of the calcium ions in the perfusing medium, nor the previous bleaching of the broken ROS did influence the effect of cGMP on the sodium efflux.

These experiments demonstrate that, at physiological levels (μM), cGMP increases the permeability of the disc membranes and suggest a similar effect also on the cell membranes. Experiments with intracellular injection of cGMP in isolated photoreceptors have so far given disappointingly inconsistent results in our hands (in collaboration with L. Cervetto and M. Capovilla), whereas other laboratories report results that seem consistent with this effect (Miller and Nicol, 1979; Waloga and Brown, 1979).

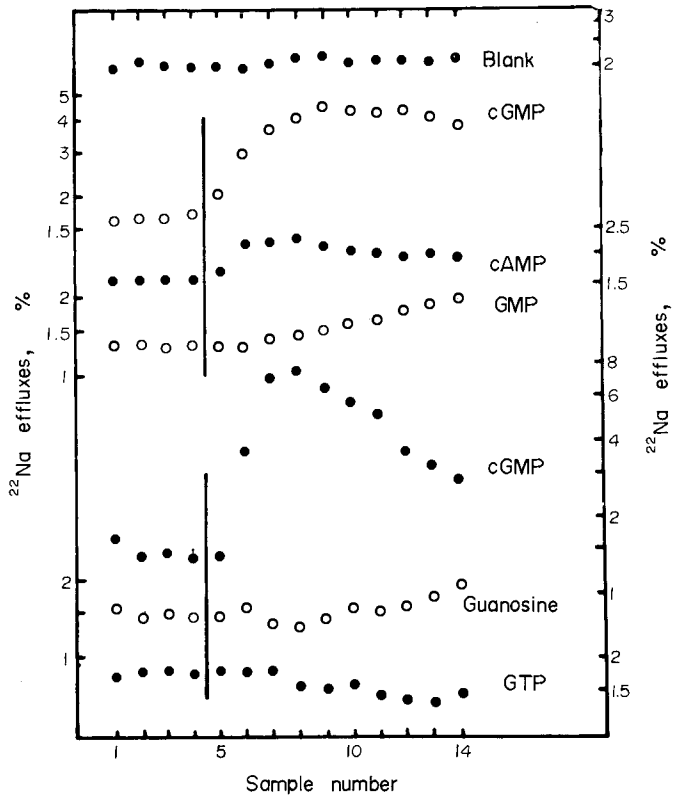

Fig. 7. Nucleotide specificity of the effect. From above: control without nucleotide, 1 mM cGMP, 1 mM cAMP, 1 mM GMP -same preparation; 1 mM cGMP, 1 mM guanosine, 1 mM GTP -another preparation; one sample every 15 s. (from Caretta and others, 1979b).

CONCLUSIONS

There is now evidence that the cGMP metabolism is involved in the early steps of photoreceptor transduction and it appears fascinating that the same type of regulation operated by proteic hormones on a variety of cells (Greengard, 1976; Nathanson, 1977) subserves an exteroceptor function in the photoreceptor cells (Bignetti and others, 1978; Bitensky and others, 1978; Yee and Liebman, 1978; Hubbell and Bownds, 1979). The precise physiological role of cGMP in the photoreceptors, however, is still undefined. Photoreceptor light adaptation, as first proposed by Bitensky and collaborators (1975, 1978), is a possibility to be considered. The biochemical process underlying this property is probably fast and the recent kinetic data on the cG-PdE do not exclude this possibility. Another hypothesis is that the excitation itself is mediated by the changes of the intracellular level of cGMP (Caretta

and others, 1979a; Hubbell and Bownds, 1979; Yee and Liebman, 1978). Electrophysiological recordings after intracellular injection of cGMP or during poisoning of the cG-PdE, presently at the beginning, will undoubtly help to solve this problem (Lipton and others, 1977).

On the basis of the information now available on the membrane permeability, we can suggest that in the dark-adapted conditions the cGMP concentration in the ROS (Woodruff and others, 1977) is sufficient to maintain the Na permeability of the cells membrane high (Caretta and others, 1979b). When the light is absorbed by the photoreceptors, the bleached rhodopsin activates the cG-PdE and the GTPase. Since, in the presence of excess GTP, the rate of hydrolysis of cG-PdE is high at physiological substrate concentrations (Caretta and others, 1979a; de Azeredo and others, 1978), the intracellular cGMP level decreases very quickly (Woodruff and Bownds, 1979) and, therefore, the Na permeability of the plasma membrane decreases. The cG-PdE and the GTPase form probably a functional complex (Bignetti and others, 1978) and, since the cG-PdE needs GTP to be light-activated and the K_m for the GTPase as well as the GTP concentration that is required for the half-maximal activation of the cG-PdE are of similar magnitude (μM), it is possible that the catalytic and the regulatory sites for GTP are the same. (Bitensky and others, 1978). If this is true, the GTPase, once activated by light, would hydrolyze the GTP bound and deactivate the cG-PdE.

However, there are some pieces of evidences that do not fit in this picture and others that are still missing. For instance, it is not clear how the cGMP influences the membrane permeability. Recently it has been advanced that light causes some low molecular protein to dephosphorylate (Hubbell and Bownds, 1979) and that a soluble cGMP-dependent protein kinase is present in the ROS (Farber and others, 1979; Lolley and others, 1977). These findings may be interpreted in the way that cGMP influences the membrane permeability through a cGMP-dependent phosphorylation of a low molecular protein responsible for the sodium permeability (Greengard, 1976; Hubbell and Bownds, 1979). A puzzling aspect is that the cGMP effect on the permeability has been seen in ROS fragments without cytoplasm, and not even the peripheral proteins seem to be necessary, since the effect is also present in broken ROS washed with low ionic solutions. Moreover, since the addition of GTP is not needed, it is possible to rule out a phosphorylation process, although tightly bound GTP may still be present. The target molecule of cGMP on the ROS membranes has yet to be found. Preliminary experiments in our laboratory tend to exclude the membrane lipids and bring into cause the proteins.

There are other two pieces of evidence that have yet to be fitted in the above mentioned picture, i.e., the role of the calcium ions and that of the light-induced phosphorylation of rhodopsin. The many data on the fluxes of calcium in the ROS (e.g., Gold and Korenbrot, 1979; Szutz and Cone, 1977; Szutz, 1979) are not univocally interpretable on the basis of the Hagins hypothesis (1972). However, it has been shown that calcium concentration influences the cGMP level in the retina (Cohen and colleagues, 1978) and in the isolated ROS (Woodruff and Bownds, 1979) and that the addition of calcium mimics the effect of light in dephosphorylating some low molecular proteins (Hubbell and Bownds, 1979). In contrast, our experiments in the broken ROS seem to be completely calcium-independent. It is also rather puzzling that opsin-kinase, soluble in the dark-adapted conditions (Miller and Paulsen, 1975; Weller and others, 1975), becomes firmly bound to the membrane upon illumination and that, later on in the dark, the capacity of the bleached membranes to bind the kinase decreases in parallel with the light-induced phosphorylating activity (Kühn, 1978).

Obviously this biochemical complexity reflects the physiological complexity of the photoreceptor function. Our data favour the simple idea that cGMP is an intracel-

lular modulator of the photoreceptor permeability.

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Neurochemistry Vol. 1, pp. 311-326.
Pergamon Press Ltd. 1980. Printed in Great Britain.

LOCALIZATION AND ROLES OF CYCLIC NUCLEOTIDE SYSTEMS IN RETINA

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ABSTRACT

The distribution of cyclic GMP, cyclic AMP and related enzymes in the vertebrate retina, together with factors regulating their levels, are described. Photoreceptor cells in retinas from all species examined contain very high levels of cyclic GMP and high activities of both guanylate cyclase and cyclic GMP phosphodiesterase. In more proximal regions of the retina, cyclic GMP is found at concentrations similar to that of brain. Guanylate kinase and GDP kinase, enzymes involved in GMP metabolism, also have increased activities in photoreceptor cell layers although their pattern of distribution does not exactly parallel that of cyclic GMP. The concentration of cyclic AMP is fairly uniform throughout the retina and at a level similar to that found in other areas of the CNS. However adenylate cyclase has an uneven distribution with particularly high activity in the inner plexiform layer. Cyclic nucleotide levels in retina may be modified by several factors. Light decreases both cyclic nucleotides in rod-dominant retinas, although we have not observed similar changes in cone-dominant retinas. Anoxia or ischemia elevates cyclic AMP and decreases cyclic GMP, similar to other areas of CNS, while incubation of retina in Ca^{++}- free media markedly increases cyclic GMP levels, an effect opposite that seen in brain tissue. Depolarization of retina with high K^{+} causes a modest elevation of cyclic AMP but has no effect on cyclic GMP, which is also significantly different from the response in brain. Cyclic AMP levels in retina however, can be elevated by dopamine which is an effect similar to that in striatum. These data indicate that there are probably multiple cyclic GMP and cyclic AMP systems in retina, some of which may be unique to this tissue.

KEY WORDS

Retina, cyclic GMP, cyclic AMP, guanylate cyclase, adenylate cyclase, cyclic GMP phosphodiesterase,cyclic nucleotide regulation.

INTRODUCTION

It is now well established that adenosine 3',5'-monophosphate (cyclic AMP) and

guanosine 3',5'-monophosphate (cyclic GMP) exist in almost all biological systems. It appears that each of the cyclic nucleotides may have several roles in tissues and perhaps even in individual cell types. The purpose of this review is to explore the idea that retina has multiple cyclic nucleotide systems, some of which are similar to those in other tissues and some which may be unique. Studies of the distribution of cyclic AMP, cyclic GMP and enzymes involved in their formation and degradation in retinas from various animals are described and factors that are known to regulate retinal cyclic nucleotide levels are discussed. In addition, possible roles of cyclic nucleotides in retinal function are considered.

DISTRIBUTION OF CYCLIC NUCLEOTIDE SYSTEMS IN RETINA

Cyclic GMP and Cyclic AMP Levels

Similar to essentially all other animal tissues, retinas from all species examined so far contain both cyclic AMP and cyclic GMP. In whole retina the average concentration of cyclic AMP is near 1-2 uM, a level found in most other areas of the CNS. In contrast, the average concentration of cyclic GMP in retina is approximately 5 uM, 1-2 orders of magnitude higher than in other regions of the CNS and substantially greater than the level of cyclic AMP (Goridis *et al.*, 1974: Ferrendelli and Cohen, 1976; Goridis *et al.*, 1977; De Vries *et al.*, 1978).

TABLE 1 Cyclic AMP, Cyclic GMP and Protein Content of Normal and Biologically Fractionated Mouse Retina

	Protein ug/retina	Cyclic AMP pmoles/retina	Cyclic GMP pmoles/retina
Normal	320 ± 4	2.5 ± 0.1	11.5 ± 0.2
Dystrophic (rdle/rdle)	110 ± 1	1.6 ± 0.1	0.17 ± 0.01
Glutamate Treated	210 ± 5	1.5 ± 0.1	9.4 ± 0.3

Values are the mean ± SEM respectively of 27 - 101 light adapted retinas.

Examination of rod outer segment preparations reveals very high levels of endogenous cyclic GMP (Fletcher and Chader, 1976; Krishna *et al.*, 1976), implying that perhaps much of this cyclic nucleotide is in the outer segment of photoreceptor cells. In support of this idea is the finding that in retinas devoid of photoreceptor cells from mice with an inherited retinal degenerative disease, levels of cyclic GMP are only 1-2% of that found in normal mouse retina (De Vries *et al.*, 1978, and Table 1). Cyclic AMP in the dystrophic retina, on the other hand, is 50-60% of the total cyclic AMP content of normal retina. In retinas from mice treated postnatally with glutamate, which causes the inner portions of the retina to degenerate while the more distal regions, including the photoreceptor cells, remain virtually intact, the levels of cyclic AMP are 50-60% of normal retinal values, whereas cyclic GMP levels are 60-85% of those found in control mice (Table 1). These data further indicate that in mouse retina most of the cyclic GMP is located in the photoreceptor cell, while cyclic AMP is more evenly distributed throughout the retina. Cyclic GMP and cyclic AMP levels also have been measured in individual layers of freeze dried retinas from rabbit (Orr *et al.*, 1976), ground squirrel (De Vries *et al.*, 1979b), and frog (de Azeredo, *et al.*, 1979). In both rabbit and ground squirrel, the levels of cyclic GMP are

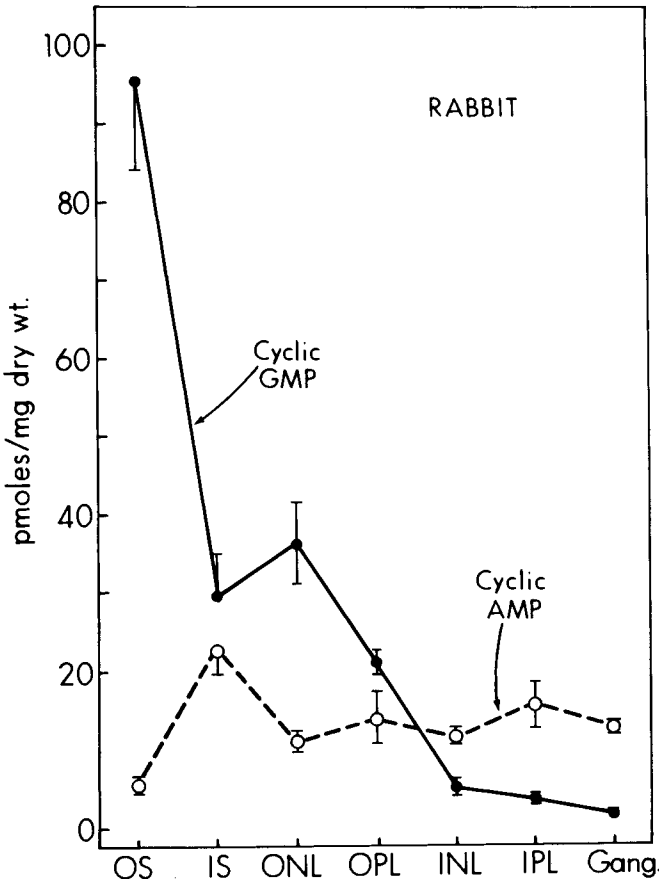


Fig. 1 Concentration of cyclic AMP and cyclic GMP in individual layers of freeze-dried retina from light-adapted rabbits. Abbreviations: OS - outer segments; IS - inner segments; ONL - outer nuclear layer; OPL - outer plexiform layer; INL - inner nuclear layer; IPL - inner plexiform layer; Gang - ganglion cell layer. Each point and vertical bar represents the mean value ± SEM, respectively, of 3-4 eyes.

highest in retinal layers containing photoreceptor cells where they are at concentrations 10-25 times that found in the inner retinal layers. Furthermore, outer segments appear to have two to three times or more cyclic GMP than other portions of photoreceptor cells (Figs. 1, 2). The concentration of cyclic AMP is more uniform throughout both retinas, with the lowest levels found in the outer segment layers. Similar distributions of cyclic GMP and cyclic AMP occur in frog retina (de Azeredo *et al.*, 1979). It is apparent therefore, that, in vertebrate photoreceptors cyclic GMP levels are very high and, unlike any other known cell, it is the predominant cyclic nucleotide. In contrast, in inner retina where cyclic GMP

levels are much lower, cyclic AMP predominates, a situation similar to other regions of the CNS.

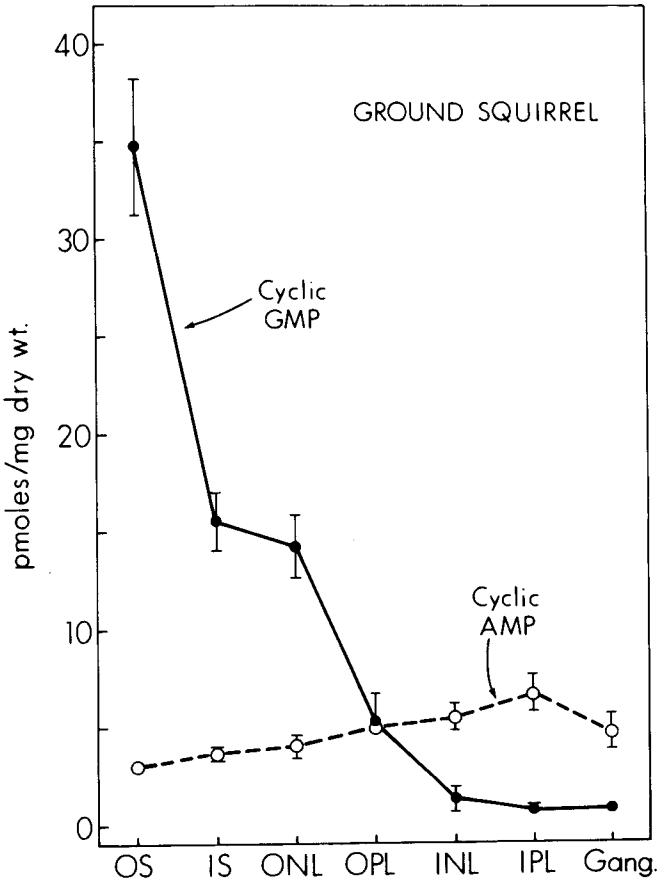


Fig. 2: Concentration of cyclic AMP and cyclic GMP in individual layers of freeze-dried retina from light-adapted ground squirrels. Abbreviations are the same as in Fig. 1. Each point and vertical bar represents the mean value ± SEM respectively, of 4 eyes.

Guanylate and Adenylate Cyclases

Early studies indicated that guanylate cyclase activity is very high in isolated rod outer segments from various species, and, in fact, is higher than that seen in whole retina homogenates and much higher than that in brain (Goridis *et al.*, 1973; Pannbacker, 1973; Bensinger *et al.*, 1974; Pannbacker, 1974). Enzyme activity in rod outer segments has been reported by several laboratories to range from 1000-8000 pmoles cyclic GMP formed/mg protein/min, while that in brain is only 10-120 pmoles cyclic GMP formed/mg prot/min. In an attempt to localize the enzyme, guanylate cyclase activity was measured in various subcellular fractions

of whole retina homogenates and the overwhelming majority of guanylate cyclase activity is associated with the outer segment fractions (Virmaux *et al.*, 1976; Zimmerman *et al.*, 1976). This distribution of enzyme activity has been confirmed in rabbit and ground squirrel retinas where guanylate cyclase activity has been measured in various layers throughout the retina (De Vries *et al.*, 1979a). Activity in the outer segment layer is about 1200-1400 pmoles cyclic GMP formed/ mg dry wt/min, which is 10-50 times that found in any other layer (Fig. 3).

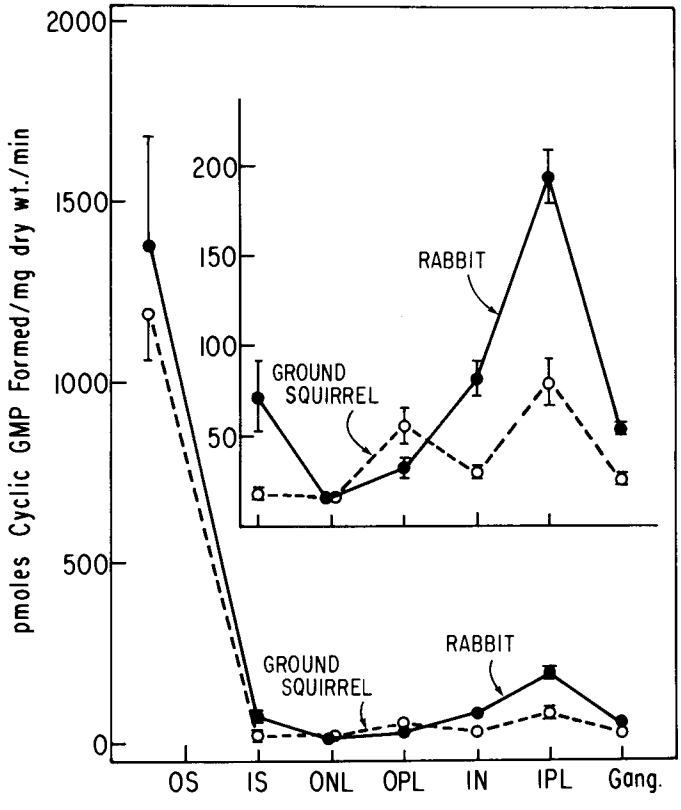


Fig. 3: Guanylate cyclase activity in individual layers of freeze-dried retina from light-adapted rabbits and ground squirrels. Abbreviations are the same as in Fig. 1. The insert is an expanded scale (5x) of activities in inner segments through ganglion cells. Each point and vertical bar represents the mean value ± SEM, respectively, of 3-4 eyes.

Both soluble and particulate guanylate cyclases have been found in a variety of tissues, including retina. Earlier studies of guanylate cyclase activities in retinal homogenates from normal and dystrophic mice suggested that at least two classes of enzymes might exist, one of which is preferentially associated with photoreceptor cells (Farber and Lolley, 1976). Later investigations indicated

that there may be at least three guanylate cyclases in retina (Troyer *et al.*, 1978). There is a soluble enzyme similar to that found in brain which has a Km-GTP of 70 uM, which is stimulated by Ca^{++} in the presence of low Mn^{++}, and which is not influenced by Triton X-100, NaN_3 or NH_2OH. In addition, there are two particulate enzymes -- one which is similar to the particulate enzyme found in brain, and one that has a greater affinity for GTP, a very high Vmax and is inhibited by Ca^{2+}. This latter enzyme seems to be unique and confined to photoreceptor cells; the other particulate enzyme and the soluble enzyme seem to be localized primarily to the inner retina.

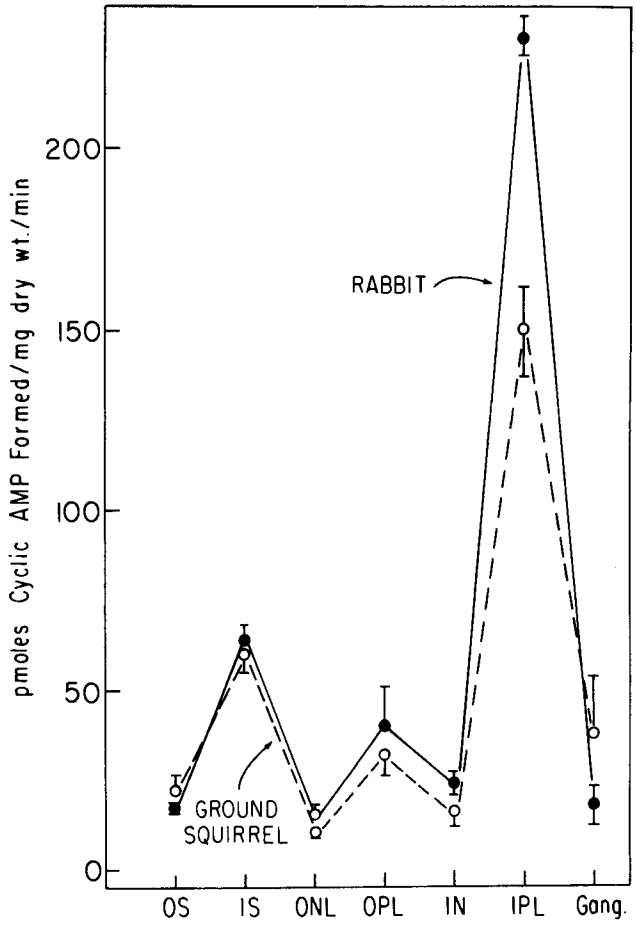


Fig. 4: Adenylate cyclase activity in individual layers of freeze-dried retina from light-adapted rabbits and ground squirrels. Abbreviations are as in Fig. 1. Each point and vertical bar represents the mean value ± SEM, respectively, of 2-3 eyes.

One of the earliest observations to stir interest in the role of cyclic nucelotides in retinal physiology was the report of very high adenylate cyclase activity in

rod outer segment preparations (Bitensky *et al.*, 1971; Bitensky *et al.*, 1972). Later reports, however, were unable to confirm these observations (Pannbacker, 1973; Hendriks *et al.*, 1973, Manthorpe and McConnell, 1974). However, several laboratories have found adneylate cyclase activity in retinal tissue from several animal species. In addition, a dopamine-sensitive adenylate cyclase in retinal homogenates has been reported (Brown and Makman, 1972;Bucher and Schorderet, 1975), with increases in enzyme activity ranging from 40-50% in mouse and rabbit to 6- to 10-fold in monkey. In order to localize enzyme activity, adenylate cyclase has been measured in two strains of dystrophic ("rodless") mice (Makman *et al.*, 1975). The specific activity of adenylate cyclase is higher in the mutant mice than in controls. Furthermore, in rats in which neonatal glutamate treatment selectively destroyed the inner retina, there is a decrease in basal adenylate cyclase activity together with a proportionate reduction in dopamine stimulated activity. Similar observations have been made in the RCS rat where degeneration of photoreceptor cells parallel an increase in adenylate cyclase specific activity (Farber and Lolly, 1977). Furthermore, direct measurement of enzyme activity in microdissected mouse retina reveals much higher adenylate cyclase activity in the

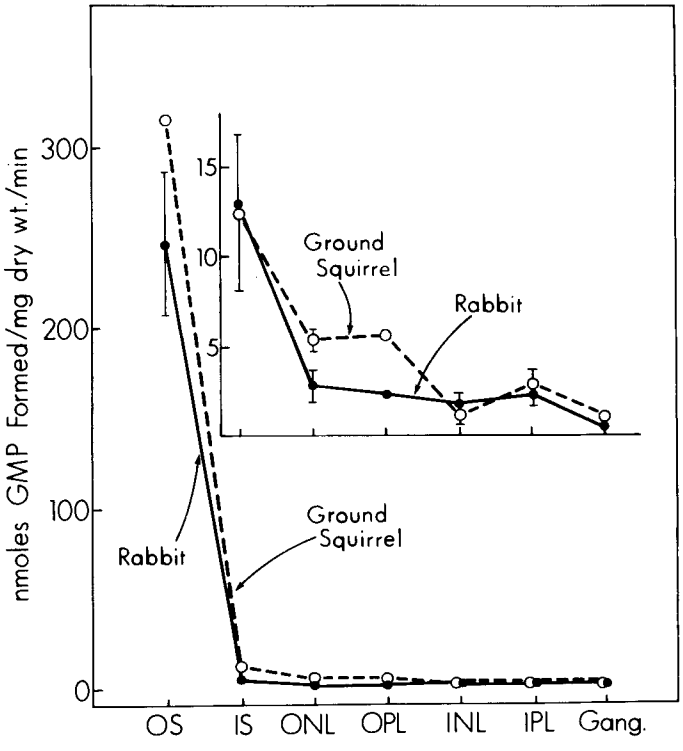


Fig. 5: Cyclic GMP-phosphodiesterase activity in individual layers of freeze-dried retina from light-adapted rabbits and ground squirrels. Abbreviations are the same as in Fig. 1. The insert is an expanded scale (10X) of activities in inner segment through ganglion cells. Each point and vertical bar is the mean value ± SEM, respectively, of 2-3 eyes.

bipolar-plus-ganglion cell layer than in the photoreceptor layer (1141 pmoles cyclic AMP formed/mg prot/min vs 35 pmoles/mg prot/min, respectively) (Lolly *et al.*, 1974). The region with the greatest adenylate cyclase activity seems to be the inner plexiform layer. In both ground squirrel and rabbit, enzyme activity in this region is 3- to 4-times as much as that in other retinal layers (Fig. 4). These data demonstrate that most of the adenylate cyclase activity is associated with the inner portions of the retina where hormonal regulation of enzyme activity is more likely to be found.

Cyclic Nucleotide Phosphodiesterase (PDE)

A high level of cyclic nucleotide phosphodiesterase activity has been observed in whole retina and in rod outer segment preparations (2-6 times that reported for brain). The enzyme associated with rod outer segments hydrolyzes both cyclic GMP and cyclic AMP, but it has a greater affinity for cyclic GMP (Pannbacker *et al.*, 1972; Goridis and Virmaux, 1974). It has been demonstrated that light activates this enzyme and that the light-activated PDE is probably the major regulator of cyclic GMP concentrations in photoreceptor outer segments (Miki *et al.*, 1973; Chader *et al.*, 1974a; Goridis and Virmaux, 1974; Manthorpe and McConnell, 1975). The cyclic nucleotide phosphodiesterase from frog rod outer segments has been purified and shown to have an approximate molecular weight of 240,000 and K_m values for cyclic GMP and cyclic AMP of 70 uM and 3 mM respectively (Miki *et al.*, 1975). In order to clarify the distribution of enzyme activity throughout the retina, cyclic nucleotide phosphodiesterase has been measured in various subcellular fractions of bovine retinal homogenates (Chader *et al.*, 1974b). In whole retina homogenates, cyclic GMP-PDE activity is 3 times that of cyclic AMP-PDE. After fractionation, the specific activity found in rod outer segments is significantly higher for both enzyme activities, while that in the nuclear, mitochondrial and microsomal fractions does not change. Cyclic GMP-PDE activity has been measured in various layers of freeze-dried rabbit and ground squirrel. (Fig. 5). Activity in the outer segment layer ranges from 148-318 nmoles GMP formed/mg dry wt/min, which is 15-300 times that found in any other layer. Cyclic AMP phosphodiesterase has also been measured in freeze-dried retinal layers. This enzyme activity in outer segments is much less than that of cyclic GMP PDE (i.e., approx. 10 nmoles AMP formed/mg dry wt/min) but still 5-10 times that of any other layer (Carter *et al.*, 1979).

Kinetic studies of cyclic GMP-PDE activity in mouse retinal homogenates indicates the presence of two classes of enzyme activity with apparent K_m-cyclic GMP values of 30 and 300 uM (Farber and Lolly, 1976). In microdissected freeze-dried rat retina, a cyclic GMP-PDE with a high K_m has been localized in the photoreceptor cell layer, while an enzyme activity with a greater affinity for cyclic GMP has been demonstrated in the bipolar-plus-ganglion cell layer (Lolly and Farber, 1975).

Other Nucleotides and Enzymes

The levels of GTP and ATP have been measured in various retinal layers. The ratio of GTP to ATP in photoreceptor cell layers is 1:1. This situation appears to be unique to photoreceptor cells, since there is no other tissue in which the levels of guanine nucleotides approaches the concentration of adenine nucleotides. In the inner retinal layers, the ratio of guanine to adenine nucleotides is only about 1:4, which is similar to that in other regions of the CNS.

The primary mechanism for the conversion of GMP to GDP is the phosphorylation of guanosine monophosphate, using ATP as the phosphate donor, in the presence of guanylate kinase. In rabbit and ground squirrel retina, the activity of this

enzyme in the inner retina is comparable to that seen in various regions of brain (Yang and Lowry, unpublished), while very high activities are found in the photoreceptor cell layers (peak activities of 46-76 nmoles GDP formed/mg dry wt/min). In the photoreceptor cell the activity in outer segments is only 5-10% of that in other portions of the cell. The distribution throughout the rest of the photoreceptor cell is characteristic for an enzyme present in the cytosol and excluded from mitochondria and nuclei. The level of activity and distribution of adenylate kinase in the retina has not been reported.

The conversion of 5'-diphosphate nucleotides to 5'-triphosphate nucleotides is catalyzed by a class of relatively non-specific enzymes, the "nucleotide diphosphokinases." This is a ubiquitous class of enzymes whose activities have been observed to be similar in most tissues studied. GDP-kinase activity in the inner retinal layers is approximately equal to that seen in various regions of mouse brain. The levels of activity in the photoreceptor cell layers are much higher (peak activities of 158-301 nmoles GTP formed/mg dry wt/min). Within the photoreceptor cell layers, however, the lowest GDP kinase activity is found in the outer segment layers. Since this enzyme is found primarily in the cytosol, the high membrane content and small cytoplasmic volume of this layer may contribute to its low specific activity.

It is apparent that the distribution of activities of guanylate kinase and GDP-kinase in the retina does not parallel that of guanylate cyclase and cyclic GMP phosphodiesterase. In fact, the levels of the former enzymes follow more closely the levels of GTP than those of cyclic GMP. This may indicate that the synthesis and degradation of cyclic GMP occurs in the outer segments, while the conversion of GMP back to GTP takes place primarily in the inner portions of the cell. This would require a "shuttle" of intermediates between the inner and outer segments of the photoreceptor. It is also possible, however, that the "cyclic GMP cycle" takes place entirely within the outer segments, and that the high levels of GTP and increased activities of guanylate kinase and GDP-kinase in the photoreceptor cell may be related to other guanine nucleotide pathways. Cyclic AMP metabolism in the retina and, in particular, in the photoreceptor cell is less well understood at this time.

REGULATION OF CYCLIC NUCLEOTIDE LEVELS IN RETINA

Effects of Light and Darkness

Some of the earliest studies of cyclic nucleotides in retina revealed that cyclic GMP levels were influenced by light and darkness. Initially it was reported that exposure of dark-adapted, isolated retinas to light produces a rapid fall in their cyclic GMP levels (Goridis *et al.*, 1974). Subsequently, it was observed that the content of cyclic GMP in isolated rod outer segments could also be reduced by illumination (Fletcher and Chader, 1976; Woodruff *et al.*, 1977). Measurements of cyclic nucleotides in individual layers of rabbit retina have demonstrated that the changes in cyclic GMP levels after long (1 hour) light- or dark-adaptation occur in all regions of photoreceptor cells, including outer segment, but not in the inner retina (Orr *et al.*,1976). A most intriguing observation has been reported by de Azeredo *et al.* ((1979). These investigators found that exposure of dark-adapted frogs to 2 min of room light not only decreases photoreceptor cyclic GMP levels but also markedly increases its levels in the outer plexiform layer and in the outer portion of the inner nuclear layer. The levels of cyclic GMP in these areas then returns to normal after a longer (1 hour) exposure to light.

Light and darkness have been reported to influence cyclic GMP levels in rod-dominant retinas from mouse (De Vries *et al.*, 1978), frog (Goridis *et al.*, 1974), rabbit (Orr *et al.*, 1976) and cow (Goridis *et al.*, 1974). In contrast, cyclic GMP levels seem to be unaffected by light and darkness in cone-dominant retinas from ground squirrel (Farber and Lolley, 1978; De Vries *et al.*, 1979b) and *Anolis* (De Vries, unpublished). At present it is unclear whether this difference is due to fundamental properties of cones and rods, species differences or some yet unresolved technical problems associated with examination of cone-dominant retinas.

Cyclic AMP levels in retina are also altered by light- and dark-adaptation. We observed that light depresses and dark elevates the levels of this nucleotide in intact mouse (Ferrendelli and Cohen, 1976; De Vries *et al.*, 1978) and rabbit retina (Orr *et al.*, 1976). In rabbit the change in cyclic AMP levels seems to occur primarily in the outer plexiform layer. Similar to cyclic GMP, cyclic AMP levels seem to be unaffected by light and darkness in the cone-dominant retinas from ground squirrel (De Vries, *et al.*, 1979b) and *Anolis* (De Vries, unpublished).

Anoxia and Ischemia

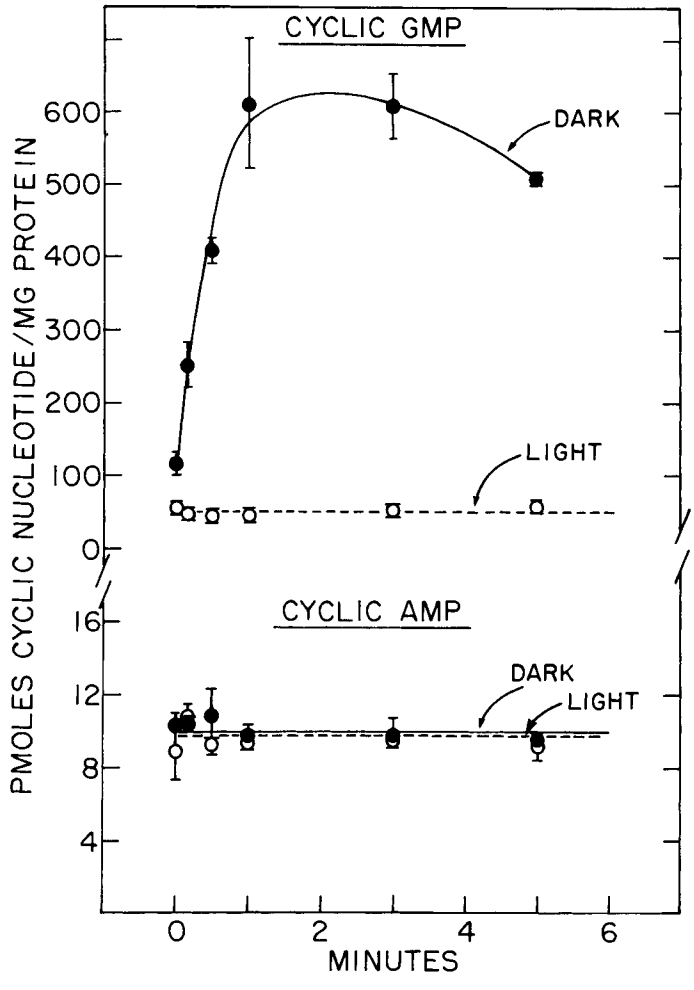
As in other regions of the CNS anoxia or ischemia has been reported to elevate cyclic AMP levels and decrease cyclic GMP levels in mouse (Mitzel *et al.*, 1978) and rabbit retina (Orr *et al.*,1976). Quantitative histochemical studies indicate that the effect of ischemia on cyclic GMP levels occurs only in layers of retina containing photoreceptor cells, whereas cyclic AMP changes occur predominantly in inner portions of the retina. Ischemia has a much more pronounced effect on both cyclic AMP and cyclic GMP levels in dark-adapted tissue.

Effect of Calcium

The fact that isolated retina maintained, in vitro, possesses many of the characteristics of retina in situ has allowed study of this tissue when its environment is modified in known ways. Using mouse retina incubated in oxygenated physiologic buffers we studied the effect of Ca^{2+} concentration on cyclic nucleotide regulation in this tissue (Cohen *et al.*, 1978). Exposure of dark-adapted retinas to Ca^{2+}-free media in darkness causes a 5 to 6-fold increase in cyclic GMP levels. This effect is not observed in light adapted retinas (Fig. 6). This rise occurs rapidly, reaching its peak in 3 min, and then cyclic GMP levels slowly fall toward normal with continued incubation. Light still causes a rapid decrease of cyclic GMP levels of retinas incubated in Ca^{2+}-free media. Suggestive that the increase in cyclic GMP levels induced by Ca^{2+}-lack occurs in photoreceptor cells is the finding that little or no effect is seen in dystrophic, rodless retinas. Ca^{2+}-lack seems to have no effect on cyclic AMP levels in retina.

These data suggest that calcium has a strong regulatory influence on cyclic GMP levels in dark-adapted photoreceptors but not in light-adapted retina nor in the process responsible for the light-induced decrease in cyclic GMP levels. In addition, the observations described above demonstrate that regulation of cyclic GMP in rod photoreceptors is much different than that in brain. In contrast to the findings in retina, brain tissue incubated in Ca^{2+}-free media has reduced levels of cyclic GMP, and most substances that elevate cyclic GMP levels in incubated brain slices have no effect in the absence of calcium (Ferrendelli *et al.*, 1976). Thus, it may be concluded that the cyclic GMP system in photoreceptor cells is substantially different than that in other nervous tissue.

Fig. 6: Effect of calcium-deprivation on cyclic AMP and cyclic GMP levels in isolated-incubated mouse retina. Retinas from light-adapted or dark-adapted mice were removed and incubated in oxygenated physiologic buffer for 30 min at 37° in the light or dark respectively. Following this they were placed in buffer containing no added calcium and 0.5 mM EGTA (0 time on the graph) and incubation was continued in the light or dark. Samples were removed at variuos times thereafter and assayed for cyclic AMP and cyclic GMP. Each point and vertical bar represents the mean value ± SEM of 4 retinas.



Cellular Depolarization

Cellular depolarization has a marked effect on both cyclic AMP and cyclic GMP levels in brain tissue. For example, seizures, which are excessive paroxysmal depolarization of neurons, increase levels of both cyclic nucleotides 3- to 5-fold in brain, *in vivo*, and agents that cause cellular depolarization such as

veratridine, ouabain, excitatory amino acids (glutamate and aspartate) and high concentrations of K^+ increase levels of cyclic AMP and cyclic GMP 10- to 100-fold in brain slices (Ferrendelli, 1976; Ferrendelli *et al.*, 1979). In intact bovine retina both ouabain and high K^+ have been shown to cause a two-fold elevation in cyclic AMP content (Brown and Makman, 1972). We examined the influence of depolarizing agents on cyclic nucleotide levels in incubated mouse retina. In intact normal retina, high concentrations of K^+ increase cyclic AMP levels 2-fold in light-adapted retinas but have no effect on cyclic GMP levels; potassium depolarization has no effect on either cyclic nucleotide in dark-adapted retinas (Ferrendelli, unpublished). Thus, cellular depolarization has an effect in retina significantly different than that in brain tissue, again indicating that cyclic nucleotide systems in retina are not identical to those in brain.

Effect of Neurotransmitters

The relationship between neurotransmitters and cyclic nucleotides is better understood and has been studied more extensively in brain than in retina. There have been no reports of neurotransmitters influencing cyclic GMP levels in retina, to our knowledge. However, a dopamine-sensitive adenylate cyclase (see above) has been identified in retinas from several animal species. In addition, dopamine has been reported to cause an accumulation of cyclic AMP in retinal tissue (Makman *et al.*, 1975; Schorderet, 1978; De Mello, 1978). Similar elevations have been reported in the presence of both epinephrine and norepinephrine (Makman *et al.*, 1975; Schorderet, 1978). Preliminary studies in our laboratory suggest that norepinephrine and adenosine can also elevate cyclic AMP levels in mouse retinas (Ferrendelli, unpublished). At the present time there are not sufficient data to make any definite conclusions about neurotransmitter-cyclic nucleotide interactions in retina other than stating that there is a dopamine-regulated cyclic AMP system. Obviously this area of retinal research deserves much additional study in the future.

CYCLIC NUCLEOTIDES IN RETINAL FUNCTION

The roles of cyclic nucleotides in retinal physiology are poorly understood. The cyclic GMP system in the outer segment of the photoreceptor cell is, clearly, the best defined, but even here its exact function has not been established. It is generally held that the rod outer segment photoresponse (i.e. a plasma membrane hyperpolarization associated with a decrease in Na^+ conductance) is mediated by an internal transmitter. Cyclic GMP has been suggested as a candidate for such a transmitter. Application of cyclic GMP, or agents which have been shown to increase intracellular levels of cyclic GMP, leads to a depolarization of the rod membrane and an increase in the amplitude of the light response (Lipton *et al.*, 1977; Miller and Nicol, 1979). Since cyclic nucleotides, through the phosphorylation and dephosphorylation of membrane proteins, have been shown to control membrane permeability in other systems, it is conceivable that cyclic GMP may be serving a similar function in rod outer segments. The presence of a cyclic nucleotide dependent protein kinase and the phosphorylation of a single endogenous protein have been demonstrated in rod outer segment preparations (Lolly *et al.*, 1977; Farber *et al.*, 1979). Whether these changes in membrane permeability are related to visual transduction or light-dark adaptation are not known. Cyclic GMP phosphodiesterase from rod disc membranes has been shown to have very high specific activity and sufficient gain and speed of activation to significantly alter cyclic GMP levels in the time required to be involved in the visual transduction mechanism (Yee and Liebman, 1978, Liebman and Pugh, 1979). Furthermore, light has been shown to cause a rapid decrease in endogenous cyclic

GMP levels of rod outer segment preparations which is also sufficient in amplitude and time course to be consistent with a role in the transduction process (Woodruff *et al.*, 1977, Woodruff and Bounds, 1979). Although it has also been suggested that cyclic GMP may have a role in adaptation, no supportive evidence has been presented to date. In fact, superfusing retinas with cyclic GMP leads to no measured alteration in receptor sensitivity (Lipton *et al.*, 1977).

Alteration of photoreceptor cyclic GMP metabolism have been implicated in pathophysiologic processes associated with hereditary degenerative disease of retina in a variety of species (Farber and Lolley, 1974; Lolley *et al.*, 1974; Lolley and Farber, 1975; Aguirre *et al.*, 1978). However, further studies will be necessary to ascertain the exact relationship between cyclic GMP and these degenerative diseases.

The role(s) of cyclic GMP in the inner retina are very poorly understood at present. The enzymes that form and degrade cyclic GMP in this region seem to be distinctly different than those in photoreceptors and cyclic GMP levels are also regulated differently. Therefore, it is highly probable that cyclic GMP has a separate function in inner retina from that in photoreceptors. The fact that a soluble and a particulate guanylate cyclase, each with distinct enzymatic properties, exist in inner retina further suggests that cyclic GMP may have more than one role in this region of the retina. Possibly, they are similar to those in other portions of the central nervous system.

Cyclic AMP may also have several roles in retina. The fact that dopamine increases cyclic AMP levels in intact retina and that a dopamine sensitive adenylate cyclase has been identified in retinal homogenates indicates a relationship between cyclic AMP and dopamine neurotransmission in retina probably similar to that in striatum and other areas of nervous tissue where dopaminergic neurons exist. The existence of cyclic AMP and activity of adenylate cyclase and cyclic AMP - phosphodiesterase in regions of retina not containing dopaminergic neurons suggest that this cyclic nucleotide has other functions. At present there is insufficient data to even guess about these other possible roles.

In conclusion, we wish to emphasize that there is now substantial, convincing data indicating the presence of several cyclic AMP and cyclic GMP systems in retina. Characterization of these systems and defining their involvement in retinal physiology and biochemistry are necessary prerequisites for understanding mechanisms responsible for normal and abnormal visual function.

ACKNOWLEDGMENTS

Studies from the authors' laboratories were supported in part, by USPHS Grants EY-02294, EY-00258, EY-00092, NS-09667, and NS-08862 and by American Cancer Society Grant BC-4S.

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Neurochemistry Vol. 1, pp. 327-336.
Pergamon Press Ltd. 1980. Printed in Great Britain.

CYCLIC NUCLEOTIDES IN ROD- AND CONE-DOMINANT RETINAS

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ABSTRACT

Rod-and cone-dominant retinas differ in their relative content of cyclic AMP and cyclic GMP. Cyclic GMP is concentrated in retinas dominated by rods and is responsive to light; cyclic AMP is enriched in those having a majority of cones. Light reduces by about 50% the content of cyclic AMP in cone-dominant retinas, but the levels of cyclic GMP are affected only minimally. Microdissection of rod-dominant retinas shows that most of the cyclic GMP is localized in photoreceptor cells whereas cyclic AMP is evenly distributed throughout the retina. In contrast, our studies of cyclic nucleotides and the morphological changes that occur in cone visual cells of the ground squirrel retina, during hibernation and iodoacetate-induced cone degeneration, suggest that cyclic AMP is localized in cone visual cells. By analogy with the rod system, cyclic AMP may modulate the intracellular metabolism of cones.

KEYWORDS

Cyclic nucleotides; cyclic GMP; cyclic AMP; cone visual cells; rod visual cells; photoreceptors; ground squirrels; hibernation; iodoacetate treatment.

INTRODUCTION

The last few years have witnessed accelerated discoveries in the field of vision. For almost a century, attention has been focused on the study of rhodopsin and its spectral changes upon bleaching. However, with rapid advances in methodology and the availability of supersensitive techniques for assaying biological compounds, new avenues of research have been opened that allow investigation of other components of visual cell metabolism. For example, an interest in cyclic nucleotides has been stimulated by the finding that cyclic AMP and cyclic GMP can modulate cellular function in several tissues. Now, it is suggested that cyclic nucleotides are involved in the regulation of the metabolism or function of visual cells.

Cyclic nucleotides are unevenly distributed within the cellular layers of the retina. Generally, the lowest concentration both of cyclic AMP and cyclic GMP are found in the pigment epithelium whereas the highest content is found in the visual cells. While distribution of cyclic nucleotides within the retina is consistent,

the relative concentration of cyclic AMP and cyclic GMP in the visual cells can vary. For example, rod-dominant retinas contain more cyclic GMP than cyclic AMP whereas the reverse is true of cone-dominant retinas. Perhaps this is not surprising since rod and cone visual cells have diverged significantly during the evolutionary process.

Rod and cone photoreceptors have distinct morphological and physiological characteristics. Rod visual cells contain the visual pigment, rhodopsin, in their outer segments; morphologically, rod outer segments are composed of disk-shaped sacks that are completely separated from the plasma membrane of the outer segment. Several types of cone visual cells have been identified with each having a distinctive visual pigment that facilitates the discrimination of color; cone visual pigments are found within the layered membranes of outer segments that are often continuous with the plasma membrane of the outer segment.

Cone and rod visual cells differ also in the process of outer segment renewal. Rod photoreceptors constantly synthesize and renew the disks of their outer segments. The disks are assembled at the base of the outer segment by a process that includes the invagination of the plasma membrane which encloses the disk stack (Nilsson, 1964; Sjöstrand, 1961). Then, the disks are progressively displaced from the outer segment base. Disks arriving at the top of the outer segment are shed in packets which are phagocytized and digested by the pigment epithelium (Young, 1971; Young and Bok, 1969).

Cone photoreceptors renew their outer segment membranes also by synthesizing protein which, after assembly into the cone membranes, becomes distributed diffusely throughout the outer segment (Young and Droz, 1968). They shed outer segment membranes in a manner similar to that of rod outer segments, but they differ in the time of the day when shedding occurs. Cones respond to high intensity light: they function during the day and shed the tips of their outer segments at dusk (Young, 1978). Rod visual cells are sensitive to low levels of illumination. They function at night and shed the tips of their outer segments at dawn (Young, 1978).

Man and most mammals have duplex retinas with a variable number of rods and cones. However, some species of animals have retinas dominated either by rods or cones. Rats, mice, rabbits, frogs and toads have rod-dominant retinas, and tree shrews, birds, lizards and ground squirrels have retinas with a majority of cones. The advantage of studying retinas that contain mostly the same kind of photoreceptors is the homogeneity of the visual cell response. Uniformity of sample also allows the characterization of the biochemistry and function of rods or cones in relative isolation.

For clarity, we will consider, first, the cyclic nucleotide content and light-sensitivity of rod-dominant retinas. Secondly, we will compare cyclic nucleotide levels and their modulation by light in cone-dominant retinas. And, finally, we will present data obtained from retinas subjected to natural or experimental conditions which affect the cone photoreceptors.

CYCLIC NUCLEOTIDES OF ROD-DOMINANT RETINAS

Rod-dominant retinas contain high levels of cyclic GMP and levels severalfold lower of cyclic AMP (Table 1). During the differentiation and development of rod-dominant retinas, the levels of cyclic GMP rise as the outer segments of rod visual cells grow and mature, indicating a concentration of cyclic GMP in rod photoreceptors (Farber and Lolley, 1977). Work with dystrophic animals, namely rd (retinal degeneration) mice and Irish setter dogs (described in chapters by Drs. Lolley and Farber and by Dr. Chader and co-workers) confirmed that cyclic GMP

was localized in rod visual cells. Moreover, microdissection studies indicated that 90% of the total cyclic GMP in rod-dominant retinas is found in the photoreceptor cells, with a great enrichment in rod outer segments. The remaining cyclic GMP (10%) is distributed across the layers of the inner retina (Orr and co-workers, 1976).

TABLE 1 Cyclic Nucleotides in Dark-Adapted Cone- and Rod-Dominant Retinas

	cAMP	cGMP	cAMP/cGMP
	(pmol/mg protein)		
Cone-Retina			
Ground squirrel	89.5 ± 5.9	11.1 ± 0.7	8.0
Western fence lizard	21.6 ± 1.6	9.6 ± 1.0	2.2
Rod-retina			
Mouse	18.1 ± 0.2	63.6 ± 0.8	0.3
Rat	10.0 ± 0.3	58.5 ± 0.7	0.2
Toad	6.5 ± 0.6	29.5 ± 1.3	0.2

The concentration of cyclic nucleotides was measured by radioimmunoassay. Values represent the mean ± SE.

In contrast to cyclic GMP, the cyclic AMP content is quite uniform throughout the layers of the rod-dominant retinas, with the lowest levels found in the outer segments, (Orr and co-workers, 1976). In rod outer segments, the concentration of cyclic AMP is only 1/5 to 1/25 the content of cyclic GMP.

Light reduces the content of cyclic GMP in dark-adapted, rod-dominant retinas, *in vitro*, or in isolated rod outer segments (Farber and Lolley, 1977; Goridis and co-workers, 1974; Fletcher and Chader, 1976; DeVries and co-workers, 1978). According to Orr and co-workers (1976), the reduction of cyclic GMP by light is observed throughout the photoreceptor cell, but it does not occur in the inner retinal layers. By contrast, the already low levels of cyclic AMP are reduced by light in the outer plexiform layer, primarily, with small changes occurring in the inner retinal layers. Cyclic AMP of the outer segments is not altered by light.

The concentration of cyclic GMP in rod photoreceptors is greater by about 300-fold than that found in brain. The modulation of cyclic GMP levels by light implies a specific role for cyclic GMP in the function or metabolism of rod visual cells. The importance of this specialization may be dramatized by the enzymatic defect in cyclic nucleotide metabolism which causes photoreceptors of *rd* mice and affected Irish setter dogs to degenerate (see chapters by Drs. Lolley and Farber and by Dr. Chader and co-workers).

Cyclic GMP might be involved in the visual process directly (Farber, Brown and Lolley, 1978) or in the phenomenon of light/dark adaptation. If cyclic GMP is a component of the phototransduction process, its modulation by light should be very rapid. The light-induced hydrolysis of cyclic GMP must be at least as rapid as the light-induced hyperpolarization of the rod visual cell. Woodruff and co-workers (1977) have shown, using isolated outer segments from frog retina, that a decrease in cyclic GMP content becomes larger, as the intensity of illumination increases, and it varies with the logarithm of light intensity at levels which bleach between 5×10^2 and 5×10^5 molecules of rhodopsin per outer segment per second. They calculated that bleaching one molecule of rhodopsin can lead to the hydrolysis of 1000-2000 molecules of cyclic GMP within 100-300 milliseconds. Thus, cyclic GMP meets some of the criteria for an internal transmitter which links photon absorption in disk membranes to decreases of sodium permeability in the outer segment plasma membrane.

CYCLIC NUCLEOTIDES OF CONE-DOMINANT RETINAS

Cone visual cells have received relatively little attention, despite the fact that they play a major role in human vision. Some of this deficiency could be justified on the basis that animals possessing a cone-dominant retina are more trouble to gather and maintain than laboratory rodents which have almost pure rod retinas.

We have studied the cone-dominant retina of the 13-line ground squirrel (Citellus tridecemlineatus) and that of the Western fence lizard (Sceloporus occidentalis).

Until not too long ago, the 13-line ground squirrel was assumed to have a pure cone retina and to display only those visual capacities traditionally associated with photopic vision. The following criteria were consistent with this conclusion: anatomical observations (Vaidya, 1964; Dowling, 1964; Hollenberg and Bernstein, 1966), physiological data (Tansley, Copenhaver and Gunkel, 1961; Dowling, 1964), photopigment studies (Dowling, 1964) and behavioral patterns (Jacobs and Yolton, 1971). Recently, with the use of more sophisticated techniques, a very small percentage of rod-like cells has been detected in the 13-line ground squirrel retina (West and Dowling, 1975; Green and Dowling, 1975; Anderson and Fisher, 1976). Still, about 96% of the photoreceptors of the 13-line ground squirrel retina are cones. This makes the retina of this ground squirrel a particularly useful tool for studying cone biochemistry. The retina of the Western fence lizard also contains a preponderence (80-90%) of cones.

The content of cyclic AMP in the retina of the 13-line ground squirrel is very high and greater by about 8-fold than that of cyclic GMP (Table 1). Its cyclic AMP/cyclic GMP ratio is 35-40 times higher than that of rod-dominant retinas. The Western fence lizard also has higher levels of cyclic AMP than cyclic GMP, with a cyclic AMP/cyclic GMP ratio of 2.2. These ratios are in contrast to those of rod-dominant retinas which are less than one.

Light appears to affect cyclic AMP metabolism in the cone-dominant retina whereas it alters that of cyclic GMP in rod-dominant retinas. For example, the content of cyclic AMP in dark-adapted ground squirrel retina is reduced approximately 55% by exposure to light, whereas cyclic GMP levels are unaffected (Table 2). Similar results are observed with the Western fence lizard (Farber and co-workers, 1979).

TABLE 2 Cyclic Nucleotide Content of Dark-and Light-Adapted Ground Squirrel Retinas and Eyes

	Cyclic AMP	Cyclic GMP
Retina (pmol/mg protein)		
Dark (n = 16)	89.5 ± 5.9	11.1 ± 0.7
Light (n = 18)	40.0 ± 2.3	9.8 ± 0.8
% reduction by light	55.3 ($p < 0.001$)	11.7 (n.s.)
Eye (pmol/eye)		
Dark (n = 7)	354.3 ± 14.6	35.5 ± 2.8
Light (n = 5)	166.7 ± 12.5	32.3 ± 2.0
% reduction by light	53.0 ($p < 0.001$)	11.5 (n.s.)

Values represent the mean ± SE.

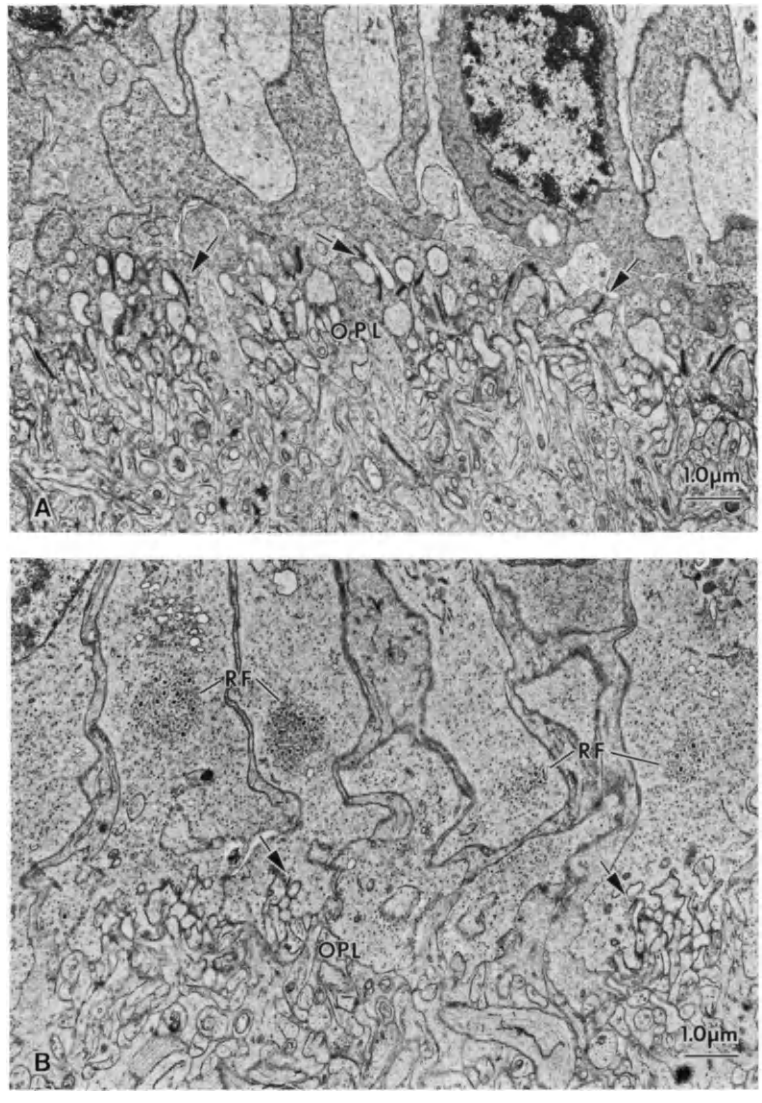

Fig. 1. Electron micrographs of the synaptic terminal of cones in an active (A) and hibernating (B) ground squirrel (x9,000). Characteristic synaptic terminals with large synaptic ribbons (arrows) are shown in (A). In (B), only a few attenuated ribbons are present in the outer plexiform layer (OPL). Ribbon fields (RF) which are aggregations of ribbon material and synaptic vesicles are displaced from the presynaptic membrane.

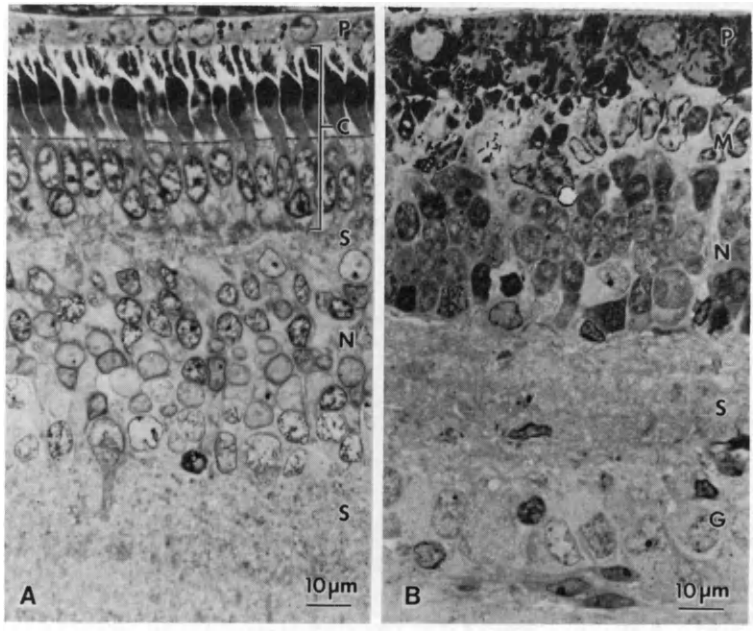

Fig. 2. Light micrographs of 13-line ground squirrel (Citellus tridecemlineatus) retinas fixed in glutaraldehyde-osmium and embedded in epoxy resin (x750). (A) Control retina showing an area extending from just above the pigment epithelium to the inner synaptic layer. (B) The full thickness of a retina fixed eleven days after an intracardiac injection of iodoacetic acid. Abbreviations: P, pigment epithelium; C, cone cell layer; S, outer (above) and inner (below); G, ganglion cell layer; M, an irregular layer of macrophages.

Retinal pigment epithelium of dark-adapted ground squirrels contains also a considerable concentration of cyclic AMP (24.3 pmol/mg protein) which is reduced by 35% after exposure to light. Moreover, the levels of cyclic GMP in the pigment epithelium are minimal and do not change during illumination. We believe that the cyclic AMP found in the pigment epithelium is contributed mainly by detached outer segments of the cones that remain with the pigment epithelium during dissection of the retina. The presence of cone outer segments in isolated pigment epithelium populations has been confirmed by electron microscopy.

The anatomy of the ground squirrel eye is different from that of rodents because the optic nerve head is large and triangular in shape. During dissection, the retina must be detached from the pigment epithelium and from the perimeter of the head of the optic nerve. In order to increase the speed of dissection and reduce the trauma imposed on the retina, we measured the cyclic nucleotide content from the intact eyes of dark- and light-adapted animals. Light reduces the cyclic AMP content of the dark-adapted eye by 53%, whereas the levels of cyclic GMP are not significantly affected (Table 2). These results are similar to those observed with isolated retina.

Freezing of the ground squirrel retina or whole eye modifies the content of cyclic AMP by a process which is unknown. It reduces the levels of cyclic AMP in the dark- or light-adapted tissues by about 60%, whereas the levels of cyclic GMP are minimally affected. This phenomenon has a bearing on the apparent distribution of cyclic AMP (and cyclic GMP to a lesser extent) in ground squirrel retinas which are prepared for quantitative histochemistry. In our microdissection studies using light-adapted retinas, the levels of cyclic AMP are similar in the photoreceptor and inner layers of the retina. In this specific instance, microdissection procedures may give misleading results.

CYCLIC NUCLEOTIDES OF HIBERNATING AND DEGENERATIVE CONE-DOMINANT RETINAS

An important tool in the work with rod photoreceptors has been the availability of animal models possessing inherited diseases which destroy the visual cells of the retina. By measuring the enzymes or substrates of metabolism before and after death of the rods, we have been able to localize cyclic nucleotide metabolism in the photoreceptor layer of the rod-dominant retina. Following a similar line of investigation, we sought comparable conditions for the ground squirrel retina. Partial or total loss of cones is found naturally in the hibernating ground squirrels, as has been described by Reme and Young (1977) and by Kuwabara (1975). As a second option, we destroyed the cone photoreceptors with sodium iodoacetate.

Upon entering or emerging from hibernation, cones undergo major changes in morphology. For example, cone outer segments become shorter (Reme and Young, 1977) or eventually disappear (Kuwabara, 1975), and the diameter of the disks decreases. The mitochondria of the ellipsoid region is reduced in number and length, and the number of synaptic vesicles and synaptic ribbons is decreased. The synaptic ribbons tend to be displaced from their normal position along the presynaptic membrane and are found in "ribbon fields" which are aggregates of short, randomly arranged segments of synaptic ribbons and synaptic vesicles (Fig. 1). We found that changes in synaptic ribbon morphology are specific to visual cells because synaptic ribbons of the bipolar terminals are unchanged during hibernation. After arousal, cones recover and restore their morphology within a few days.

During the first days of hibernation, cyclic AMP levels of the ground squirrel retina rise to values above those found in dark-adapted, non-hibernating animals. Then, as the photoreceptor cell undergoes the morphological changes associated with hibernation, cyclic AMP levels fall to about 45% of the dark-adapted value. Following one and up to three months in hibernation, retinal cyclic AMP content is

stabilized at a value (42.38 ± 1.4 pmol/mg protein) which is similar to that of non-hibernating animals in the light. In contrast, cyclic GMP levels do not change during hibernation.

Within 3 days after arousal, cyclic AMP levels return to pre-hibernation values, coinciding with the re-forming of cone photoreceptors. Thus, hibernation alters both the morphology and the metabolism of cyclic GMP in cone photoreceptors, indicating that at least 50% of the cyclic AMP content of dark-adapted, non-hibernating retinas may be localized in the cone photoreceptor layer.

Intravenous injections of sodium iodoacetate produce a characteristic visual cell degeneration in several species of animals (Schubert and Bornschein, 1951; Noell, 1952). All other cells of the retina remain undamaged morphologically, unless high doses of this poison are used. We have successfully employed iodoacetate to destroy the photoreceptors of the ground squirrel retina.

Eleven days after a single intracardiac injection of iodoacetate, intact cone cells are replaced by a layer of macrophages containing large, irregular, dense material (Fig. 2). These masses are identified by electron microscopy as the partially degraded residue of photoreceptors. In sparce patches of the retina, cones, debris and macrophages are absent altogether, so that the pigment epithelium lies directly on the inner nuclear layer. The inner layers of the ground squirrel retina remain unchanged.

The level of cyclic AMP in the dark-adapted retina of the iodoacetate-treated ground squirrel is reduced by approximately 45%, whereas cyclic GMP levels are unchanged. The levels of cyclic AMP measured in the iodoacetate-treated retina (49.6 pmol/mg protein) are in the range of those observed with light-adapted control animals. We believe that this selective change in cyclic AMP content indicates that cyclic AMP is enriched in cone visual cells of the 13-line ground squirrel retina.

CONCLUDING REMARKS

Cyclic GMP appears to be involved in the metabolism or function of normal rod-dominant retinas. In degenerative disorders, an imbalance in cyclic GMP levels leads to photoreceptor death and blindness. Therefore, cyclic GMP is an important modulator of rod photoreceptors,possibly a component of the visual transduction mechanism.

Cyclic AMP is enriched in the photoreceptors of cone-dominant retinas. It is still unclear whether it is concentrated in the cone outer segments, cell soma or synaptic terminal of the visual cell. The fact that light reduces the levels of cyclic AMP in dark-adapted cone-dominant retinas suggests an analogy between the function of cyclic GMP in rod photoreceptors and of cyclic AMP in cone visual cells. It is possible that cyclic AMP may act during visual excitation to modulate the intracellular metabolism of retinal cones.

A great deal of work must still be done in order to identify a role for cyclic AMP in cone activities. Whereas some pieces of the puzzle are already interlocked, the picture is far from complete.

ACKNOWLEDGEMENT

We wish to recognize the excellent technical assistance of Dennis Souza, Suni Kloss and Elizabeth Racz and to thank Louise Eaton and Inga Anderson for their thoughtful

help in the preparation of this chapter. This work was supported by the National Eye Institute grant EY 2651 and RCDA 1 KO4 EY144 (to DBF) and by the Medical Research Service of the Veterans Administration.

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Neurochemistry Vol. 1, pp. 337-353.
Pergamon Press Ltd. 1980. Printed in Great Britain.

THE ISOLATED RETINA OF MAMMALS: A USEFUL PREPARATION FOR ENZYMATIC-(ADENYLYL CYCLASE) AND/OR BINDING STUDIES OF DOPAMINE RECEPTORS

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ABSTRACT

Rabbit and bovine retinae were used for investigations in vitro related to dopamine receptors. Dose-dependent stimulations of cyclic AMP formation induced by dopamine (or dopamine-like drugs including ADTN and some ergot alkaloid derivatives), which can be blocked by dopamine antagonists (neuroleptics), lithium or d-LSD, were found either in intact retinae or in homogenates. The neuroleptic sulpiride was the only exception since it was devoid of inhibitory effects.

Binding studies of retinal dopamine receptors were also achieved with homogenates. By using ^{3}H-spiroperidol as a ligand, saturability (in the nmolar range), stereospecificity (by using butaclamol- or thioxanthene-isomers), tissue linearity, and pH- or temperature-dependence were all demonstrated. Furthermore, displacement curves of ^{3}H-spiroperidol stereospecifically bound generated by various dopamine- and/or catecholamine-related drugs seem to indicate that dopamine receptors are the only monoamine receptors in this tissue and that a coupling possibly exists between binding- and catalytic-sites.

These results suggest that by studying both dopamine-sensitive adenylyl cyclase and drug-induced displacement of ^{3}H-spiroperidol binding the mammalian retina is appropriate to selectively characterize the type of action of various drugs with CNS dopamine receptors.

KEYWORDS

Retina dopamine receptors; adenylyl cyclase; binding studies; neuroleptics; ergot alkaloids.

INTRODUCTION

Since the discovery of striatal and retinal dopamine receptors linked to the enzyme adenylyl cyclase (Kebabian, Petzold and Greengard, 1972; Brown and Makman, 1972), measurements of cyclic AMP-induced by various agents in intact- and in broken-cell preparations of dopaminergic areas of the CNS (including retina)

were carried out in order to study the pharmacological characteristics of these receptors (Iversen, 1975). Some discrepancies, however, have been found between the results of such studies in vitro and the potency in vivo of various neuroleptics, whose therapeutic activity is supposed to be due to the blockade of CNS dopamine receptors (Bartholini, 1978). Moreover, some neuroleptics such as sulpiride and/or other benzamide derivatives, have been found to be devoid of antagonist activity upon the dopamine-sensitive adenylyl cyclase of various dopaminergic areas (Trabucchi and others, 1975; Magistretti and Schorderet, 1978a; Theodorou and others, 1979), implying that two types of dopamine receptors may exist, namely the D1-receptors (adenylyl-cyclase dependent) and the D2-receptors (adenylyl-cyclase independent) (Kebabian and Calne, 1979). A better correlation of data obtained in vitro with neuroleptic's potencies in vivo has been then found in classical binding studies using striatal homogenates exposed to radiolabelled dopamine-agonists or -antagonists (Burt and others, 1975; Seeman and others, 1975). However, the selectivity of such binding studies might be, to some extent, hampered, since the radioligand (and its displacing agents) can also interact with striatal serotonin receptors, whose blockade (by dopamine antagonists) appears to be unrelated to the therapeutic efficacy of neuroleptics (Crow, 1979). The aim of our present paper is therefore to describe several pharmacological characteristics of the retinal dopamine-sensitive adenylyl cyclase (in relation with some still debated effects of ergot alkaloids including d-LSD) and to compare those with recent data of binding studies performed with homogenates (Magistretti and Schorderet, 1978b, 1979a, b). Finally, attempts will be made in the Discussion to prove the relevance of the use of retina, compared to the availability of other brain structures, for investigating by two experimental approaches the selective affinity of various drugs for dopamine-receptors of D1-type.

MATERIALS AND METHODS

Adenylyl Cyclase Activity (endogenous cyclic AMP) in Intact Retina

The procedure for the dissection, pre-incubation and final incubation of isolated rabbit retina was essentially the same as described previously (Schorderet, 1977a) with two exceptions: 1) the concentration of theophylline was raised from 5 to 10 mM; 2) in some experiments, one half retina served as control- and the other half as stimulated-tissue (paired data) instead of half retinae prepared in a random way from a pool of 4 to 5 rabbits (unpaired data).

Dopamine or other drugs including d-LSD were added in appropriate dilutions just before starting the final 10 min incubation. Following the 10 min heat inactivation of tissue samples and their homogenization at ice temperature, cyclic AMP and proteins were measured as before (Schorderet, 1977a).

Adenylyl Cyclase Activity (Cyclic AMP formed from Exogenous ATP) in Retina Homogenates

The assay was modified from the previous one (Bucher and Schorderet, 1975) and essentially based on the procedure described by Kebabian, Petzold and Greengard, (1972), and Clement-Cormier and others, (1975) for striatal homogenates. Briefly summarized, the isolated retinae were homogenized in a glass-Teflon homogenizer-2 retinae per 0.5 ml (rabbit) or per 2.5 ml (calf) of 2 mM tris-(hydroxymethylaminomethane)-maleate buffer, pH 7.4 containing 2 mM EGTA. Without further centrifugation and/or washings, 50 (or 40) µl of tissue suspensions were added into assay tubes containing 400 (or 410) µl of a standard-assay mixture (Kebabian and Saavedra, 1976). The tested drugs were previously dissolved into the same mixture. The assay tubes were allowed to stay for 30 min at ice temperature. For the activation

of the enzyme, 50 µl of 15 mM ATP (i.e. final concentration 1.5 mM) were added and the assay tubes were incubated at 30° for 2.5 min in a shaking water-bath. Following a 3 min period of treatment on a boiling water-bath samples were centrifuged. Then, 50 µl supernatants (in duplicate) were used for cyclic AMP measurement (Brown and others, 1971) and the sediments dissolved in NaOH-SDS (Lees and Paxman, 1972) for protein measurement (Lowry and others, 1951).

Binding Studies: Tissue Preparation

Calf or rabbit eyes were collected within 5-10 min after death of the animals and immediately placed into ice. Dissection of retina was performed by gentle separation from sclera at 4°. Tissue suspensions were prepared as described for striatum (Creese, Burt and Snyder, 1975) and retina (Magistretti and Schorderet, 1979a, 1979b) with some minor modifications. Briefly summarized, homogenization was performed using a Polytron PT-10 homogenizer (Brinkman Instrument Co) at a setting of 6 (full scale = 10) for 30 sec, in 50 volumes (weight/volume) of ice-cold 50 mM Tris-HCl buffer (pH 7.7, at 25°). The homogenate was then centrifuged at 50'000 x g for 10 min on a Sorvall OTD-2 centrifuge. The supernatant was discarded, the pellet rehomogenized and recentrifuged under the same conditions. Finally the pellets were rehomogenized in 25 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.6, at 25°) containing 10 µM pargyline, 0.1% ascorbic acid and ions as follows: 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$ and 1mM $MgCl_2$. Except where indicated the homogenates were always kept on ice.

Binding assay

For standard binding assays, 450 µl aliquots of homogenate (15-20 mg original tissue weight) were added to test tubes containing 25 µl of ^{3}H-spiroperidol (final concentration ranging from 0.025 to 3.5 nM), and 25 µl of different drugs according to the type of experiment performed. All solutions were made up fresh in 0.1% of ascorbic acid and protected from light. Except for time course experiments, samples (at least in triplicate) were incubated for 15 min in a 37° water-bath under continuous agitation. At the end of the incubation, samples were diluted with 2 ml ice-cold 50 mM Tris-HCl buffer (pH 7.7 at 25°) to allow uniform distribution on filters, rapidly filtered under vacuum through Whatman GF/B glass fiber filters and washed twice with 5 ml of the same buffer. The whole procedure took uniformly less than 20 sec. Filters were allowed to dry under vacuum for 10 min, then placed into liquid scintillation vials. 10 ml of ice-cold toluene-POPOP-PPO were added, the samples were stored for 60 min at 4°, then counted for 5 min in a Beckman LS-355 liquid scintillation counter with an efficacy of 45-49% for ^{3}H. Stereospecific binding fo ^{3}H- spiroperidol was defined as the difference between the amount bound in presence of 1 µM l-butaclamol minus that bound in presence of 1 µM d-butaclamol. At this concentration the pharmacologically inactive isomer did not displace any appreciable amount of radiolabelled ligand. Under these conditions ^{3}H-spiroperidol stereospecifically bound represents 40-50% of total binding. Protein concentration was determined for each experiment (Lowry and others, 1951).

RESULTS

Characteristics of the Dopamine-induced Accumulation of Cyclic AMP in Isolated Rabbit Retina.

Dose-response curve. Fig. 1 shows that the concentration threshold of dopamine for

stimulating the cyclic AMP formation under the experimental conditions described above was situated at about 5 x 10^{-7} M. Lower concentrations of dopamine, such as 10^{-7} M (Fig. 1) were not sufficient to generate cyclic AMP. Half-maximal stimulation, on the other hand, was obtained in the presence of 10^{-6} M dopamine, whereas maximal effects appeared to be already elicited by 5 x 10^{-6} dopamine (Fig. 1). No significant differences in cyclic AMP levels were found whether the half-retinae were taken at random for either control- or stimulated-tissues or when, from the same retina, half was used for control- and the other half for stimulated-tissue (Table 1). Finally, cyclic AMP levels measured in retinae of pigmented rabbits appeared to be similar to those of albinos (not shown).

Time-course. Fig. 2 shows that in the standard experimental conditions a maximal effect of dopamine (at 10^{-5} M) upon cyclic AMP formation was reached after 10 min of incubation. Shorter periods of dopamine treatment, such as 1 or 5 min, led also to significant increases of cyclic AMP compared to control values. These increases, however, were smaller than those obtained after 10 min of stimulation. Cyclic AMP levels in control half-retinae could vary from one experiment to another with concentrations ranging from 10 to 30 pmoles . mg protein $^{-1}$. In the latter situation (high levels of cyclic AMP in control tissues) the extent of stimulation with maximal effective concentrations of dopamine (e.g. 10^{-5} M) was also more pronounced. Thus, the means of Δ increases over controls were comparable and ranged usually from 30 to 45 pmoles . mg protein^{-1} (Figs. 1-2).

Agonist- and/or antagonist-effects of CNS acting drugs upon cyclic AMP formation in intact rabbit retinae. In previous experiments (Schorderet, 1977a), it was found that several catecholamines, such as adrenaline and noradrenaline, as well as some 2-amino-5, 6-dihydroxytetrahydronaphtalene derivatives (5,6-aminotetralins) (Schorderet and McDermed, 1978) were able to generate cyclic AMP at 10^{-4} M, whereas the same agents were found to be inactive at 10^{-6} M concentration. At the latter concentration, dopamine, epinine, apomorphine and 6,7-aminotetralin (ADTN) as well as some ergot alkaloid derivatives (Schorderet, 1976, 1978a) were still active. The potency and the efficacy of apomorphine were found to be equal to those of dopamine, without displaying antagonist activity upon the dopamine-elicited accumulation of cyclic AMP when both drugs were simultaneously applied (not shown).

Agonist effects of dopamine (and of other dopamine-mimetic drugs cited above) can be blocked in a dose-dependent manner by neuroleptics, such as chlorpromazine, fluphenazine, haloperidol, d-butaclamol and cis-thioxanthenes (Bucher and Schorderet, 1974; Schorderet, 1977a; Magistretti and Schorderet, 1978a) by lithium (Schorderet, 1977b) but not by sulpiride (Magistretti and Schorderet, 1978b) or by α-and/or β-adrenergic receptor antagonists such as phentolamine and propanolol (Schorderet, 1977a).

Slight but significant increases of cyclic AMP were also found recently in response to 10^{-4} M of d-LSD (Table 2) in contrast to several other drugs such as isoproterenol , phenylephrine, amphetamine, piribedil, S-584, 3,4-dihydroxy-phenylamino-2-imidazoline (DPI), mescaline and morphine (Schorderet, 1975, 1977a, 1978b). All these drugs were unable to stimulate cyclic AMP formation in intact rabbit retina, even at the highest concentration tested (10^{-4} M). Furthermore, d-LSD was also found to have a partial antagonist activity as in the experiment shown in Table 2.

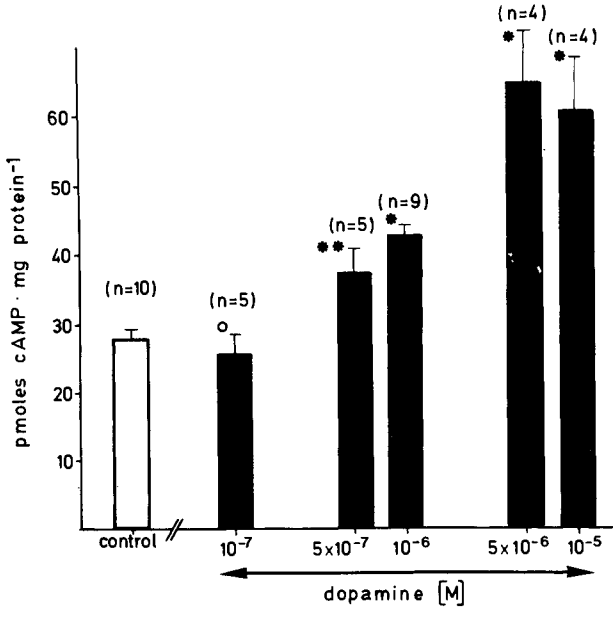

Fig. 1. Dopamine-dose response curve upon cyclic AMP formation in isolated rabbit retina. Control- and stimulated-tissues were incubated for 10 min in the presence of 10^{-2} M theophylline. Values were means ± S.E.M. **P< 0.01, *P< 0.001, Student 't' test for unpaired data.

Agonist- and/or antagonist-effects of CNS acting drugs upon cyclic AMP formation in homogenates of rabbit and/or calf retinae. Previous results (Bucher and Schorderet, 1975) were recently confirmed using homogenates of rabbit- and/or calf-retinae. Dopamine and 6,7-amino-tetralin (ADTN) were found to be the most potent agents in both species, epinine having not been tested yet under these experimental conditions. However, apomorphine appeared to be less active in broken cell- than in intact- preparations (see above). In line with that finding, ergot alkaloids such as ergometrine, bromocriptine, β-DH-ergocriptine, lisuride, etc., which have been shown previously to be very effective at 10^{-6} to 10^{-4} M concentrations to stimulate cyclic AMP production in intact rabbit retina (see above and Schorderet, 1976; 1978a) were also less potent in homogenates. Only at the highest concentration tested (i.e. 10^{-4} M) were they able to activate adenylyl cyclase (120% stimulation over control instead of 250% seen with dopamine). Serotonin was totally inactive.

On the other hand, the dopamine sensitive adenylyl cyclase of retina homogenates was blocked by chlorpromazine, fluphenazine and haloperidol, but not by sulpiride. These results were similar to those collected with intact retina (see above),

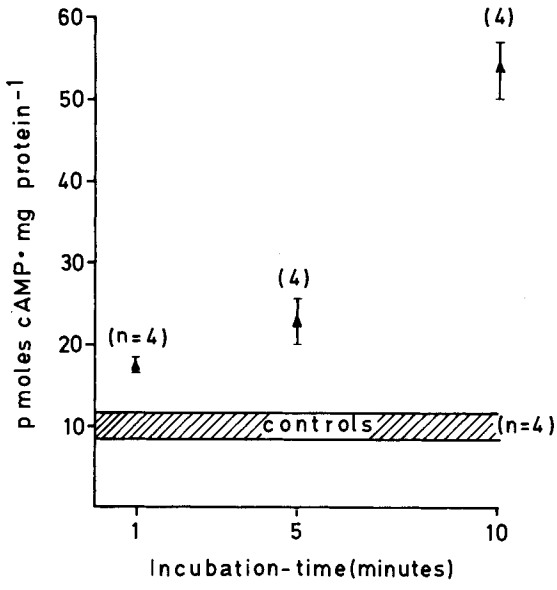

Fig. 2. Time-course of the dopamine-elicited formation of cyclic AMP in isolated rabbit retina. The concentration of dopamine (stimulated tissues) and of theophylline (controls and dopamine-stimulated tissues) were 10^{-5} and 10^{-2} M, respectively. Values were means ± S.E.M. $P<0.01$ (1-5 min), $P<0.001$ (10 min), Student 't' test for unpaired data.

except that the doses of inhibitors achieving a total inhibition of the dopamine effect (10^{-7} to 10^{-6}M) were smaller than those needed in intact tissue for the same purpose (10^{-5} to 10^{-4}M). However, apomorphine was able to display also antagonist properties, since it partially blocked (at 10^{-5} to 10^{-4} M) the effects of 10^{-5} M dopamine. The latter experiment was recently performed in calf retina homogenates in order to compare with the potency of apomorphine to induce a displacement of ^{3}H-spiroperidol binding in the same tissue (see further). Similarly, several ergot alkaloids including d-LSD were also able to antagonize the dopamine-stimulated formation of cyclic AMP in a dose-dependent manner. A detailed analysis of such inhibition is shown (for d-LSD effects) in Fig. 3. In this experiment, five different concentrations of d-LSD in the presence of a concentration of dopamine which was held constant (10^{-5} M) were applied. For comparison, two different concentrationsof fluphenazine were also tested during the same experiment. At lower concentrations of d-LSD, such as 10^{-8} and 10^{-7} M, the drug had no significant effect upon dopamine stimulation ($P> 0.2$, Student 't' test). In contrast, 10^{-7} M fluphenazine showed around 30% inhibition of dopamine effect with a highly significant value of P (<0.02). However, increasing d-LSD concentrations from 10^{-6} M up to 10^{-4} M led to significant inhibitions of dopamine stimulation (more than 80% in the presence

TABLE 1 Cyclic AMP Levels (pmoles · mg Protein^{-1}) in Dopamine-stimulated half Retinae versus Controls. Comparison between Paired- and Unpaired-tissues.

Paired half-retinae*			Unpaired half retinae**		
Control	dopamine 10^{-5} M	Δ increase	Control	dopamine 10^{-5} M	Δ increase
23.5	30.6	7.1	18.9	37.3	18.4
20.7	34.8	14.1	26.8	37.2	10.4
15.9	38.5	22.6	27.8	42.7	14.9
20.4	34.7	14.3	27.2	48.4	12.9
		Mean			
20.1	34.6	14.5	25.1	41.4	14.1

*Each line of values corresponds to a whole retina cut in two pieces, one half being used as control-, the other half as stimulated-tissue. Lines 1-2 correspond to the retinae of the first rabbit, lines3-4 to those of the second.
**Isolated retinae (from 2 rabbits) were cut in two pieces and used at random as control- or stimulated-tissues.

TABLE 2 Comparison of the Effects of Dopamine with those of d-LSD alone or with those of d-LSD in Combination with Dopamine upon Cyclic AMP Formation (pmoles · mg protein^{-1}) in isolated rabbit retina.

Controls		18.9 2.0 (n=5)	
drugs	M		
dopamine	10^{-6}	50.3 ± 3.4 (n=5)	P<0.001 v.controls
d-LSD	10^{-4}	27.2 ± 0.7 (n=5)	P<0.01 v.controls P<0.001 v.dopamine
dopamine	10^{-6}	35.5 ± 3.0 (n=5)	P<0.001 v.controls
plus d-LSD	10^{-5}		P<0.05 v.dopamine

*Five rabbits (i.e. 20 half-retinae) were used for that experiment. Ten half-retinae were for control- and d-LSD-stimulated-tissues, ten others for dopamine- and dopamine plus d-LSD-stimulated tissues (paired data).

of 10^{-4}M of d-LSD, Fig. 3). For comparison, the potency of fluphenazine was roughly 15 times higher since 5 x 10^{-7} M of this neuroleptic gave comparable inhibition than 10^{-5} M of d-LSD (Fig. 3). It is worthy to note that serotonin (up to 10^{-4} M) had no inhibitory activity upon the dopamine sensitive adenylyl cyclase.

Characterization of specific binding sites. In bovine retina, specific ^{3}H-

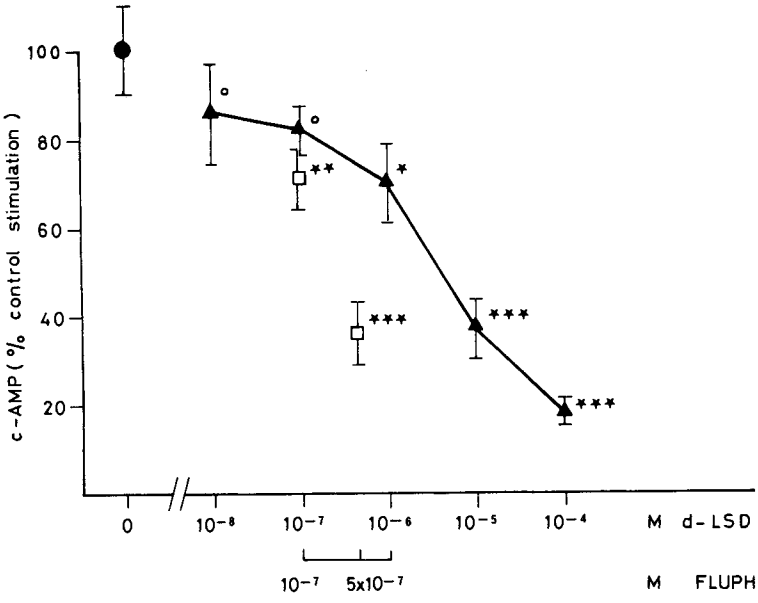

Fig. 3. Effect of various concentrations of d-LSD (▲) or fluphenazine (Fluph□) upon adenylyl cyclase activity, measured in the presence of 10^{-5} M dopamine, in a homogenate of rabbit retina. The control stimulation, i.e. the increase in enzyme activity in the presence of 10 μM dopamine, in the absence of any inhibitor (0) was of 49.8 ± 5.3 (S.E.M., n=11) and was represented as 100%. The data represent mean values ± S.E.M. for a minimum of 3 and a maximum of 6 samples for each concentration of d-LSD and for 5 samples for each concentration of fluphenazine. At the concentrations studied, none of the two antagonists altered adenylyl cyclase activity in the absence of added dopamine, 0= NS, *P< 0.05, **P<0.02, ***P< 0.001, Student 't" test.

spiroperidol binding was saturable and reached maximal levels around 2nM, in contrast to nonspecific binding which increased linearly between 0.025 and 3.5 nM (Magistretti and Schorderet, 1979b). Scatchard analysis of the saturation data yielded a K_D of 1.35 nM and a density of binding sites of 107 fmoles.mg prot.$^{-1}$. Specifically bound ^{3}H-spiroperidol increased linearly with tissue concentration between 0.5 and 3 mg of proteins . ml^{-1}. Specific binding was optimal at pH 7.1 with a sharp decline below and above this value (Magistretti and Schorderet, 1979b). Time course experiments indicated that equilibrium was reached after 10 min at 37° (not shown). No stereospecific binding was detectable after 15 min at 2° while only 25% of specific binding was observed at 22°. Pre-incubation of homogenates for 5 min at 90° completely abolished stereospecifically bound radioactivity (not

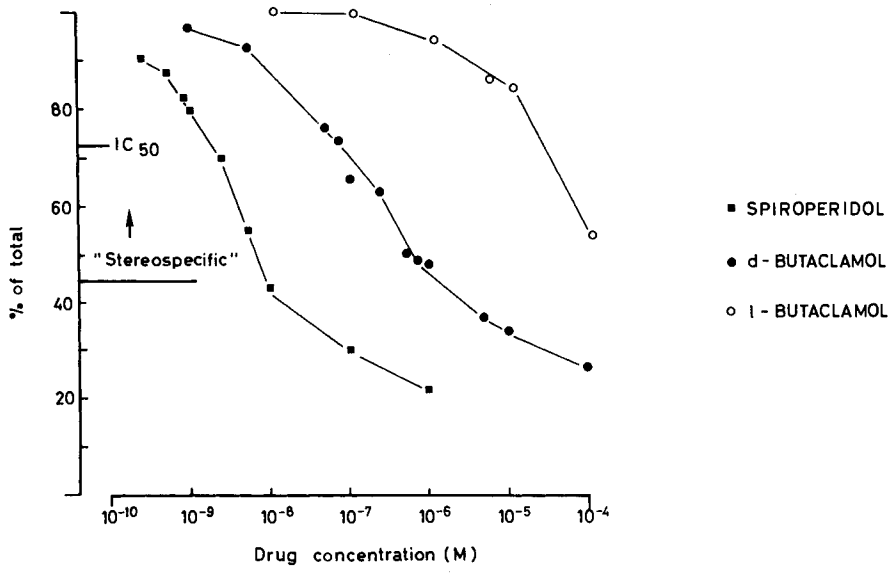

Fig. 4. Competition for ^{3}H-spiroperidol binding. Various concentrations of non-radioactive spiroperidol or geometrical isomers of butaclamol were incubated with 20 mg of tissue and a fixed concentration (0.25 nM) of ^{3}H-spiroperidol in a final volume of 0.5 ml at 37°for 15 min. Results are expressed as a percentage of total binding in the absence of added drugs and are from one experiment replicated twice. Each determination was performed in triplicate. The "stereospecific" portion of binding was defined with reference to tubes containing 1 µM d-butaclamol, as shown in the figure (taken from Magistretti and Schorderet, 1979b).

shown).

Stereospecificity. ^{3}H-spiroperidol was stereospecifically displaced by butaclamol isomers, d-butaclamol being 1'000 times more potent than l-butaclamol (Fig. 4). Another class of neuroleptics, the thioxanthene isomers, such as cis- or trans-flupenthixol and clopenthixol, also showed stereospecificity in displacing ^{3}H-spiroperidol (Fig. 5).

Pharmacological experiments. The potency of various neuroleptics, dopamine agonists and other drugs acting on CNS was tested using drug displacement curves. The IC_{50} values for stereospecific displaceable ^{3}H-spiroperidol binding are shown in

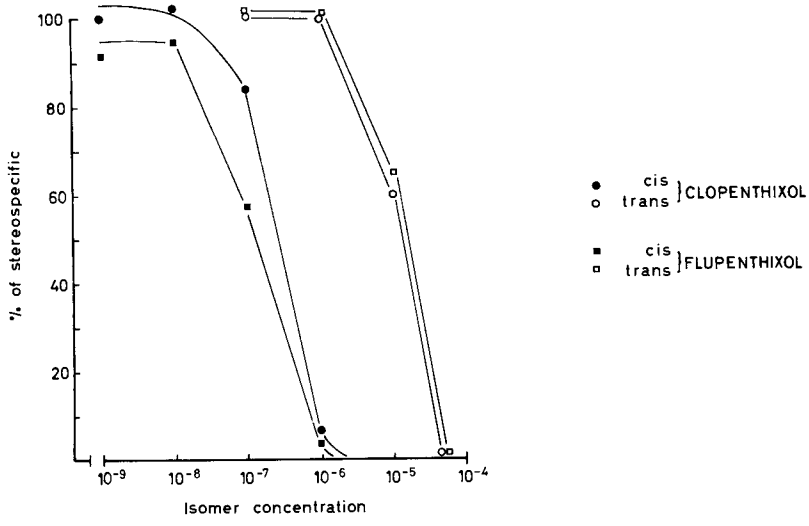

Fig. 5. Stereospecificity of ^{3}H-spiroperidol binding. Increasing concentrations of geometrical isomers of flupenthixol and clopenthixol were incubated in the same conditions as in Fig. 4. Results are expressed as a percentage of stereospecific binding defined with reference to tubes containing 1 µM d-butaclamol. Each determination was performed in triplicate (Taken from Magistretti and Schorderet, 1979b).

Table 3. For neuroleptics three orders of potency could be distinguished. The most potent inhibitors were spiroperidol, pimozide, haloperidol and d-butaclamol with IC_{50} values ranging from 1.8 to 24 nM. A second group (101.6 nM < IC_{50} < 268.7 nM) included the phenothiazines fluphenazine and chlorpromazine, cis-flupenthixol, clozapine and pipamperone. Finally, sulpiride, a benzamide neuroleptic, was almost inactive (IC_{50} = 20'000 nM).

Among dopamine agonists the potency of dopamine, epinine and ADTN was quite similar (4'700 nM < IC_{50} < 5'333 nM) and could be clearly distinguished from that of apomorphine (IC_{50} = 81.7 nM) (Table 3) (see also Fig. 6). Finally a number of compounds acting on CNS, including 5-HT and adrenergic agonists and antagonists were inactive, showing IC_{50} over 45'000 nM (Table 3) (see also Fig. 6).

DISCUSSION

Two kinds of experimental approach intended to unmask and to characterize pharmacologically the dopamine receptors of mammalian retina were described in the pre-

TABLE 3 Inhibition of ^{3}H-spiroperidol binding by drugs

DRUGS	IC_{50} (nM)
A. Neuroleptics	
spiroperidol	1.8
pimozide	16.8
haloperidol	21.3
d-butaclamol	24
cis-flupenthixol	101.6
fluphenazine	175.8
clozapine	206
chlorpromazine	239
pipamperone	268.7
trans-flupenthixol	13'000
sulpiride	20'000
l-butaclamol	28'000
B. Dopamine agonists	
apomorphine	81.7
n-methyl-dopamine(epinine)	4'700
ADTN*	4.775
dopamine	5.333
C. Inactive drugs	
5-HT(serotonin), amphetamine, 6-OH-dopamine, α-methyldopa, amantadine, harmaline, propranolol, nor-epinephrine, epinephrine, isoproterenol, phentolamine, atropine, carbachol, morphine.	> 45'000

The potency of drugs (minimum three concentrations) on stereospecific displaceable ^{3}H-spiroperidol binding was determined as described in "MATERIALS AND METHODS". IC_{50} values were calculated from three independent determinations using freshly prepared tissue and drug solutions, and performed at least in triplicate.
*2-amino-6,7-dihydroxytetrahydronaphtalene.

sent report. In the first one, cyclic AMP accumulation was measured in intact tissue or cyclic AMP formed from exogenous ATP was detected in homogenates in both cases after treatment with dopamine. It was then possible to firmly establish that rabbit- and/or calf-retina contained dopamine receptors which were linked to the enzyme adenylyl cyclase, as previously reported in these and other species (Brown and Makman, 1972, 1973; Bucher and Schorderet, 1974, 1975: Wassenaar and Korf, 1976; De Mello, 1978). In the experimental conditions described here for intact retina, a maximal accumulation of cyclic AMP was measured after 10 min of incubation, at 35°, in the presence of 5 x 10^{-6} to 10^{-5} M dopamine, epinine, apomorphine and ADTN. The incubations were performed in the presence of 10^{-2} M of the phosphodiesterase inhibitor, theophylline. This concentration of theophylline appeared to be adequate and maximally effective in that kind of experiments with intact rabbit retina (Magistretti and Schorderet, 1978a). Half-maximal stimulation of cyclic AMP increase was usually obtained with 10^{-6} M dopamine or other effective dopamine-mimetic drugs. Any agent that was not able to generate cyclic AMP, at 10^{-6} M,

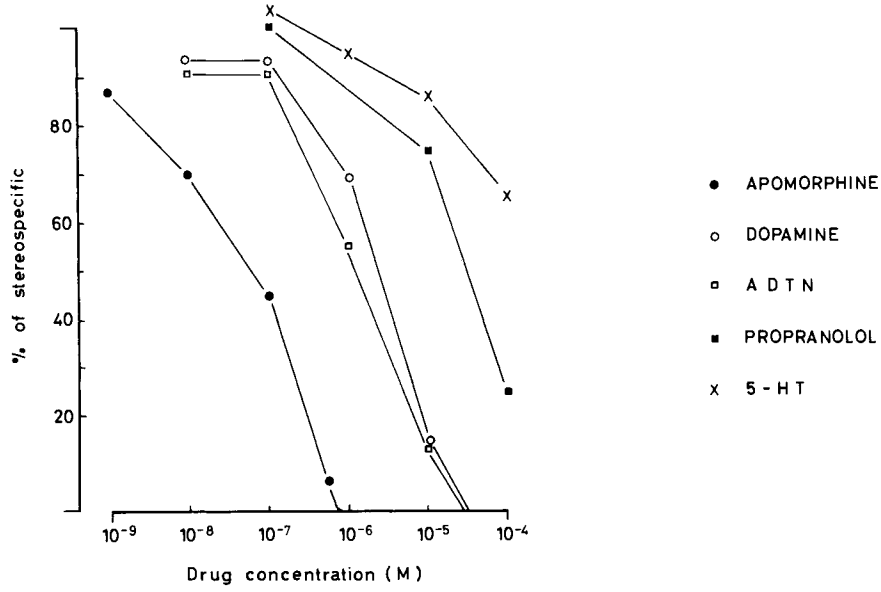

Fig. 6. Competition by non radioactive drugs. Increasing concentrations of drugs were incubated in the same experimental conditions as in Fig. 4. Results are expressed as a percentage of stereospecific binding defined with reference to tubes containing 1 µM d-butaclamol. Each determination was performed in triplicate (Taken from Magistretti and Schorderet, 1979b).

compared to significant increases detected at 10^{-4} M concentration, cannot be considered as a specific dopamine-agonist. Among these were adrenaline, noradrenaline (Schorderet, 1977a) and some 5,6-aminotetralin derivatives (Schorderet and McDermed, 1978). Therefore, attempts to reveal potential dopamine-agonist activity of various drugs using the intact retina in vitro have to be carried out in a range of concentrations going from 5 x 10^{-7} M (stimulation threshold) or 10^{-6} M (half-maximal stimulation) to 10^{-5}M. The positive effects of various drugs such as adrenaline and noradrenaline seen at higher concentrations than 10^{-6} M could be due to unspecific effects of catecholamines (and catecholamine-related drugs) upon dopamine receptors, since dopamine appears to be the only catecholamine and the only detectable monoamine in mammalian retina (Häggendal and Malmfors, 1963; Ehinger, 1976; Da Prada, 1977). The unspecific nature of the positive effects of adrenaline or noradrenaline (at 10^{-4} M) was also established by the fact that the catecholamine-induced accumulations of cyclic AMP were not blocked by α- or β- adrenergic antagonists, such as phentolamine or propranolol, but only by 10^{-4} M of a potent dopamine receptor blocking agent, such as fluphenazine (Schorderet, 1977a). Thus, intact retina preparations can be proposed as very specific and selective tools for screening potential dopamine-mimetic activity of various drugs, including new types of dopamine-agonists, such as aminotetralins (Schorderet and McDermed, 1978;

Schorderet, Magistretti and McDermed, 1978), ergopeptines and ergolines (Schorderet, 1976, 1978a).

As to a test in vitro for potential dopamine-antagonist activity of CNS acting drugs, such as neuroleptics which are supposed to block dopamine receptors of various or selected brain areas (Bartholini, 1978), the retinal homogenates appear to be more appropriate than intact tissue in two aspects: first of all, the number of samples provided by the same amount of tissue is higher with homogenates than with intact preparations. Thus, it allowed the simultaneous screening of several drugs in a wide range of concentrations, each one being tested in triplicate or quintuple samples. Secondly, the effective inhibitory concentrations of neuroleptics applied in these experimental conditions in vitro were closer to the effective plasma concentrations of the same drugs measured in the blood of patients treated for schizophrenia (Clement-Cormier and others, 1974; Seeman, 1977). As better quantitative correlation was thus found between potencies in vitro (as measured to inhibit the dopamine sensitive adenylyl cyclase in homogenates) and potencies in vivo, albeit some discrepancies still exist (Clement-Cormier and others, 1974; Seeman, 1977). However, an important difference between intact- and homogenized- retina preparations seems now to emerge. It concerned apomorphine and/or ergot alkaloid's effects. Then, in intact retina, apomorphine was an exclusive dopamine-agonist agent with a potency and an efficacy equal to those of dopamine. The same feature has been found with several ergot alkaloids, although the efficacies of those agents appeared to be less pronounced than that of dopamine and/or apomorphine (Schorderet, 1976, 1978a). Only d-LSD was shown to be a rather poor dopamine-agonist in intact tissue, whereas a significant dopamine- antagonist activity was detectable under the experimental conditions described above (see Results, Table 2, Fig. 3). In homogenates of retina, however, apomorphine was able to inhibit partially the dopamine-elicited accumulation of cyclic AMP, in contrast to the mentioned findings collected in intact retina (see above and Results). On the other hand, most ergot alkaloids tested in homogenates appeared to lose to a great extent their dopamine-mimetic activities, whereas some of those, besides d-LSD cited above, were revealed as partial dopamine-antagonists. Since the pharmacological actions of new ergopeptines and ergolines appear to be far from being completely understood at the present time (Lemberger, 1978; Müller-Schweinitzer and Weidmann, 1978), it is hoped that this type of investigations in vitro can possibly bring about new insights into the type of interactions of those powerful agents with dopamine receptors. Our data would indicate, in any case, that homogenization of the retina should alter some physico-chemical properties of the dopamine-receptors, which thereby could modify the pharmacological characteristics of those receptors. It can also be inferred that complex molecules such as apomorphine and/or ergot alkaloids would imply different type(s) of action(s) with dopamine receptors, before and after homogenization, than those observed with more simple molecules (and purely dopamine- like) such as dopamine-analogs and/or aminotetralins. Comparable detailed studies between two types of preparations were not performed yet with other dopaminergic tissues. Cyclic AMP increases induced by 10^{-4} M dopamine in slices of rat striatum, for example, were very small (Forn, Krueger and Greengard, 1974) and even much lower than those produced by isoproterenol in the same tissue. Intact rat striatum, thus, appears to be less selective for dopamine than intact mammalian retina.

The second approach was an attempt to label retinal dopamine receptors by radioactive-agonists and/or -antagonists (Magistretti and Schorderet, 1978b, 1979 a, b), according to very well described methodology already used for binding studies of striatal dopamine receptors (Creese, Burt and Snyder, 1975; Seeman and others, 1975; Seeman, 1977). The latter studies have shown a better correlation between drug activity in vivo and binding-capacity and/or displacement in vitro than that observed in striatal adenylyl cyclase investigations. An extensive study of that

kind was recently performed with bovine retinal homogenates, the radioligand ^{3}H-spiroperidol and several neuroleptics as well as catecholamine- and/or serotonin-related drugs as displacing agents (Magistretti and Schorderet, 1979b). The most potent inhibitors were those with predominant antidopaminergic properties and could be clearly distinguished from those for which a serotonergic component has been demonstrated (Leysen and others, 1978; Leysen, Gommeren and Laduron, 1979). Comparing our pharmacological results to those previously reported in frontal cortex and striatum (Leysen and others, 1978; Leysen, Gommeren and Laduron, 1978), the following considerations can be drawn: the rank order of drugs was markedly different from that shown in frontal cortex where ^{3}H-spiroperidol has been proposed to label predominantly serotonin receptors, thus revealing serotonergic affinity of drugs (Leysen and others, 1978). Yet our results would better compare with IC_{50} values of drugs for ^{3}H-spiroperidol binding in striatum. However, some differences appeared. Firstly, the distinction between neuroleptics with a predominant antidopaminergic affinity and those with a serotonergic component seemed to be more clear in retina. Taking into account the recent proposal that serotonin antagonist activity of a wide range antipsychotic drugs is uncorrelated with their antischizophrenic activity (Crow, 1979), retinal preparations should be appropriate for binding experiments related to the interactions of various drugs with dopamine receptors. Secondly, apomorphine was almost ten times more potent in retina (IC_{50} = 81.7 nM) than in striatum (Leysen, Gommeren and Laduron, 1978). This strong affinity of apomorphine stresses the dopaminergic characteristics of ^{3}H-spiroperidol binding in retina, particularly in view of the fact that 5-HT and adrenergic compounds were inactive. Moreover, the fact that apomorphine displayed an affinity similar to that of an antagonist could suggest that this drug is a mixed agonist-antagonist in bovine retina. The latter finding would nicely correlate with the fact that apomorphine was also an antagonist when it was tested as an inhibitor of dopamine sensitive adenylyl cyclase in bovine retina homogenates (see above and Results). In line with that finding it was striking to reveal that the neuroleptic sulpiride, acting possibly upon D2-type of dopamine receptors (Kebabian and Calne, 1979) was almost inactive in calf retina (IC_{50} = 20'000 nM, Table 3), whereas in striatum its potency was 20 times greater (Leysen and others, 1978). Furthermore, when sulpiride was tested upon dopamine-sensitive adenylyl cyclase in intact rabbit retina (Magistretti and Schorderet, 1978a) or calf retina homogenates (unpublished), it was totally ineffective. Therefore, two experimental evidences would disclose a positive correlation between enzymatical- and binding- studies of retinal dopamine receptors and would suggest the existence in this tissue of dopamine receptors of D1-type (Kebabian and Calne, 1979).

Taking into account the last experimental features, the model of retina can be used for appropriate pharmacological screening of selective affinity of drugs for dopamine binding-sites linked to functional adenylyl cyclase. It can also provide a useful tool, after intravitreal injections of various agents (Magistretti and Schorderet, 1979c) to investigate the development of the state of super- and/or sub-sensitivity, at the level of binding- and/or catalytic-sites of the retinal dopamine receptors and to understand possibly better the mechanism of such adaptative phenomenon of living cells.

ACKNOWLEDGEMENTS

This work was supported by SNSF grant Nr. 3.327.078.

The authors would like to express their gratitude to Miss G. Allenbach, Mr. S. Ferrara and Mr. A. Masiero for invaluable technical assistance, to Mrs. Denise Tabin for competent editorial help, and to Mr. F. Pillonel for excellent graphic illus-

trations.

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Pergamon Press Ltd. 1980. Printed in Great Britain.

ERG ALTERATIONS INDUCED BY SOUND

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ABSTRACT

The existence of efferent neurons from the CNS to the retina mediating centrifugal effects is an old and much debated hypothesis. In order to test whether the human ERG can be influenced by another sensory input, we have recorded the retina response to light when a sound was presented simultaneously. It was found that the b-wave of the ERG increased when a flash was paired with a sound. The sound-induced increment of the b-wave showed the following characteristics: a) Short time course, b) No relation to the frequency and intensity of the sound, c) Habituation specific to the frequency of the sound, d) Adaptation when the sound lasted more than 2 sec, e) Dependence on localization cues of the sound and f) Enhancement by low doses and suppression by high doses of diazepam. Further experiments on rabbits showed that it is not suppressed by ether anesthesia, by local application of atropine or by cutting the external ocular muscles. The results suggest that this effect is brought about by a reticulo-retinal pathway, the function of which is most probably to control the input of the visual route.

KEYWORDS

Electroretinogram; efferent control of visual perception; diazepam; sound-vision interaction; reticulo-retinal pathway; ether anesthesia on ERG.

INTRODUCTION

In 1956 Hernández-Péon, Scherrer and Jouvet reported that the potentials evoked by clicks and recorded from the cochlear nuclei of unanesthetized cats reduced in size when the animal's attention was drawn to another disturbing stimulus, like the sight of mice, the smell of fish or shock to the feet. This and many other similar experiments led to the belief that the CNS can modulate the input of all sensory modalities (Jouvet, 1957; Granit, 1962; Angel, 1978). This efferent control of the CNS supposedly works by inhibiting receptors or the early stages of sensory transmission, so that only selected information is allowed to pass through to the center. However, both the results of these experiments and their interpretation are open to criticism (Milner, 1970). If the signals were blocked before they reached the CNS, it would be impossible for the attention mechanisms to determine whether or not a signal was still present and, whether it has become important for the animal.

Furthermore, in most of the experiments of this kind the recordings were taken with gross electrodes which probably measure the dendritic potentials, produced by the partial depolarization of many neurons, and such potentials are expected to behave like those of the EEG i.e., to increase in a relaxed animal and to decrease during arousal or in an attentive state. If this is the case the results of this kind of experiments should be interpreted as showing that the perceptual ability of a sensory pathway is facilitated rather than inhibited, when another sensory input is stimulated. ERG is of particular value to test such a hypothesis, because it provides a means of obtaining information about the earliest stage of a sensory process. Especially, the b-wave of the ERG is considered to be a measure of retina sensitivity to light (Armington, 1974). In the present work we have been concerned with the question whether or not the human ERG changes when an auditory stimulus is presented.

METHOD

The experiments were carried out on human volunteers, and the ERG was recorded with a lens electrode of Henkes van Balen type placed on the cornea. The reference electrode was a silver EEG plate electrode fixed to the middle of the forehead. Flashes, as well as sounds, were delivered via an Alvar Soneclat. The light source was a flash tube mounted on a reflector. The electrodes were connected to the input of a differential amplifier. The results were displayed on an oscilloscope and were monitored on a tape recorder. They were reproduced from the tape onto a storage oscilloscope from which photographs were taken.

RESULTS AND DISCUSSION

We found that a sound paired with a flash caused an increased b-wave of the ERG (Fig. 1). This effect was observed only when sound and flash were presented simultaneously, which suggests that the effect of the sound on the retina should be mediated via a neural pathway, rather than by a humoral factor. Centrifugal fibres of unclarified origin and function, which run in the optic nerve and terminate around amacrine cells, have been described in birds (Cajal, 1889; Cattaneo, 1922), frogs (Rozemayer and Stolte, 1931), cat (Maturana, 1958) and also in the human retina (Becher and Knoche, 1959; Wolter and Liss, 1956). Efferent fibres of this sort may carry the effect of nonvisual stimuli from the CNS to the human retina. Concerning the origin of the efferent fibres running in the optic nerve, the by now classical experiments of Granit (1955) on cats, have shown that they are derived from the brain-stem reticular formation. Granit (1955) has reported that stimulating various loci of the reticular formation produced either inhibition or facilitation of the retina ganglion cells activity.

In our experiments we have found that the increment of the b-wave produced by sound, is unrelated to the intensity and the frequency of the presented sound. Tones from 400 up to 50.000 cps and of 70 up to 120 phons[1] had the same effect.

Habituation, which was frequency-specific, has been observed in all cases. When a sound of a given frequency was repeatedly presented, it gradually ceased to elicit an increase of the b-wave. The habituation developed after 5 to 6 successive presentations of the same tone. The subsequent presentation of a sound of a novel frequency caused disinhibition, i.e., it abolished the habituation established by the initially presented frequency. Experimental evidence provided by Sharpless and Jasper (1956), Jouvet (1961) and especially by Horn and Hill (1966) postulated that frequency-specific habituation is accomplished in the mesencephalic RAS.

[1]Phons is decibels above the threshold of hearing.

What we suggest is that in our experiments the unspecific response of the retina to the sound, as well as the frequency-specific habituation, may indicate that the effect of the nonvisual stimulus in the retina response to the light is mediated via the brain-stem RAS.

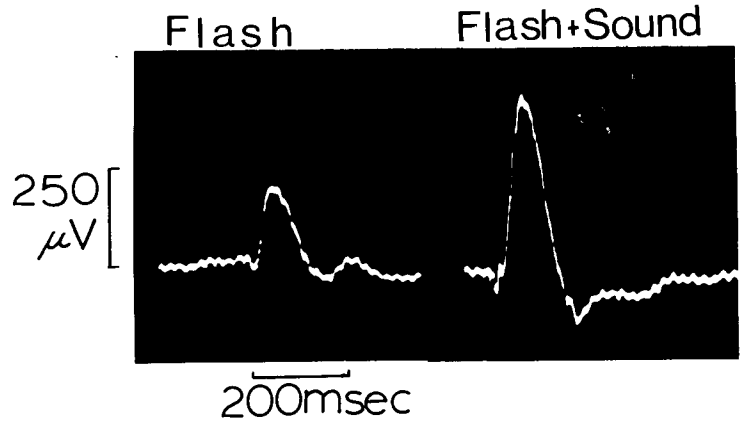

Fig. 1. Human ERG. Left trace: Response to a flash. Right trace: Response to a flash paired with a sound.

Figure 2 shows another characteristic of the response which we may call adaptation. Here an effective sound is presented for a period of 3 sec. A flash coming just before the end of the sound fails to elicit enhancement of the b-wave. Adaptation occurred always when the sound was sustained for longer than 2 sec. The adaptation should be a peripheral effect, possibly resulting from receptor fatigue or nerve accomodation of the acoustic pathway. It should be distinguished from what we described as habituation, which seems to be a type of learning integrated inside the CNS.

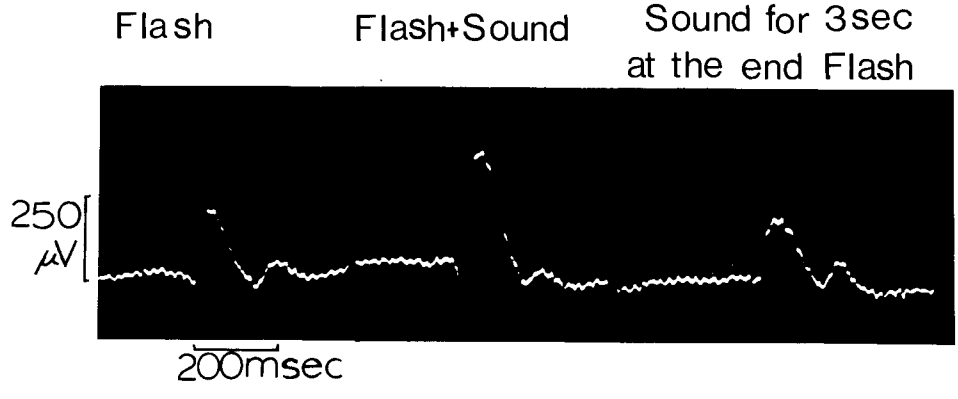

Fig. 2. Human ERG. Left trace: Response to a flash. Middle trace: The b-wave increased when the flash is paired with a sound. Right trace: The flash comes at the end of a sound which had the same characteristics as this in the middle trace but lasted 3 sec.

Figure 3 shows that the effect is maximal when the sound was presented symmetrically to both ears, less when ipsilaterally and minimal when contralaterally. It has been shown by several investigators (Poulton, 1953; Broadbent, 1954; Spieth, Curtis and Webster, 1954; Webster and Thomson, 1954) that one of the main cues the CNS utilizes in filtering sensory information and orientating selective attention mechanisms are localization cues. It is, therefore, reasonable to suppose that the ability of the auditory input to affect visual perception might be a normal behavioural mechanism of selective attention. This implies that the attention should operate on objects and situations and not on sensory modalities, as it has been supposed by other investigators. It seems logical to assume that an alert organism becomes keenly sensitive to sensory modalities, so that all cues pertaining to an object or situation can be perceived.

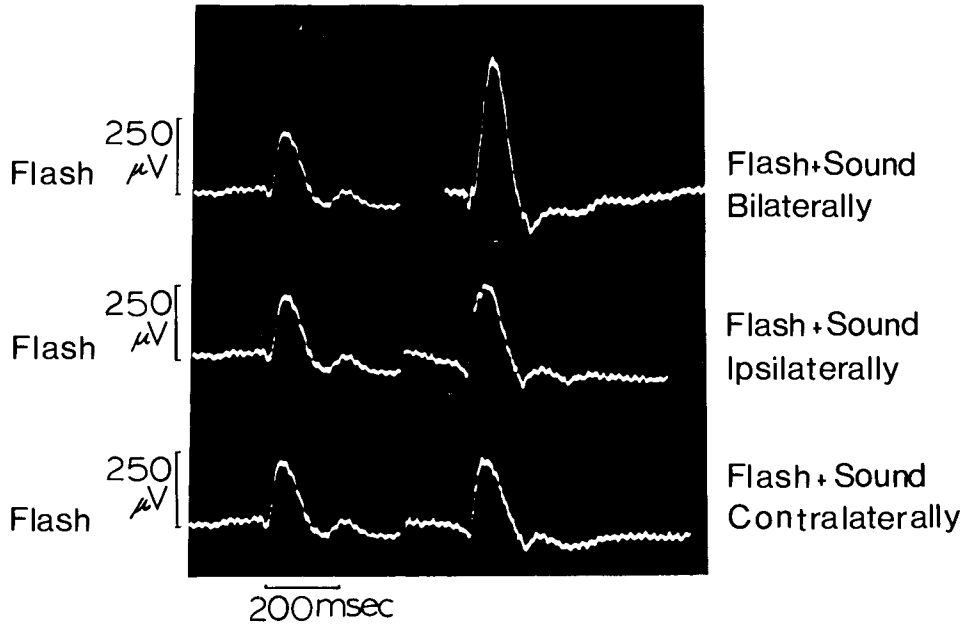

Fig. 3. Human ERG. Left: Response to light. Right: Response to light when simultaneous with a flash. Upper traces: The sound presented symetrically to both ears. Middle traces: The sound to the ipsilateral ear. Down traces: The sound to the contralateral ear.

Figures 4 and 5 show the effect of diazepam. This was tested in 12 subjects and it was found that doses of 2 and 4 mg of diazepam enhance the effect of sound on the ERG, while doses of 8 and 10 mg suppress it. It is known that low doses of diazepam increase the responsiveness of the RAS, and facilitate the afferent impulses, while high doses have the opposite effect (Ban, 1969). According to our assumption that the acoustic effect is mediated by the RAS the dose-related effect of diazepam is well in accordance with what should be expected.

At this stage we thought to test the effect of ether anesthesia and, since our volunteers were reluctant to cooperate, we switched to rabbits. We have tested so far only 5 rabbits. When the animals were unanesthetized the response was missing and only in a few trials there was a very small increment of the b-wave by sound.

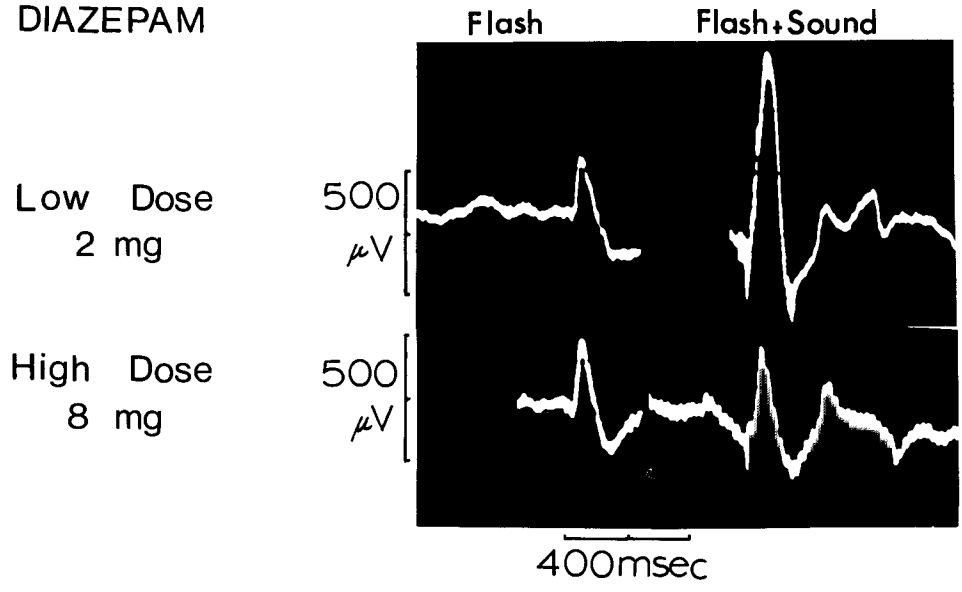

Fig. 4. The effect of diazepam on the human ERG. Diazepam administered peros at 2mg (upper traces) and 8mg (down traces). Left: Response to a flash. Right: Response to a flash paired with a sound. Records taken 15 min after diazepam administration.

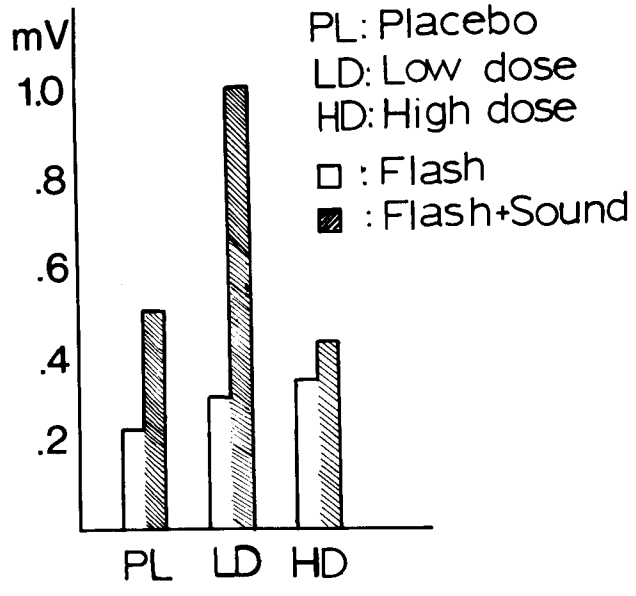

Fig. 5. The effect of diazepam on the b-wave of the ERG. Abscissa: Amplitude of the b-wave in mV. LD: 2 and 4mg; HD:8 and 10mg.

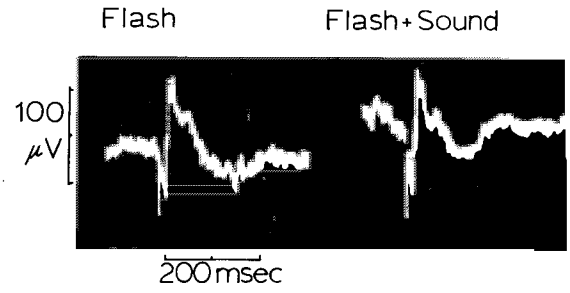

Fig. 6. ERG of an unanesthetized rabbit. Left: Response to a flash. Right: Response to a flash paired with a sound.

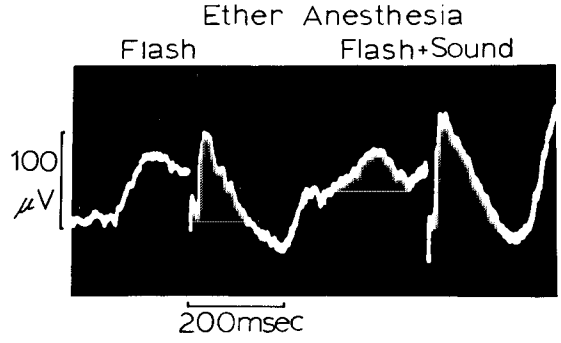

Fig. 7. ERG of a rabbit anesthetized by ether. Left: Response to a flash. Right: Response to a flash paired with a sound.

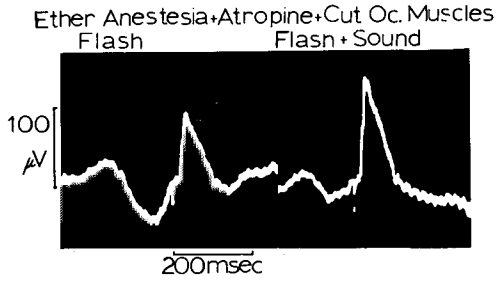

Fig. 8. ERG of an anesthetized rabbit. External ocular muscles were cut and atropine was applied. Left: Response to a flash. Right: Response to a flash paired with a sound.

We anesthetized the animals and the response appeared (Figs. 6 and 7). General anesthesia is classically attributed to a suppressed tone of the reticular ascending system. According to this view, it is difficult to understand how ether has revealed a facilitatory response. However, when we compared the amplitude of the b-waves we saw that the response of the alert animals had the same amplitude with this of anesthetized animals when enhanced by sound (Fig. 8). We think that, what is actually happening, is that unanesthetized animals were alarmed by the experimental procedure itself and the added sound could not excite much further an already highly excited pathway. Ganglof and Monnier (1956) reported that rabbits will be aroused much more effectively by the silent appearance of a human being than by strong stimuli, such as bright lights and loud noises. Moreover, a working hypothesis proposed by Brazier (1953) about the mechanism of action of anesthetic agents is that they upset the balance between inhibitory and excitatory input to the ascending reticular system. Especially on the visual pathway, Angel (1978) has found that anesthetic agents influence the transmission in the lateral geniculate by increasing reticular inhibition and reducing reticular excitation, whereas they do not affect 1st and 2nd order neurons. It is, therefore, reasonable to conclude that the neural route carrying "sound information" to the retina should be integrated at the level of 1st or/and 2nd order neurons, i.e., via the bipolar or the ganglion cells.

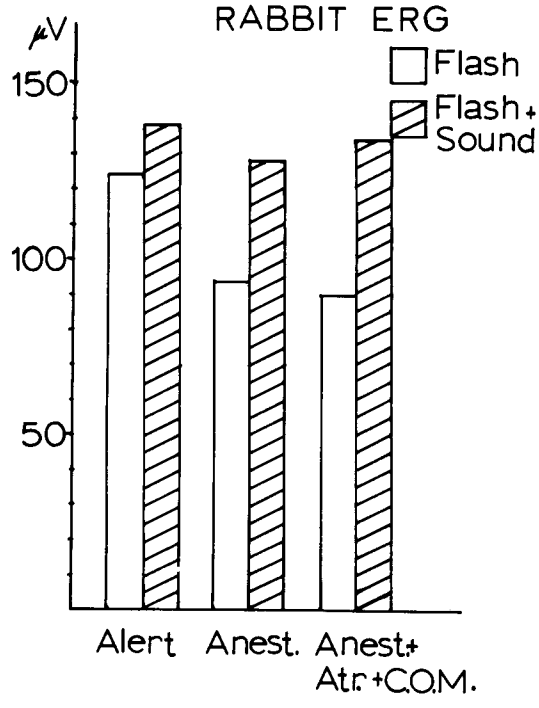

Fig. 9. Effect of sound on the rabbit ERG. Left columns: Unanesthetized animals. Middle columns: During ether anesthesia. Left columns: After cutting external ocular muscles and applying atropine. Abscissa: Amplitude of b-wave in μV.

An alternative interpretation of our findings could be that the reticular control of visual input is not exerted directly on the retina but is due to motor effects. What we suggest here, is that the auditory stimuli might have caused alarm state, and it is known that any alarm state is accompanied by pupil dilatation. Dilated pupils may facilitate the response to light, as more light is allowed to fall on the retina. Another possibility is via a motor effect on the ocular muscles. To test these possibilities we worked also on rabbits. The size of the pupil was fixed by topical application of atropine, and the eyeball was fixed by cutting the external ocular muscles. The effect of the sound on the ERG remained unaltered (Fig. 9).

CONCLUDING COMMENTS

It was found that a sound paired with a flash increased the b-wave of the ERG. This effect showed the following characteristics:

1. Short time course.
2. No relation to the frequency and intensity of the sound.
3. Habituation, specific to the frequency of the sound.
4. Adaptation.
5. Dependence on localization cues.
6. Enhancement by low doses of diazepam.
7. Suppression by high doses of diazepam.
8. Resistance to ether anesthesia.
9. It was not due to motor effects.

These results suggest that the early stages of visual perception can be influenced by sound. We believe that any other sensory modality could act on more or less the same way. The reason we chose to work with auditory stimuli was only because it was technically easier to control their parameters. An interaction of sensory messages of different modalities appears to be a commonplace situation on the functional organization of the nervous system. An effect of visual, olfactory and tactile stimuli on the auditory, visual and somatosensory receptors has been found by many investigators and in various animal species (Hernández-Péon and co-workers, 1956, 1957; Brust-Carmona and Hernández-Péon, 1959; Atweh and co-workers, 1974). The servomechanism control system of the muscle stretch reflex is the most well known example of a centrifugal control of receptors. In invertebrates a direct control of receptor excitability by the CNS is almost a general rule. Moreover, at the higher levels of all sensory pathways centrifugal control is well established (Niemer and Jimenez-Castellano, 1950; Vastola, 1960; Guillery, 1967; Hull, 1968), and strictly speaking the retina is a part of the brain which has strayed out of the skull and the optic nerve is really a track of the CNS.

Figure 10 is a drawing of the visual pathway showing its analogy to a typical cutaneous sensory pathway. The ganglion cells are second-order neurons and the optic nerve is the equivalent of the dorsal columns. In the cutaneous sensory pathway, reticular fibres end in the dorsal column nuclei and may cause inhibition (Hernández-Péon, Scherrer and Velasco, 1956).

Our results suggest that a similar reticulo-retinal pathway should control the input of the visual route. Such a pathway has been confirmed in pigeons by Holden (1968) and Cowan (1970). They have shown that the avian brain exerts control through amacrine cells, which seem to end to the presynaptic terminals of bipolars. GABA is the putative neurotransmitter on the axoaxonic synapses between amacrine cells and the presynaptic terminals of bipolars. Concerning the influence of diazepam, Gahwiler in 1976 has shown that diazepam is an extremely potent GABA antagonist. In explants of rat cerebellum the effective molecular concentration of

diazepam was 10^{-10}M, whereas picrotoxin and bicuculline were effective at molecular concentrations of 10^{-5} and 10^{-9} M respectively.

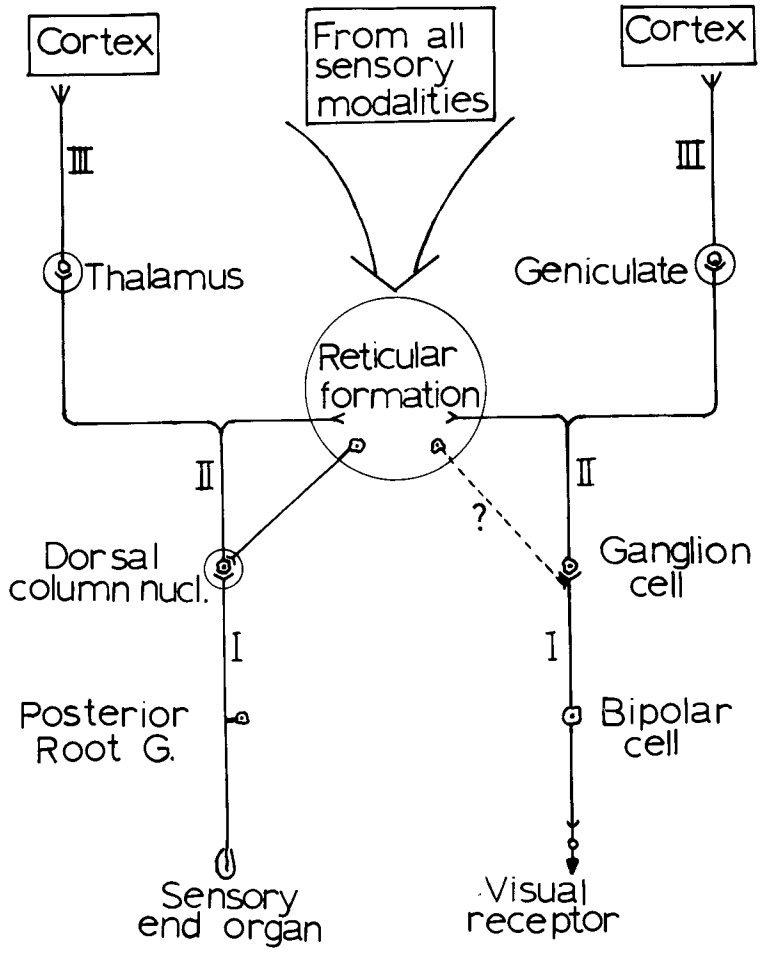

Fig. 10. Schematic diagram showing the analogy of the visual pathway (right) to the cutaneous sensory pathway (left). The dashed line shows the hypothetical pathway via which the effect of the sound on the retina might be brought about.

In conclusion, we suggest that a reticulo-retinal pathway should exert a constant, tonic inhibitory effect on the transmission from bipolar to ganglion cells, a sort of gain control on the input of the visual pathway. Such a control may shift the level of the output frequency of the visual pathway and set it to a new level implied by the integrated behavioural pattern of the animal.

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THE ACTION OF PSYCHOTROPIC DRUGS ON ADENYLATE CYCLASES AND PHOSPHODIESTERASES IN THE RAT RETINA

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How can I look backward into the past or forward into the future? How will the sensors connected with my emotions bring me to the sources of my existence? Anyway, I am aware of the utmost importance of the process of seeing to which I will confine myself at first.

Joh. S. Wassenaar

ABSTRACT

In this overview we demonstrate differences in the mode of action of DA and NA on cAMP-synthesizing systems by using psychotropic compounds such as fluphenazine and haloperidol. Evidence exists that this catecholamine induced cAMP-synthesis occurs in post-synaptic receptor-coupled adenylate cyclases. Our results reveal the expected inhibitory action of the psychotropic drugs on the DA- and NA-stimulated adenylate cyclases. We present, however, also evidence for a drug-induced inhibition of the phosphodiesterase activity involved in the NA-induced cAMP synthesis. This drug-induced inhibition of P.D.-ases was not found in the DA-governed enzymatic process. We also measured the DA- and NA-responses at various low potassium-ion concentrations (0-6 mM). Our data on this point suggest a specific membrane conformation modulated by potassium ions, which change the readiness of DA- and NA-receptors to accept the catecholamines involved. An elevation of the potassium-ion concentration up till 15 mM produced a relatively high cAMP-formation, which was suppressed by various amounts of psychotropic compounds. Our results point to different cell types in the rat retina, all containing the structure for cAMP-synthesis and cAMP-breakdown.

KEYWORDS

cAMP-synthesis; dopamine and noradrenaline; neuroleptic drugs; adenylate cyclases and phosphodiesterases; cAMP-breakdown.

INTRODUCTION

The vertebrate retina is a special structure of the central nervous system in which dopamine (DA)- containing cells are localized at the level of the inner nuclear layer in the innermost cell row among the amacrine cells in a ratio of about 1 DA-cell: 10 amacrines (Ehinger, 1977). The presence of other catecholamines in the retina is questionable though Drujan and coworkers (1965; 1968) report adrenaline to be present in the toad retina. Noradrenaline (NA) seems to be absent or nearly absent in the vertebrate retina, though Ames and Pollen (1969) and Straschill and Perwein (1969) found electrophysiological changes with NA and with DA applied on ganglion cells of rabbits and cats. Moreover, Hedden and Dowling (1977; these Proceedings) showed the modifying influences of DA on the electrophysiology of several cell types in the retina (fish). Together with dopaminergic properties of characteristic retinal cells, a DA-sensitive-cAMP-synthesizing system is present in the retina (Brown and Makman, 1972; 1973; Bucher and Schorderet, 1974; Wassenaar and Korf, 1976; Schorderet, 1977b; Schorderet and coworkers, 1978, these Proceedings). This system appears to be comparable to other dopaminergic-cAMP-synthesizing systems in brain (Makman and coworkers, 1975). However, it is known (Brown and Makman, 1972), that not only DA but also NA, both added exogenously, are capable of increasing cAMP-levels in the retina considerably. In the next pages we shall offer evidence for a special sensitivity of adenylate cyclases and phosphodiesterases for neuroleptic drugs. These enzymes seem to belong exclusively to the NA-cAMP-system. Potassium ions seem to modulate the DA- or NA-driven cAMP-synthesis in the rat retina.

PREPARATION OF RETINAE; INCUBATION CONDITIONS

The processes of cAMP-elevation by DA or by NA in the retina can be mimicked by application of the amines to intact retinae incubated in vitro or to rough retinal homogenates. In our experiments we isolated retinae from the eyes of albino-female-Wistar rats (160-180 g), 2 resp. 4 min after sacrifice and we transferred the intact retinae to pre-incubation vessels (40 min, 37°C) and thereafter to incubation vessels (2 min, 37°C) (Fig. 1). The vessels contained 25 ml Krebs-Ringer with or without a neuroleptic drug (in pre-incubations and incubations), with or without a high or low phosphodiesterase-inhibitor concentration (in incubations) and with or without DA or NA (in incubations). Incubation media were continuously gassed with 5% CO_2 and 95% O_2. Incubations were stopped by transferring the incubated retinae into 1 ml of 50% icecold acetic acid and after homogenization and subsequent evaporation, the remaining residues were dissolved and used to determine the overall-protein content (Lowry and coworkers, 1951) and to determine cAMP with a protein-binding assay (Gilman, 1970). Histological examination of the retinae after 42 min of the total incubation period did not show gross changes compared to the histological appearance of retinae fixed directly after isolation. After a net incubation-time of 2 min and in the presence of 5.10^{-5} M of one of the amines, a just maximal cAMP-level was obtained. Our data with high concentrations of phosphodiesterase-inhibitor show a responsive NA-sensitive cAMP-synthesizing system giving rise to relatively high levels of cAMP (about 200 pmoles cAMP per mg protein) whilst with the DA- cAMP- system much lower cAMP-levels are reached at similar incubation conditions (about 100 pmoles cAMP per mg protein). Besides this, we found an easy stimulation of the cAMP-synthesis with potassium ions (10-15 mM). The standard concentration of K^+ -ions in the Krebs-Ringer solution was 6.24 mM K^+.

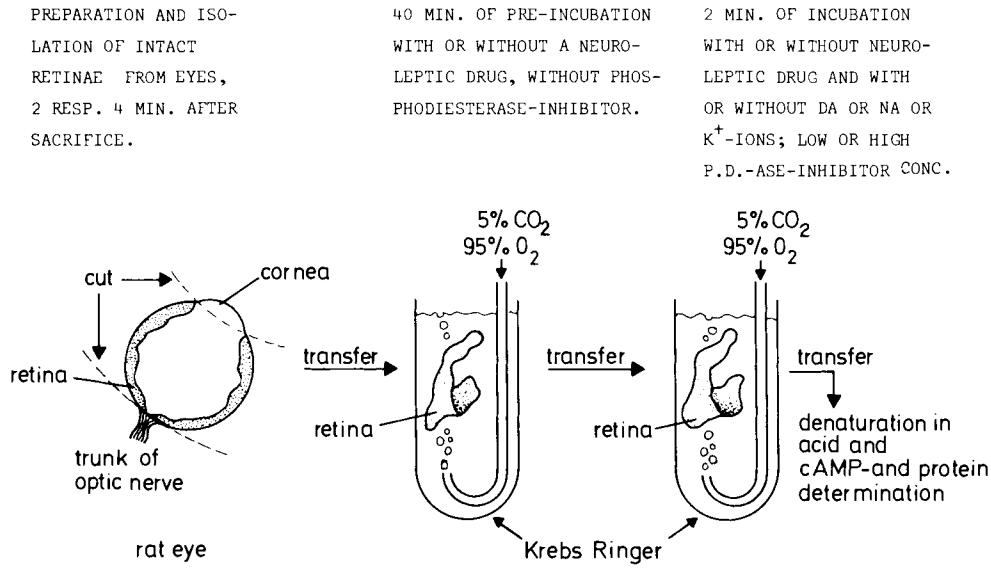

Fig. 1. Schematic presentation of the incubation of rat retinae (total incubation period: 42 min). After the 2 min incubation-period, we denaturated the retinae in 1 ml of 50 % icecold acetic acid and after homogenization we determined cAMP-levels (Gilman, 1970) and protein (Lowry and coworkers, 1951).

APPROACH

With the dissected rat retina incubated in vitro we have the opportunity to study effects of drugs on related enzymes in their most adequate environment: the living membrane surrounding the living cell in an appropriate extra-cellular and thus intracellular condition. In our studies, this holds for the catecholamine-receptor coupled adenylate cyclases, which increase cAMP-synthesis upon receptor occupation by DA or NA. There is much evidence that this DA- (and NA-?)response is localized at a post-synaptic region (Kebabian and coworkers, 1972; Clement-Cormier and coworkers, 1974; Iversen, 1975). However, quite a number of reports point not only to an antagonism of neuroleptic drugs on these receptors and subsequent inhibition of the adenylate cyclases coupled to these receptors, but the inhibitory influence of neuroleptic drugs on phosphodiesterase activity in rough homogenates of brain tissue has been mentioned as well (Honda and Immamura, 1968; Uzunov and Weiss, 1971; Weiss and coworkers, 1974; Amer and Kreighbaum, 1975; Chasin and Harrow, 1976). Considering the living retinal cell: if neuroleptic drugs do inhibit both the synthesis of cAMP achieved by adenylate cyclases (A.C.-ases) and breakdown of cAMP achieved by phosphodiesterases (P.D.-ases), these inhibitory actions must be opposite concerning their effects on the amine induced cAMP-levels. In other words: cAMP-levels in the presence of neuroleptic drugs might be the result of the balance between variable stimulation of A.C.-ases and variable high or low inhibited P.D.-ase activities. We worked on this hypothesis and we realized that we are only able to

observe this kind of differences of cAMP-levels and thus to demonstrate inhibitory actions of neuroleptic drugs on both enzymatic processes, if the inhibition-sensitivity of both enzymes is different as such. Working on the possible inhibitory influence of neuroleptic drugs on P.D.-ases, we distinguished between the classic neuroleptics, haloperidol (HALO) and fluphenazine (FLU) on the one hand and classis P.D.-ase inhibitors as theophylline, papaverine, caffein and RO-20-1724 on the other hand. Low or high concentrations of these inhibitors determine the actual P.D.-ase activity. This activity might be extra inhibited at low P.D.-ase inhibitor concentrations by neuroleptic drugs as HALO or FLU. So as to get an experimental answer to these questions, we kept the catecholamine-receptor coupled A.C.-ase stimulation in one series of experiments constant by adding one fixed concentration of one of the catecholamines in the incubation medium. We varied the P.D.-ase inhibitor concentration in the presence or absence of neuroleptic drugs and we measured the resulting cAMP-levels. In Fig. 2 we present an overview of the experimental headlines.

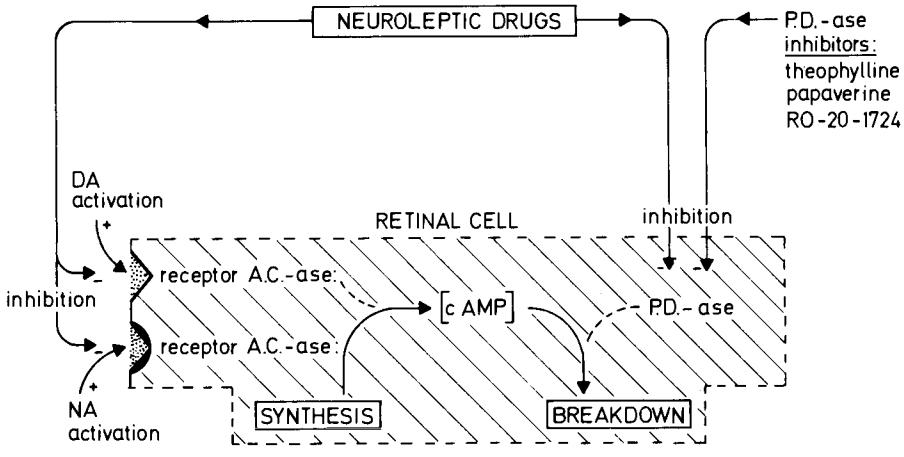

Fig. 2. Schematic overview of experimental strategy outlined under Approach.

RESULTS

High P.D.-ase Inhibition and Neuroleptic Drugs; Partial Inhibition of the NA-response

Looking at the DA- and NA-induced cAMP-elevations we found a concentration of 10^{-2} M of the P.D.-ase inhibitor theophylline sufficiently high to obtain reproducible and relatively high catecholamine induced cAMP-levels. After addition of 10^{-4} M FLU and of 10^{-4} M HALO complete inhibition of the DA-response and partial inhibition of the NA-response was found (Fig. 3). Similar results with the P.D.-ase inhibitors papaverine and RO-20-1724 were obtained.

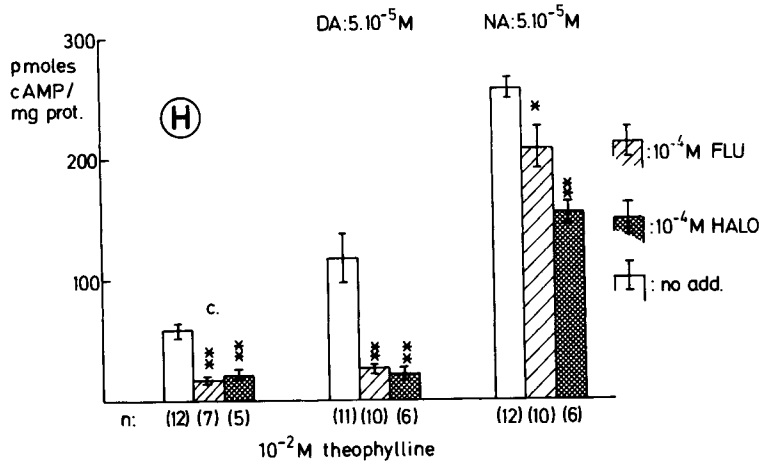

Fig. 3. DA- and NA- responses at high P.D.-ase inhibition (H) by 10^{-2}M theophylline. cAMP-values: mean ± S.E. M. Significant differences (Students-t-test) within one group with respect to the value without HALO or FLU (no add.); *P<0.025; **P<0.005. Partial inhibition of the NA-response by HALO and FLU and complete inhibition of the DA-response is clearly present.

Enhancement of the NA-responses; Low P.D.-ase Inhibition and Neuroleptic Drugs

Measuring catecholamine-responses and using low P.D.-ase inhibitor concentrations, for example 2.10^{-3}M theophylline (Fig. 4), we obtained responses with DA and with NA, which were much lower than the responses obtained with high P.D.-ase inhibitor concentrations (compare Figs. 3 and 4). In Fig. 4 we offer the result of the DA-response being inhibited by 10^{-4} M HALO and of the NA-response being enhanced by this drug.

Our hypothesis under Approach points already to this enhancement: we assumed that the lower cAMP-levels at low P.D.-ase inhibition with DA and with NA probably reflect the effect of a higher P.D.-ase activity, because of the low inhibition of the enzyme. A neuroleptic drug as HALO inhibits some extra P.D.-ase. Thus the P.D.-ase activity decreases and consequently the NA-induced cAMP-level increases: enhancement of the NA-response has been obtained.

Surprisingly enough this enhancement is not observed when DA is used as the inductor of cAMP synthesis. We extended our findings comparing DA- and NA-responses by using a series of concentrations of neuroleptic drugs at low P.D.-ase inhibition (2.10^{-3}M theophylline). In Fig. 5 we see that the enhancement of the NA-response already occurs between 10^{-7}M and 10^{-6}M FLU or HALO. Inhibition of the DA-response occurs at concentrations between 10^{-5}M and 10^{-4}M of FLU or HALO. The receptor coupled A.C.-ases become obviously susceptible for the neuroleptic drugs at these higher concentrations of FLU and HALO.

Studying the P.D.-ase inhibitor RO-20-1724, it appeared that at 10^{-4}M and at 10^{-5}M of this inhibitor partial inhibition of the NA-response by 10^{-4}M FLU or by 10^{-4}M HALO is obtained. At 10^{-6}M of this P.D.-ase inhibitor a clear enhancement is caused by FLU and by HALO (Fig. 6). Now the higher P.D.-ase activity is obviously inhibited extra by 10^{-4}M FLU. The result of this experiment confirms strongly our hypothesis.

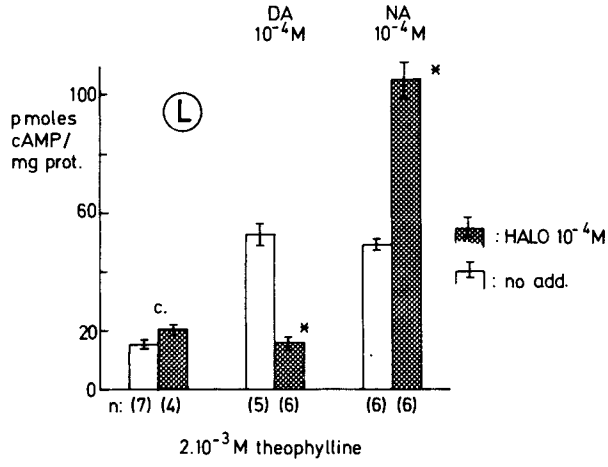

Fig. 4. DA- and NA-responses at low P.D.-ase inhibition (L) by 2.10^{-3}M theophylline. cAMP-values: mean ± S.E.M. Significant differences (Students-t-test) within one group with respect to the value without HALO (no add.): *P<0.005. Complete inhibition of the DA-response by HALO and the enhancement of the NA-response is clearly shown.

Next, we started to study the effects with the P.D.-ase inhibitor papaverine (Fig. 7). We found 10^{-4}M of this P.D.-ase inhibitor sufficiently low to obtain the enhancing effects of the NA-response by FLU. In this condition of low P.D.-ase inhibition 10^{-7}M FLU appears already to be an appropriate P.D.-ase inhibiting concentration, increasing the cAMP-level compared with the cAMP-level with 5.10^{-5}M NA alone. 10^{-5}M FLU exerts a higher P.D.-ase inhibition causing a higher cAMP-level than with 10^{-7}M FLU. Compared with the effect at 10^{-5}M FLU we find with 10^{-4}M FLU a slightly lower but still enhanced NA-response (Fig. 7). The combination of low P.D.-ase inhibition at 10^{-4}M papaverine with 10^{-4}M FLU might give a cAMP-elevation as the result of the counterbalance between extra-inhibition of the P.D.-ases involved, giving rise to an elevation of the cAMP-level, and inhibition of receptor-coupled A.C.-ases involved, giving rise to a decrease of the NA-induced cAMP-synthesis. Fig. 7 might show elegantly the difference in inhibition-sensitivity of P.D.-ases and A.C.-ases of the NA-system for neuroleptic drugs.

Finally, we found that an enhancement of the NA-response by neuroleptic drugs can be evoked even in the absence of a P.D.-ase-inhibitor (Table 2). This result offers extra support for our hypothesis directed to the simultaneous inhibition

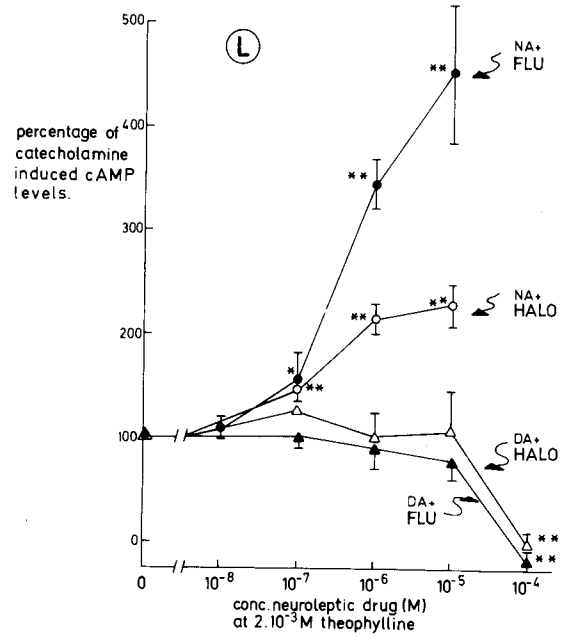

Fig. 5. DA- and NA-induced, neuroleptic drug affected cAMP-levels. cAMP values: mean ± S.E.M. (n= 4-12) in percentages of the pure DA- or NA-response at low P.D.-ase inhibition (L) by 2.10^{-3}M theophylline. Significant differences (Students-t-test) with respect to the pure DA-response (38 pmoles cAMP per mg protein) or with respect to the pure NA-response (58 pmoles cAMP per mg protein for the HALO-curve and 62 pmoles cAMP per mg protein for the FLU-curve):**P< 0.01 and *P<0.05. Enhancement of the NA-response and inhibition of the DA-response, both by HALO and by FLU, are clearly shown.

of A.C.-ases and P.D.-ases by neuroleptic drugs.

Results with Potassium Ions

In order to investigate the role of potassium-ions on the assumed catecholaminergic-postsynaptic-receptor coupled A.C.-ases together with the P.D.-ase coupled enhancement of the NA-response, we varied the K^+-ion concentration in the incubation medium. In one group of experiments we increased the K^+-concentration up to 50 mM. Increasing K^+-ion concentrations appear to induce increasing cAMP-elevations. This phenomenon of cAMP-synthesis induced K^+-ions, which is well known in brain slices (Huang and coworkers, 1972; 1973; Shimizu and Daly, 1970; 1972; Shimiza and co-workers, 1974) and in the retina (Brown and Makman, 1972), might be due to direct or indirect activation of A.C.-ases by the K^+-induced depolarization of A.C.-ase-containing membranes of cells. One of the ideas for an indirect activation of A.C.-

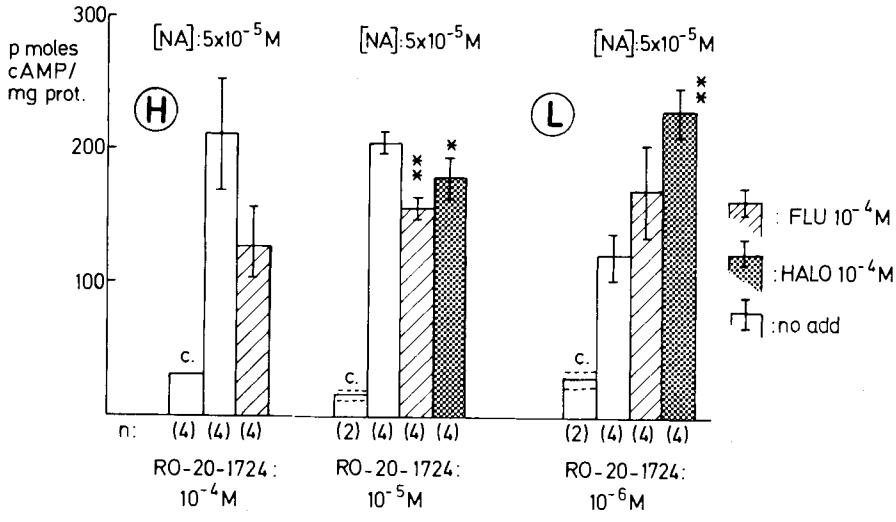

Fig. 6. NA-responses, affected by 10^{-4}M FLU and by 10^{-4}M HALO, at a low (L), a medium and a high (H) P.D.-ase inhibition using the P.D.-ase inhibitor RO-20-1724. Significant differences calculated as in Fig. 3. *P<0.05; **P<0.005. One can clearly see the partial inhibition of the NA-response at 10^{-4}M and at 10^{-5}M RO-20-1724. At 10^{-6}M RO-20-1724 we find a neuroleptic drug induced enhancement of the NA-responses.

ases is that membrane depolarization might give rise to an activation of the Na^+, K^+-ATP-ase resulting in a loss of ATP and a formation of adenosine. The adenosine formed is then said to migrate to a compartment, containing sufficient ATP. At the outside of the cell membrane of this compartment adenosine-affinity sites might be occupied by adenosine causing cAMP-synthesis (Huand and Daly, 1974a; Pull and Mc Ilwain, 1976).

The neuroleptic drugs FLU and HALO were found to inhibit the K^+- induced cAMP- elevation at low and high P.D.-ase inhibition (Table 1). A P.D.-ase coupled enhancement of the K^+-response at a low P.D.-ase inhibition could not be shown (Table 1). The NA-response was again enhanced at a low P.D.-ase inhibition at 15 mM K^+, but also at 4 mM K^+ and at 2 mM K^+. The enhancement of the NA-response at different K^+-concentrations could be observed even in the absence of a P.D.-ase inhibitor (Table 2). The enhancement of the NA-response could not be found in a K^+-free medium (Table 2).

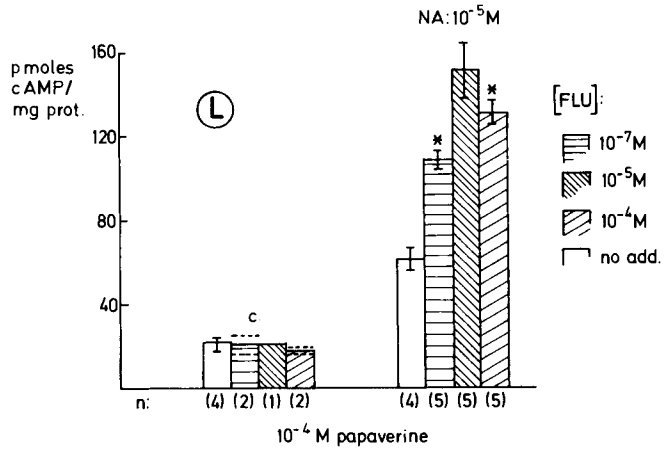

Fig. 7. NA-responses, affected by FLU, at a low (L) P.D.-ase inhibition using the P.D.- ase inhibitor papaverine. Significant differences calculated as in Fig. 4. The enhancement of the NA-response at 10^{-7}M FLU and at 10^{-5}M FLU is clearly visible. At 10^{-4}M FLU equilibrium of the counter-balance between A.C.-ase-inhibition and P.D.-ase-inhibition might have been reached.

DISCUSSION

Confirmation of the Hypothesis about the Involvement of P.D.-ases and A.C.-ases in the NA-cAMP-System

The rather high concentration of FLU and HALO (10^{-4} - 10^{-5}M) at which the antagonism on the catecholamine-receptor coupled A.C.-ases was seen, might be caused by the fact that we are working with the intact tissue of the rat retina. Entrance of the drugs occurs into all membrane structures of the tissue. Cell connections between these structures may hamper the movement of the agents applied to the tissue. Brown and Makman (1973) working with retinal homogenates found a nearly complete inhibition of the DA-induced cAMP-formation with 2.10^{-6}M FLU, which possibly shows a more easy arrival of the neuroleptic drugs at DA-receptors than we find in our intact retinae incubated in vitro.

The explanation for the effects of psychotropic drugs not being inhibitory for A.C.-ases and P.D.-ases primarily (Huang and Daly, 1974b; Pull and McIlwain, 1976), but having effects on cAMP-levels by their ability to cause depletion of A.T.P. and resultant formation and efflux of adenosine, looks to be questionable within the context of our findings. As lined out under Approach our hypothesis concerning the inhibitory action of neuroleptic drugs both on receptor-coupled A.C.-ases and on P.D.-ases can be checked in an intact cell system if both enzymatic processes are differently sensitive for the drugs. Again the difference in sensitivity found, might point to differences in penetration in several parts of the tissue. It might however be considered as a difference in sensitivity of the two enzymatic

TABLE 1. Inhibition of the K^+-Response by Neuroleptic Drugs

	theophylline	
	2.10^{-3}M (L)	10^{-2}M (H)
6.24 mM K^+ (normal)	21.4 ± 0.3 (9)	43.3 ± 7.3 (4)
20.0 mM K^+	46.3 ± 6.2 (4)	114.9 ± 10.9 (7)
+ 10^{-4}M FLU	n.d.	23.8 ± 0.6 (4)
+ 10^{-4}M HALO	n.d.	23.6 ± 1.3 (4)
+ 10^{-5}M HALO	29.3 ± 2.3 (4)	n.d.
30.0 mM K^+	62.5 ± 6.2 (4)	132.2 ± 12.5 (9)
+ 10^{-4}M HALO	17.9 ± 1.3 (4)	21.0 ± 2.3 (4)

K^+-induced cAMP-synthesis at a low (L) and a high (H) P.D.-ase inhibition; cAMP-values (pmoles cAMP per mg protein) as the mean ± S.E.M.; n, (between brackets), is the number of retinae used. Only inhibition and no enhancement of the K^+-response by neuroleptic drugs can be observed.

processes in the intact living cell membranes and moreover it can possibly be explained as a difference in compartmentalization of A.C.-ases and P.D.-ases either in the same cell or in different cells or cell types. These three explanations might be true or partly true; anyway, our results have undoubtedly offered strong evidence for the existence of a neuroleptic sensitive P.D.-ase belonging to the NA-cAMP-system. The NA-cAMP-system might be comparable to the adrenaline-cAMP-system as we found exactly the same enhancing effects of the adrenaline-response at low P.D.-ase inhibitions as we found with NA.

The absence of any P.D.-ase involved enhancement of the DA-response suggests the presence of a special NA-P.D.-ase compartment at which DA or DA-induced-cAMP does not have access.

Partial Inhibition of the NA-response by Neuroleptics

The partial inhibition of the NA-response (Figs. 4 and 6) found at incubation conditions with high P.D.-ase inhibition compared to the complete inhibition of the DA-response by these drugs might point to the presence of two types of NA-receptors: firstly, DA-receptor-coupled A.C.-ases might also be sensitive for NA and for neuroleptic drugs and secondly, a special type of NA-receptor-coupled-A.C.-ases might exist; they are only sensitive for NA, not for DA and they are possibly non-sensitive for neuroleptic drugs. Considering the partial inhibition with NA, these latter receptors are then responsible for the still existing NA-induced cAMP-level. This should be indicative of a difference in compartmentalization of the DA-sensitive-receptor coupled A.C.-ases and the NA-sensitive A.C.-ases. This explanation for the partial inhibition of the NA-response, compared to the complete inhibition of the DA-response, closely resembles the statements of Forn and coworkers (1974) and of Harris (1976). The difference in response quality is probably also an important indication of two different physiological roles for DA and NA.

Enhancement of the NA-response found with FLU and HALO, occurred also with (+)- and (-)-butaclamol and with cis- and trans-flupentixol. This means in our hypothesis

TABLE 2. The NA-Response at Different K^+-ion Concentrations at Low-inhibited and Actual P.D.-ase activity

	LOW P.D.-ase inh. * (2.10^{-3}M theophylline)		ACTUAL P.D.-ase activity* (no P.D.-ase inhibitor)	
15 mM K^+	31.5 ± 1.4	(4)	13.4	(2)
+10^{-5}M FLU	28.0 ± 7.2	(3)	15.3 ± 2.9	(3)
+10^{-5}M NA	131.0	(2)	96.0	(2)
+NA + FLU	233.0 ±12.1	(4)	200.4 ± 27.9	(4)
4 mM K^+	21.9	(2)	13.2	(2)
+10^{-5}M FLU	9.4	(2)	16.2	(2)
+ 5.10^{-5}M NA	63.8	(2)	53.9	(1)
+NA + FLU	268.9 ± 0.9	(4)	231.1 ± 45.4	(4)
2 mM K^+	18.5	(2)	11.3	(2)
+10^{-5}M FLU	19.8	(2)	10.0	(2)
+ 5.10^{-5}M NA	75.4	(1)	86.1	(2)
+NA + FLU	313.4 ±15.4	(4)	156.4 ± 57.8	(4)
0 mM K^+	6.8	(2)	7.5	(2)
+10^{-5}M FLU	6.1	(2)	6.9	(2)
+ 5.10^{-5}M NA	34.7	(2)	21.9	(2)
+NA + FLU	29.5 ± 2.6	(4)	21.3	(2)

*Values (pmoles cAMP per mg protein) as the mean ± S.E.M. (4 determinations); ±S.D. (3 determinations). The FLU induced enhancement of the NA-response is clearly shown. The enhancement is absent at 0 mM K^+.

that we found inhibition of the NA-P.D.-ase by both stereoisomers of butaclamol and of flupentixol. Moreover, we did not observe this in other brain areas occurring stereoselective antagonism against DA-receptors. Instead, we found antagonism by all of the stereoisomers used. Then the stereoselective action is probably absent in the albino-rat retina, and our findings are in contrast with Schorderet and co-workers (1978), who found stereoselective antagonism using stereoisomers against the DA-induced cAMP-synthesis in the intact rabbit retina.

K^+-induced cAMP-synthesis and the Absence of a P.D.-ase involved Enhancement of the K^+-response at a Low P.D.-ase Inhibition

Our preliminary findings with K^+-ions touch again the complex role which K^+-ions play in the extra-cellular and thus also in the intra-cellular environment in the tissue. With increasing K^+-concentrations the membranes of cells are depolarized and cAMP-synthesis occurs. Though depolarization might activate A.C.-ases in

general, the pure presence of K^+-ions might activate A.C.-ases or stimulate the formation of adenosine. Then the adenosine formed might activate adenosine-receptors resulting in cAMP-formation (Huang and Daly, 1974a, b). We found neuroleptic drugs to inhibit K^+-responses both at low and at high P.D.-ase-inhibition. No P.D.-ase involved enhancement of the K^+-response at low P.D.-ase-inhibition could be observed (Table 1). It is too early to take the risk of an interpretation of this, but if we consider the presence of the enhancement of the NA-response and the absence of an enhancement of both the DA-response and the K^+-response, then, as the DA-response, the K^+-response might be located in a special cell-type in which a neuroleptic sensitive P.D.-ase is not present. But it remains puzzling why we do not find an enhancement of the NA-response in the absence of K^+-ions. Does this mean that the P.D.-ases of the NA-system only work in the presence of K^+-ions? But what might then be the cause that the K^+-response does not show a neuroleptic induced enhancement at a low P.D.-ase inhibition? Do various P.D.-ases exist, some acting at the K^+-induced cAMP-levels and some others acting at the NA-induced cAMP-elevation? This question may partly be answered by the knowledge about the existence of multiple forms of P.D.-ases. These isoenzymes have been demonstrated in brain and in the retina (Farber and Lolley, 1973; Weiss and coworkers, 1974; Weiss, 1975).

The ideas about the existence of various isoenzymes might have relevance for the concepts outlined before. In contrast to the elevation of cAMP by an increase of the K^+-concentration, decrease of K^+-ions in the incubation media lowered the cAMP level (Table 2). How can this effect be understood from the hyperpolarization of membranes. Enhancement of the NA-response at low P.D.-ase inhibition and at a low K^+-concentration of 2 mM is still present (Table 2). However this enhancing effect disappears at 0 mM K^+. The NA-response, still being present at 0 mM K^+, is very low at a high P.D.-ase inhibition: about 50 pmoles cAMP mg protein; the NA-response at 6.24 mM K^+ (standard concentration) and at a high P.D.-ase inhibition is about 200 pmoles cAMP per mg protein. For the DA-response these cAMP-values are about 40 resp. 100 pmoles per mg protein. The decrease of 75% of the NA-response at 0 mM K^+ tempts to localize the major part of the NA-response, i.e. of the NA-receptor, in a K^+-sensitive cell compartment for example in the Müller-cells. These cells are glia cells and hence more susceptible for variation of K^+-ions (Hertz, 1973). Then the question if this also fits for the DA-receptors remains open. Anyway, the readiness for DA- and NA-affinity sites or -receptors for acceptance of DA and/or NA looks to be lowered in the absence of K^+-ions; the selectivity of the different receptors appear to remain the same under this condition (results not shown). In conclusion: are the P.D.-ases under consideration located at the innerside of the cell membrane or are the enzymes soluble in the cellplasma? How do neuroleptic drugs then affect these enzymes? Does the work of this kind of research indicate P.D.-ases instead of A.C.-ases to be key role enzymes for the therapeutic action of neuroleptic drugs? And if so, do these drugs have their therapeutic value for men by the increase of cAMP-levels in brain instead of by the decrease of them (Hess and coworkers, 1975)? Thus, one of the messages from this work points to the involvement of P.D.-ases and A.C.-ases in the mode of action of neuroleptic drugs (Huang and Daly, 1974b). This involvement is present in the retina and holds possibly for other brain areas as well. However, this involvement seems to occur only in the NA-cAMP-system. The retinal DA-system might have another physiological position by a different localization in

the retina or by another sensitivity of the DA- P.D.-ases compared to NA- P.D.-ases.

ACKNOWLEDGEMENT

I acknowledge the attention of the members of the Departments of Neurophysiology and Biological Psychiatry. I thank Mrs. G.Wortmann-Even for typographical, Mr. T. Deddens for graphical and Mr. B. Tebbes for photographical assistance.

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Neurochemistry Vol. 1, pp. 381-392.
Pergamon Press Ltd. 1980. Printed in Great Britain.

COMPOSITION AND BIOSYNTHESIS OF MOLECULAR SPECIES OF RETINA PHOSPHOGLYCERIDES

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ABSTRACT

Recent studies on the fatty acid composition and labeling by glycerol of individual molecular species of retinal phosphoglycerides are presented. Docosahexaenoate-containing species are important constituents of retina phospholipids. 22:6 and saturated fatty acids appear in similar amounts in hexaenes (nearly 50% each). Very high percentages of 22:6 and other polyenoic fatty acids are found in molecular species containing negligible amounts of saturated fatty acids, indicating the occurrence of dipolyunsaturated phosphoglycerides, including didocosahexaenoate. On the other extreme, fully saturated species occur. 22:6-containing species are synthesized at high rates in most phosphoglycerides including phosphatidylinositol, which is mainly formed by tetraenoic classes. Oligoenes and saturates are synthesized de novo and once formed they may serve as precursors of others (e.g. tetraenes) by acyl-exchange reactions. Propranolol induces rapid modifications in the de novo biosynthesis of retina glycerolipids. Oligoenes and saturates are the most stimulated species in PI and PS, and the most inhibited ones in PC. In PE, the labeling of these species is stimulated, while the polyenes are inhibited. This remarkable example of metabolic heterogeneity in a single phosphoglyceride opens new possibilities in the study of the regulation of retinal membrane lipid biosynthesis.

KEYWORDS

Retina, glycerophospholipids, molecular species, glycerol uptake, docosahexaenoate, arachidonate, polyenes, lipid synthesis, propranolol.

INTRODUCTION

Photoreceptor cells and especially their rod outer segments, have been the main subject of studies on retinal biochemistry to date, due to the development of methods for obtaining high purity-ROS preparations and to the interest in the mechanisms of phototransduction. Several investigations have dealt with the composition of phosphoglycerides and various aspects of their metabolism, but these topics are far from being completely understood. During the past few years we have been studying minor lipids like diacylglycerols, free fatty acids and phosphatidic acids from amphibian and mammalian retinas (Aveldaño and Bazán, 1974, 1977). Differences in the total

levels and fatty acid composition of these lipids in retina as compared with other tissues gave an insight about the structural and metabolic heterogeneity of retina phosphoglycerides. Parallel studies were carried out on the de novo biosynthetic pathways in the toad (Pascual de Bazán and Bazán, 1976) and the cattle retina in vitro (Giusto and Bazán, 1979a). Every phospholipid class, as well as neutral glycerides, were efficiently labeled with radioactive glycerol. The time course of the incorporation of this precursor showed a rapid labeling of phosphatidic acid, followed by that of phosphatidylinositol and diacylglycerols. Subsequently, interesting differences between cattle and toad retinas were found: in the former the biosynthetic pathway quantitatively favoured the route PA→ DG→ TG, whereas in the toad the biosynthesis of phosphatidylinositol predominated (Bazán and co-workers, 1976a). Integrity of the retina seemed a sine qua non requisite for an efficient lipid labeling, since the synthesis of some of them was abolished and showed a different pattern in cell-free preparations (Bazán and co-workers, unpublished).

The entire retina in vitro has also proven a useful model to study the effects of drugs on the metabolism of phospholipids in the CNS (Bazán and coworkers, 1976b). On the other hand, as shown in another chapter of this book, amphiphilic drugs may represent excellent tools to search for the regulation of lipid metabolism in the tissue. In retinas incubated with amphiphilic cationic drugs, an acute stimulation of acidic phosphoglyceride synthesis (PA, PI and PS) and a drastic inhibition of that of triacylglycerols and phosphatidylcholine takes place, phosphatidylethanolamine being inhibited to a lesser extent. Thus this was an interesting model to approach the problem of metabolic heterogeneity of retina phosphoglycerides. It is a well known fact that each phosphoglyceride class has its own defined fatty acid composition, which suggests some degree of selectivity of the biosynthetic enzymes towards certain molecular species of precursors. As an example, phosphatidylinositol contains exceedingly high amounts of arachidonate and stearate in the cattle retina, whereas phosphatidylethanolamine is mainly formed by docosahexaenoate and stearate This suggests that tetraenes are the major molecular species of PI and hexaenes those of PE. Which are the reactions leading to this specific composition? Do the biosynthetic enzymes select tetraenoic species of PA to be used in the route PA→ CDPDG→ PI on one hand and hexaenoic PAs for the route PA→ DG→ PC(PE)on the other? In other tissues like liver and brain the time-course of phosphatidylinositols labeling by various precursors injected in vivo showed that tetraenes may be synthesized by exchanging arachidonate for the fatty acid in the 2-position of glycerol in less unsaturated molecules of PI (Holub and Kuksis, 1971a, 1971b; Mac Dougall and others, 1975). Are these reactions operative in retina? This chapter summarizes some pieces of our recent work on the composition and biosynthesis of retina glycerolipid molecular species. It is shown that amphiphilic drugs can affect the labeling of the species of each phosphoglyceride to different extents, suggesting that some of them may be primarily involved in the de novo pathways.

FATTY ACIDS AND MOLECULAR SPECIES OF RETINA GLYCEROLIPIDS

The major fatty acids in cattle and toad retina glycerolipid classes and in some molecular species are presented in Table 1. High levels of docosahexaenoate characterize the composition of retinal phosphoglycerides as it has been known for some years (for references see Daemen, 1973). This fatty acid is highly concentrated in toad retina glycerolipids, probably reflecting the contribution of the outer segments of rods. The high percentage of 22:6 in phosphatidylinositol (25%) is surprising and contrasts with that found in cattle retina, where it only accounts for a 4% of the acyl groups. In both retinas, phosphatidic acid contains high levels of arachidonate and particularly of docosahexaenoate. This indicates that at least part of the polyenoic species of phosphoglycerides may be synthesized de novo by using the corresponding hexaenoic species of the precursor. The composition of diacylglycerols shows a further relative enrichment in hexaenoic molecular

TABLE 1 Percentages of representative Fatty Acids in Retina Glycerolipids and in major Molecular Species of Phosphatidylcholine (PC) and Phosphatidylethanolamine (PE).

		Cattle					Toad				
Glycerolipid		16:0	18:0	18:1	20:4	22:6	16:0	18:0	18:1	20:4	22:6
A)											
PA		18 ± 2.8	26 ± 2.1	11 ± 0.6	10 ± 0.6	17 ± 3.8	5 ; 7	17 ; 18	11 ; 10	16 ; 14	37 ; 36
DG*		18 ± 1.2	26 ± 1.6	9 ± 1.0	27 ± 1.8	7 ± 1.3	19 ± 2.0	20 ± 1.1	5 ± 1.0	7 ± 1.0	42 ± 4.0
PI		8 ± 0.6	34 ± 0.2	5 ± 0.2	44 ± 0.6	4 ± 0.4	13 ; 13	21 ; 23	9 ; 9	25 ; 25	25 ; 26
PS		1 ± 0.1	35 ± 1.4	7 ± 0.7	2 ± 0.2	39 ± 1.3	0.6;0.8	13 ; 13	1 ; 1	2 ; 2	61 ; 58
CL		6 ± 0.6	3 ± 0.5	17 ± 2.3	7 ± 1.0	8 ± 1.6	-	-	-	-	-
TG*		24 ± 1.9	17 ± 2.0	18 ± 2.0	5 ± 0.7	17 ± 4.2	17 ± 2	7 ±0.7	22 ± 4.0	4 ±0.2	18 ±1.9
PC		38 ± 2.1	15 ± 0.7	17 ± 0.2	5 ± 0.1	17 ± 0.9	23 ; 23	14 ; 16	16 ; 15	4 ; 4	28 ; 28
PE		8 ± 0.7	24 ± 1.6	5 ± 0.5	8 ± 1.0	42 ± 1.3	2 ; 3	10 ; 10	8 ; 8	6 ; 6	53 ; 50
B)											
Saturated	PC	80 ± 0.5	15 ± 0.5	-	-	-	70	8	-	-	-
	PE	45 ± 2.0	27 ± 3.0	-	-	-	48	16	-	-	-
Monoenoic	PC	35 ± 0.8	11 ± 0.5	48 ± 1.0	-	-	42	4	33	-	-
	PE	26 ± 7.0	28 ± 4.8	43 ± 5.7	-	-	28	7	36	-	-
Tetraenoic	PC	20 ± 0.6	21 ± 0.6	7 ± 0.3	37 ± 1.0	-	19	23	11	39	-
	PE	6 ± 0.7	32 ± 0.3	8 ± 1.3	45 ± 1.4	-	9	29	12	43	-
Hexaenoic	PC	14 ± 0.4	25 ± 0.5	3 ± 0.1	0.3± 0.04	54 ± 0.9	13	29	4	0.4	49
	PE	9 ± 1.2	29 ± 0.3	5 ± 0.6	0.3± 0.1	55 ± 1.9	6	23	15	0.4	50
Supraenoic	PC	3 ± 0.4	2 ± 0.3	2 ± 0.5	3 ± 0.1	77 ± 1.9**	4	4	4.5	9	60**
	PE	3 ± 1.3	5 ± 3.0	2 ± 0.7	4 ± 0.3	65 ± 3.2	0.4	0.3	0.6	3	65
Supraene I, microsomal PC***		-	-	-	-	95			N.D.		

Lipids were isolated by TLC (Rouser and others, 1971; Rodríguez de Turco and Bazán, 1977; Bazán and Joel, 1970). PCs and PEs were resolved by argentation-TLC after converting the phospholipids into acetyl-diglycerides (Parkes and Thompson, 1973,1975). Fatty acids were analysed by GLC. All the fatty acids in the samples were considered for the 100% values. *, Aveldaño and Bazán, 1974. **, other highly unsaturated fatty acids (22:4,22:5,24:6, etc) were also present. ***, the subfraction of the supraenes remaining at the origin of the $AgNO_3$-TLC plates.

species, as compared to PA, in the toad retina. This is consistent with the idea of selectivity suggested above, since it is evident that phosphatidate phosphohydrolase is more active towards hexaenoic than towards other species of PA on a percentual basis. In the cattle retina, on the other hand, arachidonate-containing species predominate in diacylglycerols. This is quite an amazing feature since tetraenes are not the major species in the lipids known to be formed from diacylglycerols, namely PC, PE and TG, and may reflect the contribution to the diglyceride pool of degradative reactions involving tetraenoic phosphoglycerides (Aveldaño and Bazán, 1974). A relationship between phosphatidylinositol and diglycerides has been previously proposed in the rat brain (Keough and others, 1972). Diacylglycerols enriched in arachidonate and stereate are rapidly produced in brain shortly after decapitation (Aveldaño and Bazán, 1975). Other reactions, such as the "back reaction" of choline (ethanolamine) phosphotransferase (Kanoh and Ohno, 1976) could contribute to this complex pool.

The molecular species of phosphatidylcholine and ethanolamine were separated by argentation chromatography after converting the phosphoglycerides into acetyldiglycerides (Parkes and Thompson, 1973, 1975). These non polar derivatives offer the advantages of giving far better and more reproducible resolution into molecular species than the intact phosphoglycerides, besides requiring lower activation temperatures. About eight groups of molecular species were identified in retina: saturates, monoenes, di-, tri-, tetra-, penta-, hexa-, and "supra"-enes. The saturates, containing exclusively saturated fatty acids, accounted for 18% of the phosphatidylcholines and 1% of the phosphatidylethanolamines (Table 2). It is evident from Table 1 that a great proportion of PC saturated species is dipalmitoyl-PC, i.e. 16:0 bound to positions 1 and 2 of the glycerol backbone, since palmitate accounts for an 80% of the fatty acids of this fraction. Other subspecies in the saturates may be 18:0-16:0 as well as other minor possibilities. In phosphatidylethanolamine, saturated molecular species are very low (Table 2), and their composition, (Table 1b), indicates that dipalmitoyl species are lower in PE than in PC on a percentual basis.

Monoenes are formed by a monoenoic fatty acid (oleic, palmitoleic, etc) and a saturated one, giving rise to phospholipids containing one double bound per molecule. These species are important constituents of phosphatidylcholine, and are very low in PE (Table 2). The saturated fatty acids are palmitic and stearic, the former predominating in monoenoic-PCs and the latter in monoenoic-PEs (Table 1b).

Major subspecies in the dienoic fraction are monoenoic-monoenoic and saturated-dienoic fatty acid combinations. The tetraenoic species are predominantly formed by arachidonate-stearate and arachidonate-palmitate pairs (Table 1b), the former predominating in PE and the second being more important among tetraenoic PCs. Other combinations like 18:1-20:3 are also probable constituents of this fraction. Major subspecies of the hexaenoic fraction may be docosahexaenoate-stearate and docosahexaenoate-palmitate on the basis of the fatty acid composition shown in Table 1b. Combinations like 18:1-22:5 may also occur in this fraction. Hexaenes are the predominant species of cattle retina phosphatidylethanolamines and the second in importance among chemical classes of PC (Table 2). Hexaenoic phosphatidylcholines predominate in the toad retina.

Molecular species even more unsaturated than the hexaenes occurred in the phosphoglycerides of both toad (Aveldaño and Bazán, 1977), and cattle retina. These species, which make up a 6% of the phosphatidylcholines, contribute to a 13% of the phosphatidylethanolamines in the mammalian retina. In the amphibian they form nearly half of the phosphatidylethanolamines (Table 2). Their fatty acid composition shows very high levels of docosahexaenoate (Table 1b) as well as of other polyenoic acyl groups, whereas the content of saturated fatty acids is very low. This indicates that some of the species that form this fraction are dipolyunsaturated, some of which may contain docosahexaenoate in both positions of the glycerol backbone.

TABLE 2 Molecular Species Composition of Retina Phosphoglycerides

	Phosphatidylcholines		Phosphatidylethanolamines	
Species	Cattle (4)	Toad (1)	Cattle (4)	Toad (1)
Saturates	18.3 ± 0.8	7.7	1.0 ± 0.1	2.0
Monoenes	28.5 ± 0.7	30.1	3.9 ± 0.4	1.8
Dienes	4.7 ± 0.2	4.9	2.7 ± 0.3	1.5
Tri- + Tetraenes	11.9 ± 0.2	12.3	12.0 ± 0.5	14.3
Pentaenes	3.4 ± 0.4	2.8	3.2 ± 0.5	3.1
Hexaenes	26.9 ± 1.0	38.2	63.9 ± 2.0	28.1
Supraenes	6.3 ± 0.2	4.3	13.0 ± 1.9	49.2

Results are expressed as weight %, and were obtained after resolving the acetyldiglycerides on silver ion-TLC and subjecting the methyl esters derived from each species to GLC. Methyl-nonadecanoate was used as internal standard.

In one case in which these species were separated, it was possible to find heptaenoic molecular species (mainly containing 18:1 and 22:6) all the way down to a species remaining at the origin of the $AgNO_3$-TLC plates which contained 95% docosahexaenoate. The pattern of molecular species found in retina phosphoglycerides is therefore more complex than the one reported for most tissues, going from fully saturated to exceedingly unsaturated molecules. Since the latter are not present in other neural tissues in signigicant amounts, it is possible that these species of phosphoglycerides are highly concentrated in the outer segments of photoreceptors. Triacylglycerols containing docosahexaenoate in the three positions of the glycerol backbone were reported to occur in the tapetum lucidum of the sand trout (Nicol and others, 1972). However this is the first case, to our knowledge, that didocosahexaenoyl-phosphoglycerides are found in microsomes from a mammalian tissue.

LABELING OF MOLECULAR SPECIES BY RADIOACTIVE GLYCEROL

The polyenoic molecular species take up relatively more radioactivity from ^{14}C-glycerol in the toad than in the cattle retina glycerolipids. This is in agreement with the higher proportion and higher unsaturation degree of phosphatidic acid and diglycerides in the toad retina. The distribution of radioactivity in phosphatidylcholines and -ethanolamines (Fig. 1) is similar to the distribution of mass (Table 2) suggesting no large differences in specific activities of the species in both animals. This was not the case for phosphatidylinositol, at least in the cattle retina, since a predominant proportion of the radioactivity appeared in the fraction including penta-, hexa-, and supraenes. Taken together, these species comprise less than 15% of the total mass of the lipid, which indicates their very high biosynthetic rate.

The "supraenoic" fraction is synthesized in retina at considerably high rates (Fig. 1). These species are labeled in diacylglycerols at early incubation times, suggesting that supraenoic phosphoglycerides may be synthesized from the corresponding species of 1,2-diacylglycerols. These species also occur and are labeled from glycerol in phosphatidylinositol and phosphatidylserine (Figs. 4 and 5).

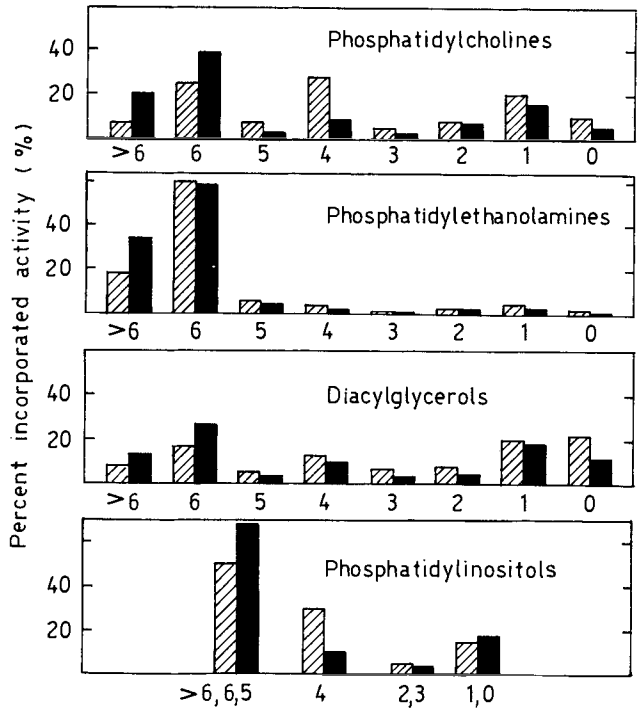

Fig. 1.- Comparison of the distribution of radioactivity from labeled glycerol among molecular species of cattle (▨) and toad (■) retina glycerolipids. The retinas were incubated for 30 min in the medium of Ames and Hastings (1956) in the presence of 5 µCi each of ^{14}C-glycerol. The molecular species of PC, PE and DG were resolved as acetyldiglycerides (Parkes and Thompson, 1973, 1975) and the PIs by spotting the intact phosphoglyceride (Holub and Kuksis, 1971 c).

The time-course of labeling of PCs and PEs shows that the polyenes are synthesized at relatively higher rates than the oligoenes (Fig. 2). This is also the case in diacylglycerols, where monoenes and saturates are produced at nearly linear rates while the uptake of radioactivity in the polyenes began to be decreased. In spite of the fact that tetraenes are the major fraction of diglycerides, they are not the most labeled, as observed in rat brain (MacDougall and others, 1975).

When retinas were incubated in the absence of labeled glycerol, after a preincubation with the precursor (Fig. 3) total diglyceride radioactivity rapidly dropped, indicating prelabelled diacylglycerols were being used for the biosynthesis of PE and PC. Again, the biosynthesis of polyenoic species of phosphoglycerides is faster than that of oligoenes, which are still increasing at 40 min incubation.

It has been shown in rat liver that 2-linoleyl-PC and 2-hexaenoyl-PE are the major products of the de novo pathway from 1,2-diacylglycerols (Kanoh and Ohno, 1976).

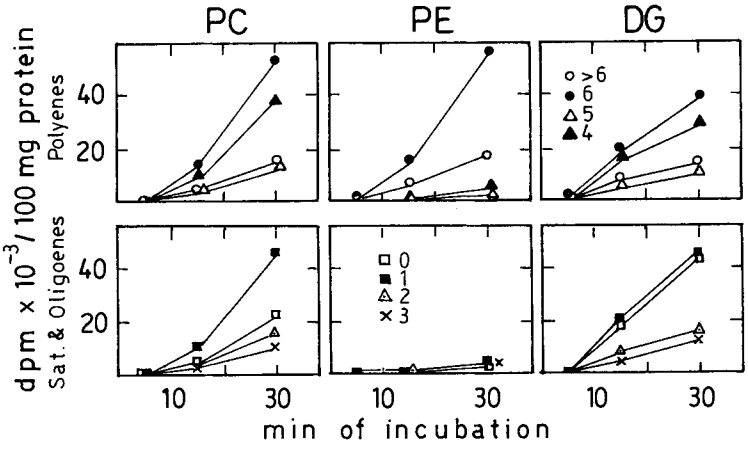

Fig. 2.- Incorporation of radioactivity from ^{14}C-glycerol in cattle retina phosphatidylcholines, -ethanolamines, and diacylglycerols. The retinas were incubated for the specified intervals in the presence of the precursor (5 µCi/retina).

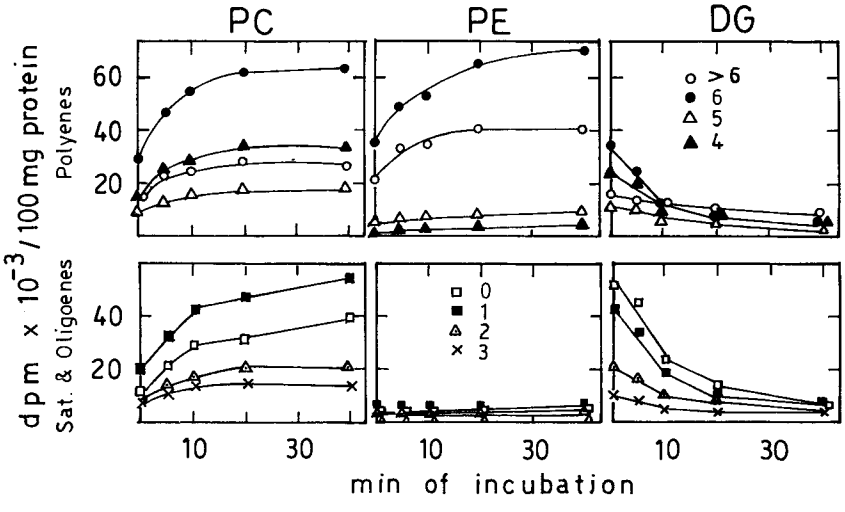

Fig. 3.- Labeling of phosphatidylcholines, -ethanolamines, and diacylglycerols after removing radioactive glycerol. Cattle retinas were incubated for 15 min with 5 µCi/retina of ^{14}C-glycerol. After extensive washings, incubation continued in glycerol-free media.

The data in figs. 3 and 4 suggest that hexaenoic phosphoglycerides may be synthesized de novo in the retina, as suggested by their high rates of labeling and by the fact that hexaenoic diglycerides are exhausted faster than other species upon withdrawal of glycerol. The same possibility is suggested from the high levels of docosahexaenoate in phosphatidic acid (Table 1 and Giusto and Bazán, 1979b). The changes in diacylglycerols do not account for the changes in phosphatidylcholines and-ethanolamines, but it should be kept in mind that an important proportion of the diglycerides are used for the synthesis of triglycerides in the cattle retina. Preliminary data suggest that saturated species of TG concentrate most of the glycerol in our in vitro model.

In separate experiments carried out with ^{3}H-glycerol, the distribution of the label among phosphatidylinositols changed as a function of time after removal of the precursor (Fig. 4). After a maximum taking place at 5 min, the percentage of radioactivity in oligoenes and saturates shows a continuous drop, while that in the tetraenes is increased. This suggests that tetraenoic phosphatidylinositols may be synthesized in retina from oligoenoic species trough acyl-exchange reactions introducing arachidonate as shown in liver and brain (Holub and Kuksis, 1971a, 1971b; MacDougall et al., 1975).

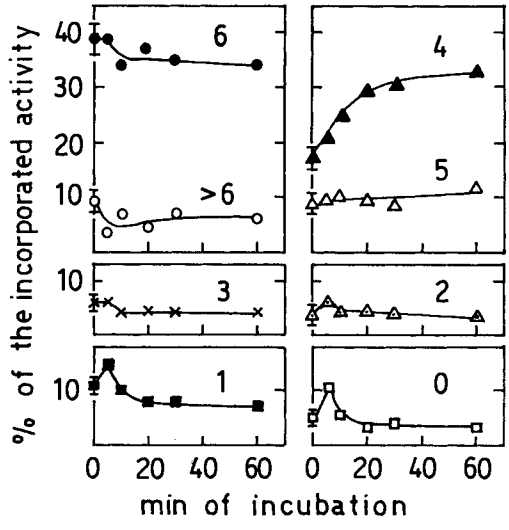

Fig. 4.- Distribution of radioactivity among molecular species of phosphatidylinositol, after removing radioactive glycerol. The retinas were incubated with ^{3}H-glycerol during 10 min. Then it was washed out and incubation proceeded for an additional period of 40 min.

EFFECT OF PROPRANOLOL ON THE LABELING OF MOLECULAR SPECIES OF RETINA PHOSPHOLIPIDS

Short-term incubations of retinas with propranolol result in a stimulated synthesis of phosphatidylinositol and phosphatidylserine (Ilincheta and others, this book). Table 3 shows that the incubation for only 10 min with the drug causes a drastic unbalance in the synthesis of molecular species of both lipids, saturated and oligoenoic species being more stimulated than the polyenes.

TABLE 3 Effect of Propranolol on the Percentage of Radioactivity appearing in the Saturated + Oligoenoic Phosphatidylinositols and Phosphatidylserines.

	Controls (4)	Propranolol (4)
Phosphatidylinositols	25.4 ± 3.1	46.2 ± 3.7
Phosphatidylserines	39.9 ± 7.5	69.9 ± 4.4

The retinas were incubated for 10 min in the presence or absence of propranolol (0.5 mM). ^{3}H-glycerol was added and incubation proceeded for an additional period of 10 minutes. Lipids were extracted and molecular species resolved on $AgNO_3$-TLC, in the form of acetyldiglycerides. The radioactivities appearing in the polyenes on one hand and in the saturates + oligoenes on the other were added, and percentages calculated.

The changes elicited by propranolol in the labeling of phosphatidylinositols, -serines, -cholines and -ethanolamines in a microsomal preparation obtained after incubating bovine retinas for 30 min in the presence of glycerol are shown in Fig. 5. The labeling of monoenoic and saturated PCs is inhibited 15 fold while the other species are inhibited about 10 fold as compared to controls. The stimulation of these species in PI is about 7 fold, while the rest of the species undergo a 3 to 5 fold stimulation during this period. The highest changes in saturated and oligoenoic species appear in phosphatidylserine: they are increased about 10 times in the presence of other species stimulation of 2 fold.

In contrast with phosphatidylcholine and triacylglycerol, the inhibition of the labeling of phosphatidylethanolamine by propranolol is weaker. Moreover, depending on the time of preincubation with the drug, no inhibition at all has occurred in some experiments. When the effect of propranolol was surveyed in phosphatidylethanolamines (Fig. 5), the polyenes were inhibited while the saturates and oligoenes were stimulated. This is a remarkable example of metabolic heterogeneity in a single phosphoglyceride and with a single precursor, and suggests a different metabolic origin of molecular classes of phosphatidylethanolamine.

The inhibited species could mainly arise from diacylglycerols through ethanolamine phosphotransferase activity as it is probably the case for phosphatidylcholines through choline phosphotransferase. Due to the definite stimulation of the oligoenoic+saturated species in phosphatidylserine, which attain very high specific activities if the mass involved is considered, a precursor-product relationship among

both phosphoglycerides in retina is suggested, specifically involving these species. ^{3}H-labeled serine is actively taken up by phosphatidylserine and phosphatidylethanolamine, perhaps through phosphatidylserine decarboxylation, the overall process being stimulated by propranolol (Ilincheta and others, this volume).

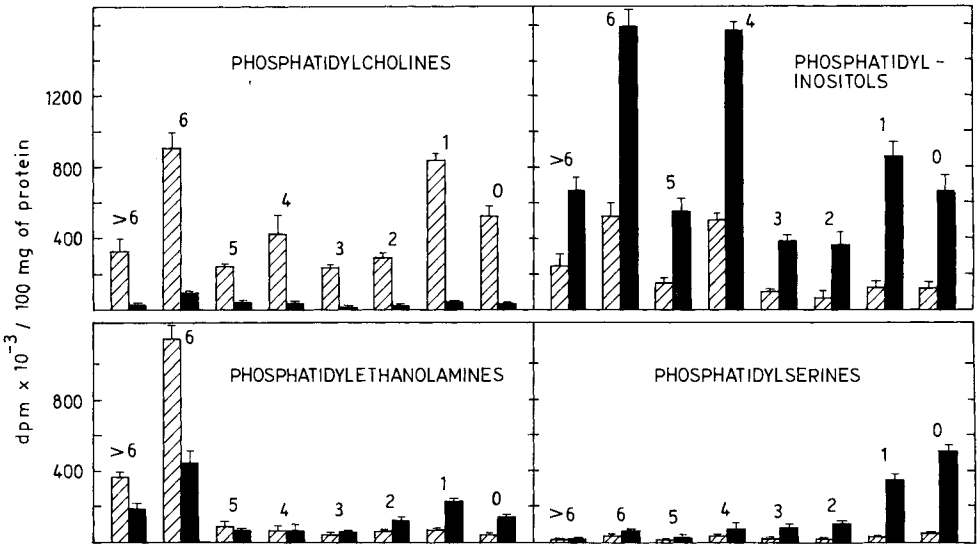

Fig. 5.- Effect of propranolol on the labeling of molecular species of microsomal phosphoglycerides obtained from cattle retinas incubated 30 min with ^{3}H-glycerol. The retinas were preincubated for 10 min before the addition of glycerol; the incubating media contained no $CaCl_2$ and 0.5 mM (■), or no (▨) propranolol. Microsomes were prepared, lipids were extracted and molecular species resolved.

When an entire tissue like the retina is incuabated 30 min with an ubiquitous precursor like glycerol, besides assesing de novo synthesis the contribution from one or more of the following reactions to the final distribution of radioactivity among species of phosphoglycerides may be expected: a) acyl-exchange reactions (any phospholipid); b) N-methylation (species of PE→PCs); c) reversion of the biosynthetic routes (radioactive PCs or PEs→labeled DG, which in turn could be recycled); d) decarboxylation (PCs→PEs); e) base-exchange reactions (PCs↔PEs↔PSs); f) other reactions. The specificities of some of these reactions have been investigated in other tissues but the retina has still many answers to give, by using suitable precursors and pharmacological tools, on the regulation of the intermediary metabolism of its lipids.

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Neurochemistry Vol. 1, pp. 393-403.
Pergamon Press Ltd. 1980. Printed in Great Britain.

SYNTHESIS AND BREAKDOWN OF DIFFERENT SIZED RETINAL PROTEINS IN DARKNESS AND DURING PHOTIC STIMULATION

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ABSTRACT

Rabbit retinas were maintained in vitro in medium resembling cerebrospinal fluid and were exposed to ^{3}H-leucine or ^{14}C-leucine in double-labeling experiments designed to measure rates of protein turnover and to determine the effects of photic stimulation on protein synthesis and degradation. The retinas were solubilized, and the proteins separated according to size by polyacrylamide gel electrophoresis. The gels were cut into 95 slices and each slice differentially counted. Protein content of the slices was estimated from Coomassie Blue staining, and molecular weight (MW) from distribution along the gel of MW standards.

The retinas appeared to function nearly normally in vitro for many hours. Protein renewal was rapid and reproducible at 0.55 ± 0.01 (S.E.M.) %/h and remained quite constant for at least 7 h. Synthesis and degradation were approximately equal. Two retinas were maintained in vitro at 37°C for 52 h, and showed good preservation of morphology, electrophysiological response to light, and protein synthesis.

Total synthesis of new polypeptides was at the rate of 103 nmole per g of protein, per h; there was a sharp peak in the 33,000 to 43,000 MW range. Proteins in every size group were very heterogeneous with respect to breakdown coefficients (i.e. longevity) which were the prime determinants of the amount of each protein present. Fractional renewal showed a highly significance ($p < 0.0001$) correlation with MW, due apparently to a reduction in maximal longevity as size increased. Neither synthesis nor degradation was significantly affected by intense continuous light or flashing light, though the latter increased uptake of 2-deoxyglucose by 38%.

KEYWORDS

Retina; rabbit; in vitro; polyacrylamide gel electrophoresis; protein synthesis; protein degradation; photic stimulation; 2 deoxyglucose

INTRODUCTION

Reactive cells in retina undergo large and sometimes sustained changes in their level of activity in response to photic stimulation. And they exhibit correspond-

ingly large changes in the chemical reactions responsible for the physiological function. Yet, in spite of these shifts in portions of their metabolism, the cells are able to continue to respond reproducibly and, in some instances, to modify their response in an adaptive fashion. As in other tissues, this is probably accomplished by appropriate adjustments in the activity of critical enzymes and transport proteins. Feedback reactions between function and protein metabolism assume a particular interest in tissues such as retina and other portions of the nervous system in which there is a requirement for high-precision performance.

Study of these phenomena imposes severe demands on the experimental preparation. It must include all of the components making up the operational entity, in their normal functional array. It must be accessible to the introduction and removal of labeled substrates. And the investigator must have control of function over a major portion of the physiological range. The isolated retina appears almost uniquely capable of meeting these requirements, and we have been trying for some years to realize some of the advantages it offers. This report will first summarize experiments to develop, validate, and characterize the preparation and will then briefly describe some preliminary applications.

Our goal was to develop methods for maintaining rabbit retina *in vitro* that would make it possible to demonstrate: (1) protein synthesis at normal or near-normal rates; (2) balance between synthesis and breakdown under control conditions; (3) stability over several hours (to permit measurements of breakdown as well as synthesis; (4) continued responsiveness to photic stimulation. In order to survey the responses of different types of proteins, we developed modifications of existing methods for promptly solubilizing all of the proteins and for separating them into 95 fractions according to size by gel electrophoresis. We then sought to use this experimental system to characterize retinal protein turnover in a general way and to see how synthesis and degradation were affected by marked, sustained changes in the level of physiological activity.

METHODS[1]

Isolation and incubation were carried out in a warm (37°C) room under very dim red light. Eyes removed from dark-adapted anesthetized rabbits were hemisected and the vitreous removed. The eye cup was everted over the rounded end of a Teflon rod and the retina gently separated from the pigment epithelium with a small glass rod. Choroid and sclera were cut from around the optic disc, leaving the retina attached to a short segment of optic nerve which served as a handle. Incubations were in 6 ml (for isotopic labeling) or 20 ml of medium, contained in small or large glass "boats" (Fig. 1) whose rocking was adjusted to create shear between tissue and medium, with minimal mechanical damage. The medium was equilibrated with a gas phase flowing through the top of the boat that contained 5% CO_2, 40% O_2 and was saturated with water vapor. A thermostated water bath maintained boat temperature at 37 ± 0.1°C. In an effort to match the fluid that had bathed the retina *in vivo*, the medium contained 48 defined constituents and ½% serum (Table 1). The concentrations of the defined constituents were based on those reported for human CSF. Leucine was always 11 μM, but the unlabeled leucine was sometimes replaced by L-[U-^{14}C] leucine (300 Ci/mol) or L-[4,5(n)-^{3}H] leucine (3000 Ci/mol). Labeling pulses were ½ h. Retinas used to measure synthesis were harvested promptly after labeling, having been returned to unlabeled medium for ¼ h for removal of labeled free leucine

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[1] The methods described here are those used in the experiments involving electrophoresis and differ somewhat from those used in the previously published studies that are cited.

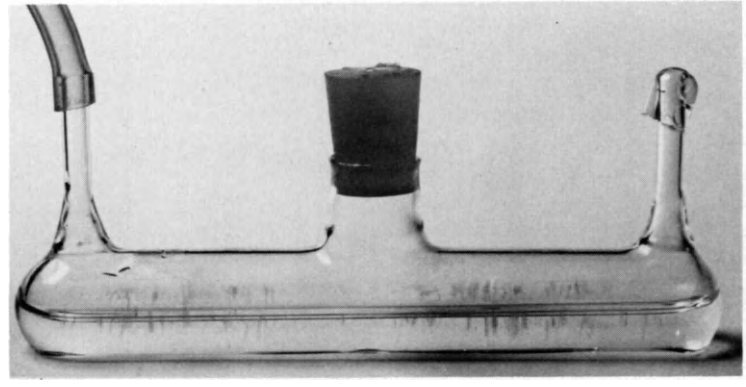

Fig. 1. Boat with 20 ml of medium. Stopper and inlet tube for gas are of silicone rubber. Light plastic cap covers gas outlet. Boat was tipped by rocker through 3^{o} in long axis, at 1 Hz.

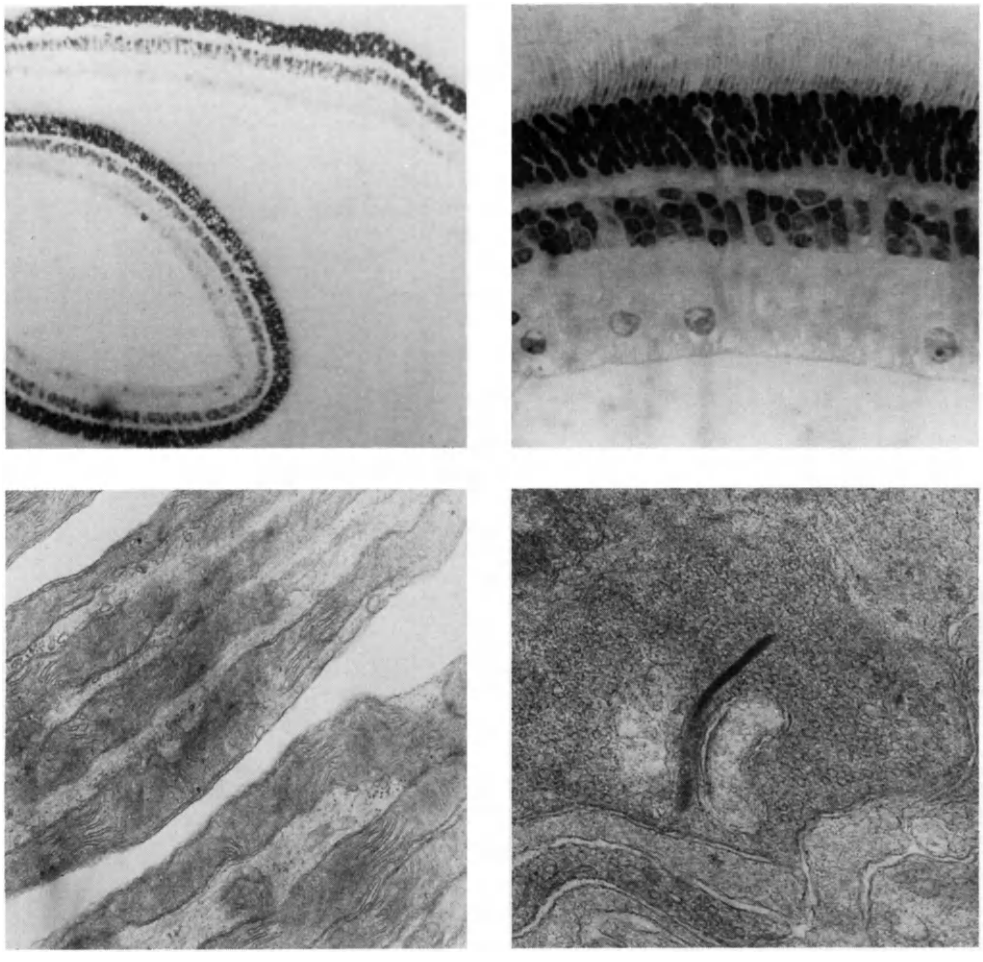

Fig. 4. Examples of regions showing well preserved morphology after 52 h *in vitro*. Light micrographs are magnified 85 x and 360 x. Electronmicrographs show mitochondria of receptor cell inner segments on left at 13,000 x magnification, and receptor cell synapse on right at 17,000 x.

TABLE 1 Composition of Control Medium

Electrolytes	Na^+	143.0	Cl^-	125.4		
m-equiv/l	K^+	3.6	HCO_3^-	22.6		
	Ca^{++}	2.3	$H_2PO_4^-$	0.1		
	Mg^{++}	2.4	$HPO_4^=$	0.8		
			$SO_4^=$	2.4		
		151.3		151.3		
Amino Acids	ASP	0.9	VAL	15.0	LYS	20.0
µM	THR	28.0	(CYS)2	0.2	HIS	12.0
	SER	24.0	MET	2.6	TRY	2.4
	GLU	7.0	ILEU	4.4	ARG	20.0
	PRO	0.6	LEU	11.0	TAU	6.0
	GLY	6.0	TYR	8.0	ASPn	6.4
	ALA	27.0	PHE	8.0	GLUn	500.0
Vitamins	D-Ca Pantothenate		0.21	Thiamine HCl		0.30
µM	Folic Acid		0.23	Biotin		0.41
	Nicotinamide		0.82	Choline chloride		5.02
	Pyridoxal HCl		0.49	i-Inositol		150.9
	Riboflavin		0.027	Ascorbic Acid		102.2
Purines & Pyrimidines µM	Hypoxanthine	6.0		Uridine	3.0	
	Cytidine	3.0		Thymidine	1.0	
Other	Glucose 6mM		Na pyruvate 0.12 mM		Horse serum 0.5%	
	pCO_2 36 torr		pO_2 285 torr			

($t_{\frac{1}{2}}$ = 1.2 min; cf. Ames, Parks, and Nesbett, 1976) and completion of peptide chains initiated with the label. Retinas used to measure degradation were returned to unlabeled medium for 1¼ or 4 h before harvesting. Retinas were sometimes photically stimulated during the incorporation of labeled leucine and sometimes during degradation of the labeled proteins. Most of the experiments involved double-labeling—sometimes in the same retina (exposed to 1 label under one condition and to the other label under a second condition), and sometimes involving 2 retinas which were then combined for analysis.

Measurements and calculations. At harvesting, the retina was cut from the nerve stump to fall into liquid N_2, then lyophilized and weighed. The tissue was solubilized completely within 5-10 min by a combination of sodium dodecylsulphate, EDTA, urea, dithiothreitol (followed later by iodoacetamide), sonication and heat to 100°C. Samples were removed for measuring the labeling of total protein and for polyacrylamide gel electrophoresis, performed by a modification of the method of Fairbanks and co-workers (1971). The gels were stained with Coomassie Blue, aligned by staining pattern, and up to 12 of them were cut simultaneously into 95 slices each by a multiblade slicer. Labeling medium, solution of total protein, and solubilized gel slices were differentially counted with correction for quenching. Leucine incorporation was calculated from the radioactivity of the tissue specimens and specific activity of the medium, with correction for the difference in specific activity between medium and intracellular fluid (cf. Ames and Parks, 1976). Approximate molecular weight (MW) of protein in each slice was calculated from the distribution of 6 MW standards that were subjected to electrophoresis with and

without the retinal proteins. Changes in density of protein along the gel were estimated from densitometer readings of the Coomassie Blue staining, and these data were used to calculate the fractional distribution of protein among the slices.

The methods used in these experiments have been described elsewhere in greater detail (Ames, Parks and Nesbett, 1979a, b).

RESULTS AND DISCUSSION

Characteristics of the in vitro preparation. Data on overall protein metabolism have been previously described (Parks, Ames and Nesbett, 1976). Leucine was incorporated into the total protein of the isolated retina at the rate of 2.03 ± 0.04 (S.E.M.) μmole/g dry wt. of retina/h. Divided by the leucine content of retinal proteins (369 μmol/g dry wt.), this gave an average renewal rate of 0.55%/h which is similar to published figures for protein synthesis in mammalian CNS in vivo. Synthesis depended on the presence of physiological levels of nutrients in the medium. For example, it fell 34% when the amino acids were omitted (Fig. 2). From measurements of net changes in 8 essential amino acids in the medium during 1 h of incubation, the average value for the ratio of (release from protein)/(incorporation into protein) was calculated to be 0.98 ± 0.07 (S.E.M.), showing that breakdown approximately equalled synthesis. Measurements of labeled leucine incorporation were made for up to 7 h in vitro, and showed that synthesis was constant during this period.

The isolated retina appeared to remain in a near physiological state for much longer than 7 h. In a recent study, retinas were maintained in the rocking boat for 52 h at 37°C, with changes of medium every 6 h. Microelectrode recordings (Fig. 3) of ganglion cell response to photic stimulation remained quite normal with respect to the number of responsive cells, the vigor of their reponses, and the functional classes of ganglion cell observed. The only evident change with time was a subtle reduction in the extent to which lateral interactions could be demonstrated, i.e., center-surround antagonism and directional sensitivity. Even after 52 h in vitro, protein synthesis was reduced by only 1/3, so that the fractional renewal had fallen from 0.55%/h to 0.36%/h (2 experiments). Morphology (Fig. 4) remained nearly normal, to both light and electron microscopy, in many areas. There were regions of abnormal structure due, at least in part, to mechanical damage from the repeated flexing. In retinas kept immobile in a superfusion chamber (see Ames and Pollen, 1969), Dr. Richard Masland has observed excellent preservation of structure and (in 1 experiment) electrophysiological responses, after 50 h in vitro, that were indistinguishable from those of freshly isolated retina.

Electrophysiological recordings from isolated retina had shown marked increases in ganglion cell activity with photic stimulation. However, we sought a more integrated measure of the retina's response for the protein studies; and for this purpose used the uptake of ^{14}C-2 deoxyglucose (^{14}C-DG), measured either by its loss from the medium or its accumulation in the tissue. In preliminary experiments, we varied the parameters of photic stimulation and obtained a maximal, sustained response with 10 msec flashes at 4 Hz with intensity of 1 ft. lambert. This stimulus increased ^{14}C-DG uptake from 24.9 ± 1.0 ml of medium cleared per g dry wt. per h in darkness to 34.3 ± 2.1 with the stimulus—an increase of 38% ($p=0.005$) for the retina as a whole, and presumably a much larger percentage increase for the cells most affected by the flash. This stimulus was used in the experiments on protein metabolism.

On the basis of the characteristics cited above, the in vitro retina appeared to be a suitable test object for the studies proposed.

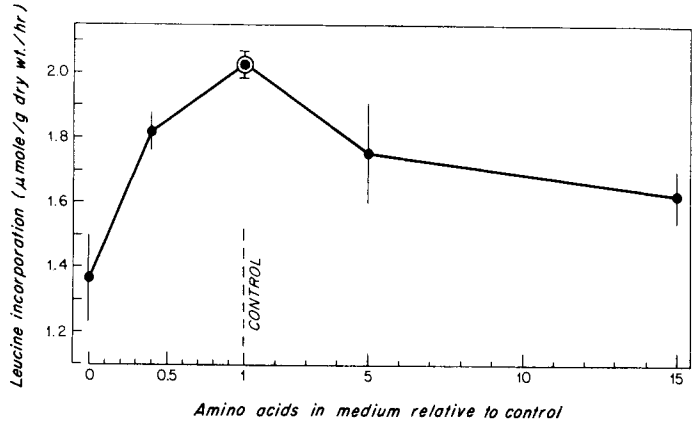

Fig. 2. Effect of the level of amino acids in the medium on protein synthesis. All of the amino acids except leucine were varied together from 0 to 15 x their control concentrations, as shown on abscissa. Open circle with vertical bar shows mean ± S.E.M. for 20 controls. Closed circles with thin lines show mean and range for test retinas. Reduction in leucine incorporation was significant ($p < 0.01$) when the other amino acids were 0,0.04 and 15 x their normal concentrations. From Parks, Ames, and Nesbett, 1976.

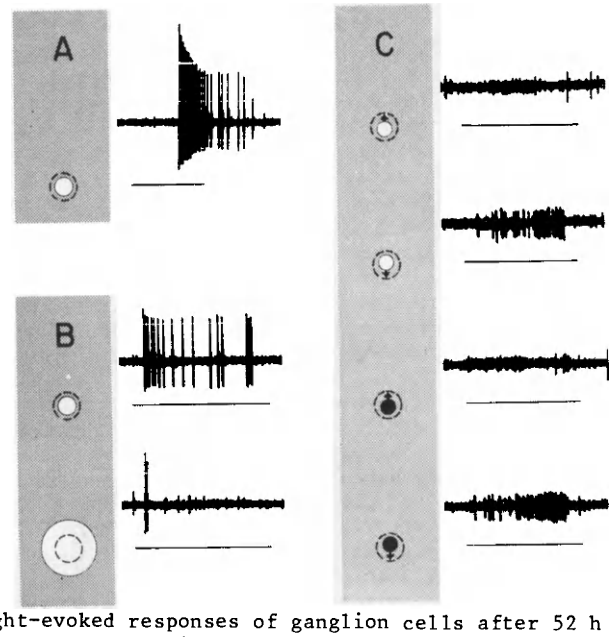

Fig. 3. Light-evoked responses of ganglion cells after 52 h in vitro. Line beneath record marks duration of stimulus, and diagram to the left shows its relation to central portion of receptive field (indicated by dashed circle). A, On-center cell firing rapidly in response to light spot in the center of its receptive field. B, Another on-center cell showing surround antagonism when light spot is enlarged to include receptive field surround. C, Directionally sensitive cell responding to downward, but not upward, movement of stimulus whether it be a light spot (above) or a dark spot (below). Stimulus duration 90 msec in A, 800 msec in B and C.

Synthesis and degradation of different sized retinal proteins. Synthesis was measured in 31 retinas that were harvested promptly after labeling and then subjected to gel electrophoresis. Leucine incorporation into protein increased rapidly as molecular weights rose to about 40,000, then fell progressively as molecular weights rose further to above 200,000. By dividing the amount of newly synthesized protein, determined by leucine incorporation, by the molecular weight attributable to each slice, an estimate could be made of the number of new polypeptides being formed per unit time. Total synthesis was calculated to be at the rate of 103 nmole per g of retinal protein, per h. There was a broad peak between 20,000 and 65,000 MW, with the most rapid synthesis between 33,000 and 43,000 at 2 nmole per g, per h, per 1000 increment in MW.

The fractional renewal rate for the proteins in each slice was calculated by dividing the amount of new protein in the slice, estimated from the labeled leucine incorporation, by the total protein in the slice, estimated from the Coomassie Blue staining. These data revealed a highly significant ($p < 0.0001$) direct correlation between fractional renewal and molecular weight. In a steady state system, the fractional renewal per unit time is a direct reflection of the breakdown coefficient. Delinger and Schimke (1971) first reported an association between size and degradative rate, and it has since been found rather consistently for proteins from a variety of tissues (see reviews by Goldberg and Dice, 1974, and Goldberg and St. John, 1976).

Double labeling techniques were used to measure the loss of labeled protein following the labeling pulse. Overall loss was 16% in 1 h and 23% in 3 3/4 h. Separating the proteins on the gel revealed significant differences in the rates of degradation of newly synthesized proteins as a function of their size. It will be noted that the fraction of the newly synthesized protein that was degraded per h (16%) was about 30 fold greater than the fraction of total protein that was synthesized per h (0.55%) in a system in which total degradation was approximately equal to total synthesis (see above). This is to be expected if the proteins are heterogeneous with respect to turnover, since the short labeling pulse preferentially labels the proteins with short half lives, which then contribute disproportionately to the breakdown measurement. Comparing the rate of degradation of the newly synthesized proteins with the rate of overall synthesis not only served to demonstrate heterogeneity but also provided some degree of quantitation. That is, the single measure of synthesis and the 2 measurements (at 1 h and 3 3/4 h)of degradation of newly synthesized protein permitted the contents of each slice to be described in terms of 2 subgroups, one representing the fast turnover proteins and the other the slow (see Appendix). This analysis showed that the contents of all the slices were markedly heterogeneous with respect to turnover, with half-times for turnover of the slow subgroup ranging from 50 to 1000 fold greater than half-times for turnover of the fast subgroup. Rates of synthesis varied much less than the breakdown coefficients, so that the latter were the prime determinants of the amount of protein present. As MW increased, the breakdown coefficient of the slow group increased (Fig. 5), but not of the fast subgroup. This indicates that the correlation between MW and turnover, observed for the total contents of the slices (see above), reflects a reduction in the maximal longevity of proteins as their MW increases. This interpretation is consistent with data reported by Dice and Goldberg (1975). The fact that the relationship shown in Fig. 5 is quite constant over the entire molecular weight range and extrapolates nearly to the origin suggests that there is iome rather non-specific mechanism of attrition whose rate is proportional to the size of the protein.

Effect of photic stimulation on synthesis and degradation. In some experiments, one retina was exposed to flashing light (described above) while incorporating leucine carrying one label, and its sister retina remained in darkness while incorporating

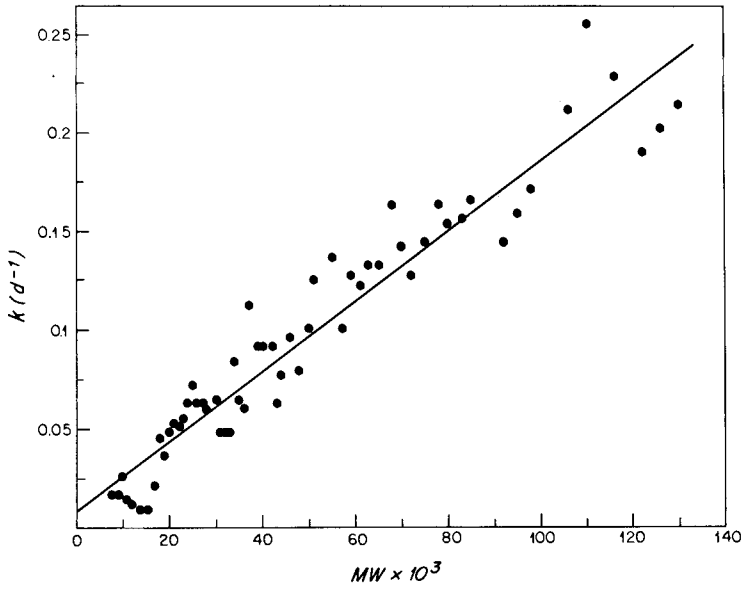

Fig. 5. The fraction of protein being degraded per day has been plotted against molecular weight. The proteins in each gel slice were characterized (see text) in terms of 2 subgroups, one representing the more slowly turning-over species and the other the more rapidly turning-over species. The breakdown coefficients (k's) shown here are those for the slow subgroup of each slice. Molecular weights (MW) were assigned to the slices on the basis of the distribution along the gel of MW standards. Line was fitted to data by regression analysis. Correlation between k and MW was highly significant ($p<0.0001$).

leucine with the other label. ^{3}H-leucine and ^{14}C-leucine were alternated as test and control labels in successive experiments. The 2 retinas were then combined for solubilization, fractionation, and differential counting. A third, "equivalence retina", was incubated in a 1:1 mixture of the 2 labeling media being used that day and then analyzed with the test-control pair to determine the ratio of isotopes in each slice if the stimulus had no effect. In calculating rates of synthesis in these experiments no correction was made for the reduction in specific activity of leucine in the intracellular fluid, it being assumed the same for test and control (a realistic assumption since, as will be shown, the stimulus had no measurable effect on protein breakdown). The flashing light had no effect on the rate of labeled leucine incorporation into total protein, which was 1.33 $\pm$ .022 (S.E.M.) nmole x g^{-1} x h^{-1} in the controls and 1.31 $\pm$ .022 in the stimulated retinas. And it had no effect on leucine incorporation into any of the 95 fractions separated on the gel, the largest difference between test and control for any slice being 1.7%, with a difference of 3% being required for significance at the $p \leq 0.05$ level.

In other experiments pairs of retinas were labeled in darkness, one with ^{3}H-leucine and the other with ^{14}C-leucine; they were then maintained for a subsequent 4 h in unlabeled medium with one now exposed to flashing light and the other continuing in darkness. The flashing light had no significant effect on degradation of total protein, there being 1.11 $\pm$ .029 (S.E.M.) nmole x g^{-1} x h^{-1} of protein-incorporated leucine remaining in the controls and 1.13 $\pm$ .050 in the test retinas. And it had no

effect on degradation of the proteins in any of the 95 fractions, the largest difference between test and control being 2.7% and not significant.

In still other experiments, retinas were labeled with one isotope in darkness and then, sometime later, with the other isotope while being exposed to intense, continuous illumination. Retinas, similarly labeled but in darkness throughout, served as controls. Though the light promptly bleached the rhodopsin (to the investigator's eye), it had no significant effect on the degradation of protein carrying the first label or on incorporation of the second label.

Thus, large and sustained changes in the retinas' functional activity had no demonstrable effect on either the synthesis or degradation of protein. Most remarkable, perhaps, was the similarity in synthesis between retinas driven by flashing light and the controls that remained quiescent in darkness. Though flashing light increased the activity of some of the cells in the retina enough to cause a 38% rise in overall energy metabolism (as assessed by 2 DG uptake), test and control retinas did not differ by more than 1.7% in the synthesis of protein in any of 95 size groups. We interpret these findings as evidence that protein synthesis and degradation are well protected from the metabolic shifts that accompany activity, and also as evidence that changes in synthesis and degradation do not play an important role in adapting cells to changes in their level of function. The latter is not surprising as it would represent a cumbersome method of modifying the activity of an enzyme or transport protein. The most common mechanism for changing the activity of proteins in response to a change in functional state is post-translational modification. Some additions to the experimental methods described here would be required to detect most types of post-translational modification. This would appear to be a promising area for future research.

ACKNOWLEDGEMENT

This study was supported by Research Grants HL 06664, RR05486 and EY02245 from the National Institutes of Health. Dr. Ames is recipient of a Research Scientist Award (MH 03769) from the National Institute of Mental Health.

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APPENDIX

Heterogeneity of proteins in each slice; partitioning into a fast and slow subgroup. From the amount of labeled leucine incorporated and the intensity of the Coomassie Blue staining, it was possible to calculate the fraction of protein in each slice that had been synthesized in ½ h ($f^{\ell}_{\frac{1}{2}}$); and, from the fraction of labeled protein remaining 1 h (r_1) or 3 3/4 h ($r_{3\frac{3}{4}}$) after the tissue had been returned to unlabeled medium, it was possible to calculate the fraction of the protein in each slice that had been synthesized in ½ h and then degraded in 1 h ($f^{\ell}_{\frac{1}{2}}(1-r_1)$ or in 3 3/4 h $f^{\ell}_{\frac{1}{2}}(1-r_{3\frac{3}{4}})$. If breakdown is a first order reaction, the fraction of a protein degraded in time t can be expressed as $(1-e^{-kt})$ in which k is the breakdown coefficient; and, if synthesis and breakdown are in balance, this expression also describes the fraction synthesized in time t. Our data, then, would have been expected to comply with the following 3 equations if the proteins in each slice had been homogeneous with respect to turnover:

(1) Fraction synthesized in ½ h: $$f^{\ell}_{\frac{1}{2}} = (1-e^{-\frac{1}{2}k})$$

(2) Fraction synthesized in ½ h, then degraded in 1 h: $$f^{\ell}_{\frac{1}{2}}(1-r_1) = (1-e^{-\frac{1}{2}k})(1-e^{-k})$$

(3) Fraction synthesized in ½ h, then degraded in 3 3/4 h: $$f^{\ell}_{\frac{1}{2}}(1-r_{3\frac{3}{4}}) = (1-e^{-\frac{1}{2}k})(1-e^{-3\frac{3}{4}k})$$

As indicated above, there was unmistakable evidence of heterogeneity within the slices, and the data therefore did not comply with the above equations. However, they could be accounted for by the following, similar equations that describe the system in terms of 2 subgroups, one representing the slowly turning over constituents and the other the fast and in which f^s is the fraction of the protein in the slow subgroup; $1-f^s$ the fraction in the fast; k^s is the coefficient for the slow; and k^f the coefficient for the fast.

(4) $$f^{\ell}_{\frac{1}{2}} = f^s(1-e^{-\frac{1}{2}k^s}) + (1-f^s)(1-e^{-\frac{1}{2}k^f})$$

(5) $$f^{\ell}_{\frac{1}{2}}(1-r_1) = f^s(1-e^{-\frac{1}{2}k^s})(1-e^{-k^s}) + (1-f^s)(1-e^{-\frac{1}{2}k^f})(1-e^{-k^f})$$

(6) $$f^{\ell}_{\frac{1}{2}}(1-r_{3\frac{3}{4}}) = f^s(1-e^{-\frac{1}{2}k^s})(1-e^{-3\frac{3}{4}k^s}) + (1-f^s)(1-e^{-\frac{1}{2}k^f})(1-e^{-3\frac{3}{4}k^f})$$

The solution of these 3 simultaneous equations provided unique answers for f^s, k^s and k^f. Though describing the contents of a slice in terms of 2 groups is still an oversimplification, it provides an indication of the degree of heterogeneity, the relative number of fast and slow components, and some of the characteristics of the fast and slow components.

Neurochemistry Vol. 1, pp. 405-426.
Pergamon Press Ltd. 1980. Printed in Great Britain.

MORPHOLOGICAL AND BIOCHEMICAL ABNORMALITIES IN A MODEL OF RETINAL DEGENERATION: CANINE CEROID-LIPOFUSCINOSIS (CCL)

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ABSTRACT

Human ceroid-lipofuscinosis is marked by blindness, dementia, ataxia, and premature death. A canine model for this disease exists in English setters whose clinical, pathological and biochemical changes resemble the human disorder. In both syndromes, autofluorescent lipopigments, i.e.; lipofuscin and ceroid ("granular", "fingerprint" and/or "curvilinear bodies") are found in the nervous system, viscera, retina, and pigment epithelium (RPE).

Retinal neurons of affected animals between 6 and 22 months of age, contain a variety of abnormal intracellular pigment inclusions. Pigment epithelial cells also contain distinctive cytosomes.

Electroretinograms from affected animals showed a reduction in b-wave amplitude. Leukocyte, retinal, and RPE peroxidases, were decreased in affected animals, and also showed age-related changes. In the normal canine eye, peroxidase was associated with fractions containing plasma membranes and melanolysosomes. Improved fractionation techniques localized normal peroxidase to "heavy" fractions (1.24 - 1.28 g/ml), and peroxidase was decreased in these fractions in CCL animals. A new particle containing hexosaminidase, galactosidase, and acid lipase was observed in affected animals. When retinal homogenates from CCL dogs were injected into the vitreous of rabbit eyes they completely abolished the ERG recording. No such change was observed with homogenates from unaffected animals.

The accumulation of large numbers of dense bodies in the retina and RPE in dogs with CCL, along with a decrease in peroxidase, suggests an impairment of degradative mechanisms. Furthermore, ceroid appears to be cytotoxic to the retina and RPE. The relationship of these cytotoxic properties to the accumulation of ceroid in the eye, is the subject of our future research.

KEYWORDS

Ceroid lipopigments, fingerprint-curvilinear bodies, ERG changes, autoxidation, peroxidase, cytotoxicity.

INTRODUCTION

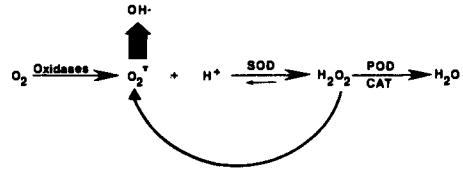
Human neuronal ceroid lipofuscinosis (NCL) or Batten-Stengel disease, is marked by early blindness, dementia, limb ataxia and premature death (Zeman and colleagues, 1970, Batten, 1903, Stengel, 1826). Clinically, the disorder can be separated into several sub-groups which are distinguished by age of onset, length of clinical course, and to some degree by the type of morphological autofluorescent lipopigments which predominate in the central nervous system, viscera and retina. Thus, in the infantile form (Santavuori, Hattia, Rapola, 1974) there is a granular form of ceroid lipopigment; in the late infantile form (Jansky, 1908; Bielskowsky, 1913) there is a predominance of curvilinear profiles, and in the juvenile form (Zeman and colleagues, 1970) there are primarily inclusions with fingerprint patterns. All types have excess lipofuscin as well. The various types of lipopigments are thought to represent end products of oxidations or peroxidation, especially the polyunsaturated fatty acids of membranous lipids, which may cross link with malonaldehyde to form autofluorescent Schiff base bridges. (Zeman and colleagues, 1970; Chio and Tappel, 1969). The adult form of NCL, or Kuf's disease, has massive accumulations of lipofuscin-like lipopigments and membranous bodies. (Chou and Thompson, 1970).

The ophthalmological findings in NCL may appear similar to those observed in retinitis pigmentosa. However, these two diseases can be differentiated and these distinguishing features will be discussed in the text. Based on the few cases reported, where the eye has been studied microscopically, the blindness results from retinal degeneration (Stock, 1908) which can be classified into three groups; damage to ganglion and inner nuclear layers, damage to outer nuclear layers, or damage to all layers of the retina (Greenfield and Holmes, 1925). All patients with NCL show irregular deposition of melanin granules, and vacuolization in the pigment epithelium (Givner and Roizin, 1944; Klein, 1954; Wotler and Allen, 1964). Ceroid in the form of both fingerprint and curvilinear profiles are present in all layers of the retina but these granules are most prominent in the ganglion cells (Goebel, Fix and Zeman, 1974). The accumulation of pigment may be related to cell death. The central portions of the retina where clinically, vision is lost first accumulate pigment granules early. (Green, 1971). The pigment epithelial cells (RPE) in Goebel's three cases were flattened, and irregular in shape. The numbers of melanin granules were reduced in some cells, but others contained mature granules, melanolysosomes and residual bodies. Phagocytized outer segments were not observed. Retinal degeneration and dementia are not prominent features of Kuf's disease, although macular degeneration has been reported (Kitagawa, Nishimura and Maketa, 1962). Recently another case was reported with extensive deposition of curvilinear profiles and lipofuscin in the retina (Dom and colleagues, 1979).

Initially, patients with NCL develop abnormalities related to central vision (Green 1971). The electroretinogram (ERG) is usually abnormal before any other clinical neurological signs are apparent and is extinguished rather quickly. (Zeman and colleagues, 1970; Copenhaver and Goodman, 1960). In patients having reduced vision, red-green color defects can be demonstrated with the anamaloscope and by chromatic adaptation, however, the blue cone response is intact. (Hansen, 1979). These findings and the observations that night vision is retained even in the advanced stages of NCL, clinically differentiates it from retinitis pigmentosa, where these findings are just the reverse. Furthermore, in retinitis pigmentosa the degeneration begins in the periphery rather than in the central retina as with NCL (Zeman and colleagues, 1970; Hansen, 1979).

Massive amounts of autofluorescent "ceroid" lipopigments are also found in the central nervous system, viscera and neural retina in a strain of English setters. This disease, termed canine ceroid-lipofuscinosis (CCL), has been proposed as a model for human NCL (Koppang, 1973/74; Armstrong and colleagues, 1978a). The formation of autofluorescent materials appears to involve free radicals (Tappel, Fletcher and Deamer, 1973; Roubal, 1970), and free radicals have been shown to be

cytotoxic (Pryor, 1976). Free radicals are particularly damaging to rod outer segments (Kagan and colleagues, 1973). Our laboratory has studied certain enzymes important in the intracellular production of free radicals and peroxides (i.e., peroxidase, catalase and superoxide dismutase and glutathione peroxidase).



A deficiency of human leukocyte peroxidase (Armstrong, Dimmitt and Van Wormer, 1974) and thyroid peroxidase (Armstrong and colleagues, 1975) has been reported previously in human NCL. A similar decrease in leukocyte peroxidase has been reported in the CCL dog (Patel and colleagues, 1974, Siakotos and Armstrong, 1975). In the normal canine retina, catalase, glutathione peroxidase, p-phenylenediamine (PPD) mediated peroxidase, and superoxide dismutase are present. These enzymes serve to protect against the formation of damaging free radicals and peroxides (Armstrong and colleagues, 1979; Armstrong, Siakotos and Koppang, 1978). These enzymes vary with the cell layer examined. Rod outer segments contain only superoxide dismutase, while the pigment epithelium has both PPD-peroxidase and superoxide dismutase (Armstrong and colleagues, 1979). Catalase activity in the pigment epithelium is demonstrable primarily by histochemical techniques (Leuenberger and Novikoff, 1975; Robison and Kuwabara, 1975). In the CCL dogs, only the maturational profile of PPD-peroxidase in the retina and pigment epithelium were decreased in whole tissues (Armstrong and colleagues, 1978). When the retina and pigment epithelium of these animals were fractionated on sucrose gradients, the PPD-peroxidase was lower than normal in all subcellular organelles where this enzyme is normally located (Siakotos and colleagues, 1978). Furthermore a new particle containing high titers of hexosaminidase, B-galactosidase and acid lipase was found, which was not present in tissues from control littermates. The present report summarizes the pertinent findings, supports our thesis that the CCL dogs provide a reasonable model for determining disease mechanisms in this type of retinal degeneration, and their potential in future approaches to therapy.

METHODS AND MATERIALS

Affected and unaffected English setters were obtained from Dr. Koppang's kennel in Oslo, Norway. At various ages, the dogs were shipped to the University of Colorado Health Sciences Center in Denver, Colorado. All animals were examined periodically for evidence of visual failure and other neurological dysfunction. Affected and unaffected dogs had been precisely identified by brain biopsy at 3 months of age by Dr. Koppang.

The method of leukocyte peroxidase recovery, purification and disruption by freezing and thawing to release soluble peroxidase has been previously described (Armstrong, Dimmitt and Van Wormer, 1974). The enzyme was assayed in 0.15 M phosphate, pH 7.4 and peroxidase measured using 1 mM hydrogen peroxide and 8.75 mM p-Phenylenediamine as the co-substrate chromogen. The reaction mixture was incubated at 25°C. One unit of enzyme activity is defined as micromoles of phenylenediamine converted /minute/mg protein at an optical density of 485 nm.

In one set of experiments, electroretinograms were obtained by conventional techniques(Armington, 1974), on three young (6-8 month) and two adult (22-24 month) dogs with CCL. One young dog and two adult unaffected littermates served as controls. The dogs were examined and recordings were made from once to three times.

Three dogs were also examined in another ERG facility. After one hour of dark adaptation, dogs were sedated with an intramuscular injection of 5 mg Acepromazine, followed 15 minutes later by 10 mg/kg of Diabutol, given I.V. over a period of 2-3 minutes. Sedation to suppress movement was difficult to obtain, particularly in affected animals. Mydriacyl (1%) ophthalmic solution was applied topically to dilate the pupils. A Burian-Allen electrode was placed under the lid and nictitating membrane to record the ERG signals. The eye was fixed in the primary position and diffused light from a Xenon discharge tube was used as the stimulus at 750,000 candle power. The signal was amplified by type 122 Tektronics preamplifiers with a band pass of 1-60 Hz.

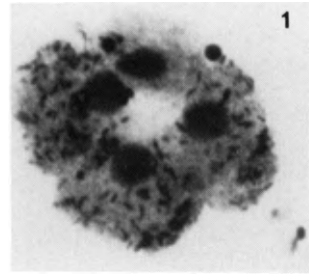
For electron microscopic and biochemical tissue studies, animals were sacrificed by electric shock. The eyes were immediately removed, cornea and iris cut away and the vitreous expressed by gentle pressure. A 5 mm corneal trephine was used to remove a single block of intact retina-choroid-sclera. The specimen was placed immediately into 4% phosphate buffered glutaraldehyde, fixed for 24 hours, rinsed, sectioned, osmicated, and embedded in Araldite. Thick and thin sections were cut, counter-stained with uranyl acetate and lead citrate and examined.

The isolation of dog pigment epithelial cells was as previously described, and consisted of brushing the RPE away from the eye cup, then purifying the cells through a series of four sucrose washes. (Armstrong and colleagues, 1978b). Typical cells are shown in Fig. 1. Preparation of retinal, ROS and RPE tissues for biochemistry and sub-cellular fractionations were as previously described (Armstrong and colleagues, 1978a, Siakotos and colleagues, 1978).

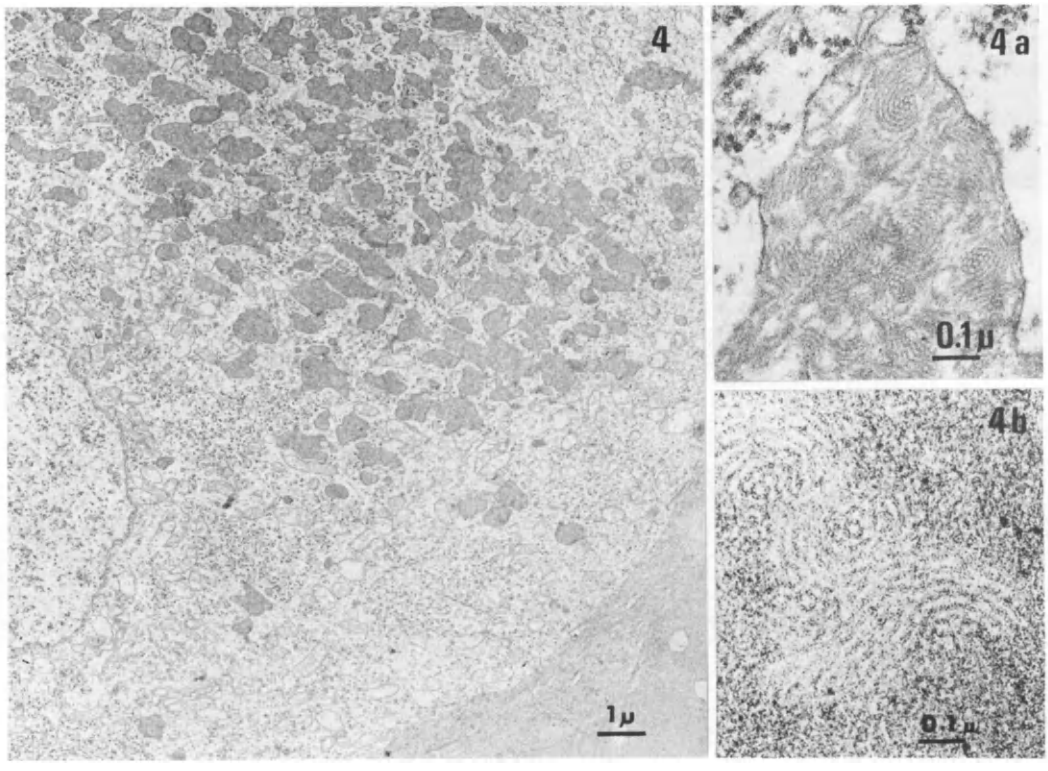
RESULTS

Neurological and ophthalmological observations on the six and eight month old dogs with CCL were indistinguishable from the age-matched control animals. This agrees with our experience over the past 5 years with a total of 40 dogs. Only the results of the previous brain biopsy allowed precise identification of the affected animals. Affected dogs usually remained clinically normal until approximately 12-14 months of age, and reduced visual acuity was usually a late, and variable manifestation of the progressive cause of the disorder.

In contrast to the young animals, the adult dogs with CCL, were quite abnormal on neurologic and ophthalmologic examination. Older affected dogs have a peculiar, unsteady gait frequently falling, and exhibit a muzzle ataxia particularly when feeding. Dogs with CCL have not survived beyond 26 months of age and are usually unable to walk or stand in the terminal stages of the disease. Reduced visual acuity is obvious by the dog's lack of response to visual threat, a dull, staring look, uncertainty in movement, and their frequency of running into obstructions. The typical appearance of a dog with CCL (Fig. 2b) and an unaffected dog (Fig. 2a) are shown in the next page.



Experiments were carried out to determine if retinal extracts from dogs with CCL were toxic to the rabbit retina. In two separate experiments, New Zealand rabbits were injected directly into the vitreous, using a 31 gauge needle. ERG recordings were made before the injection, and from 1 to 17 days thereafter. Recordings were compared to the control rabbit eye, which was injected with a retinal homogenate from an unaffected littermate.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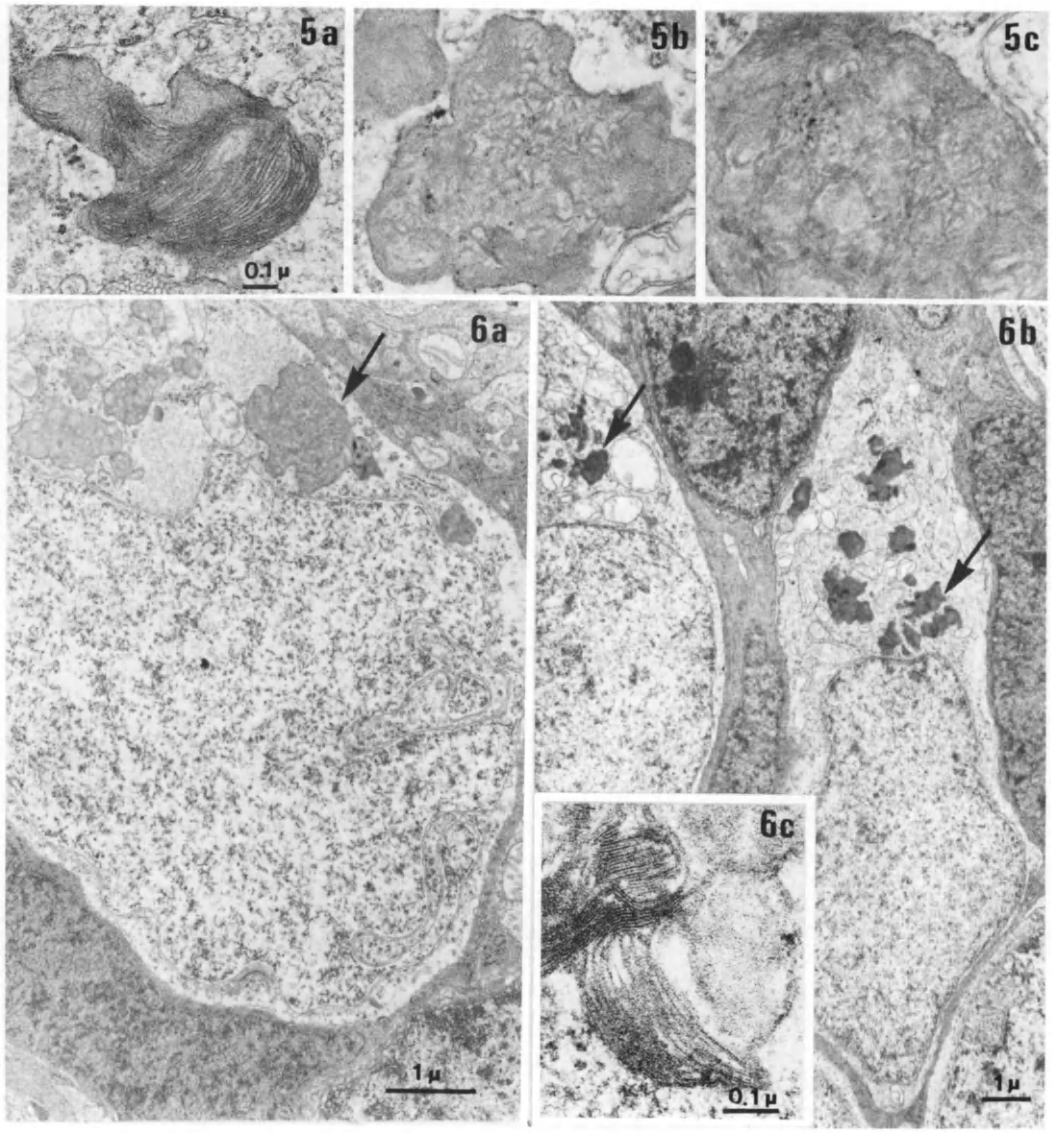
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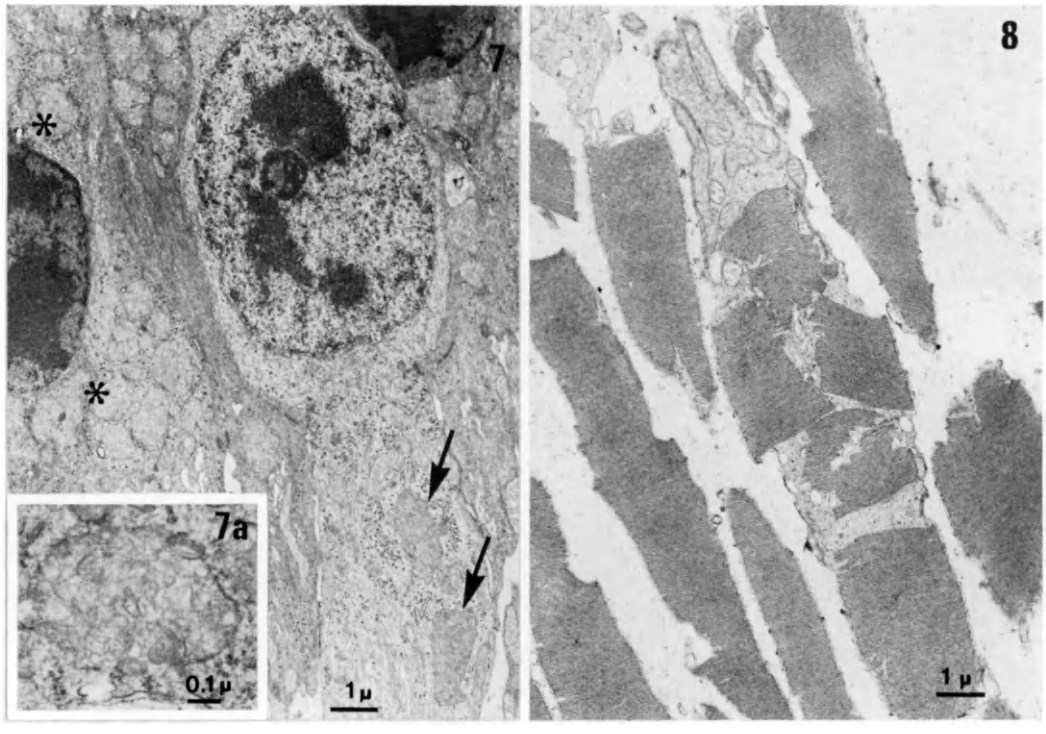
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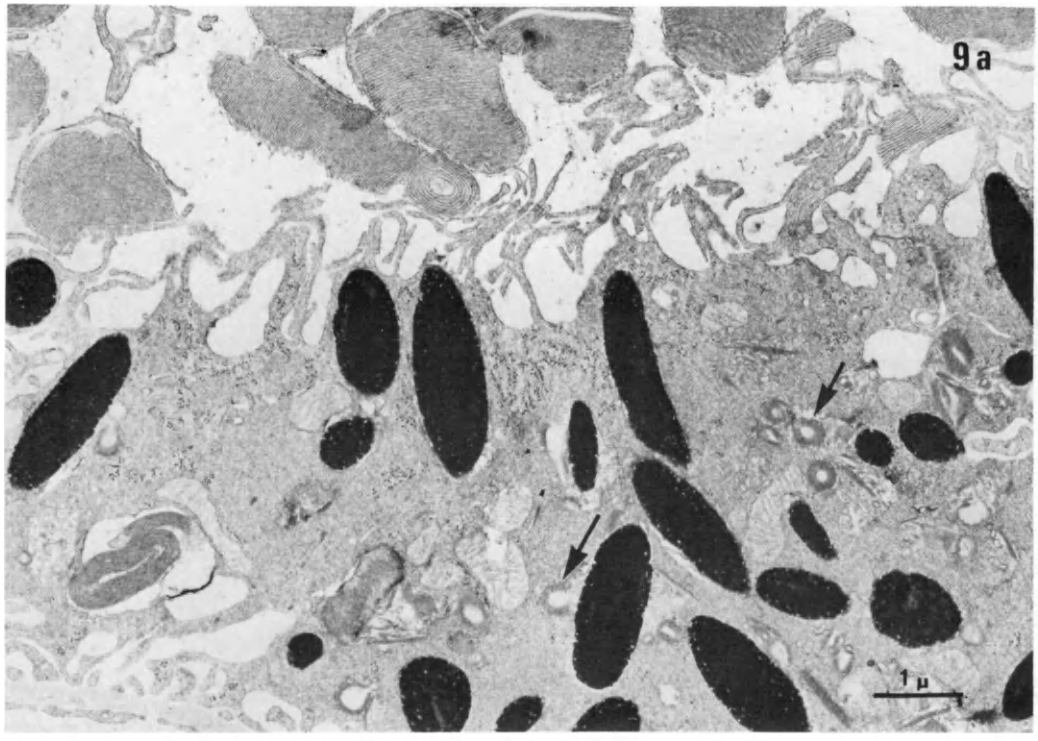
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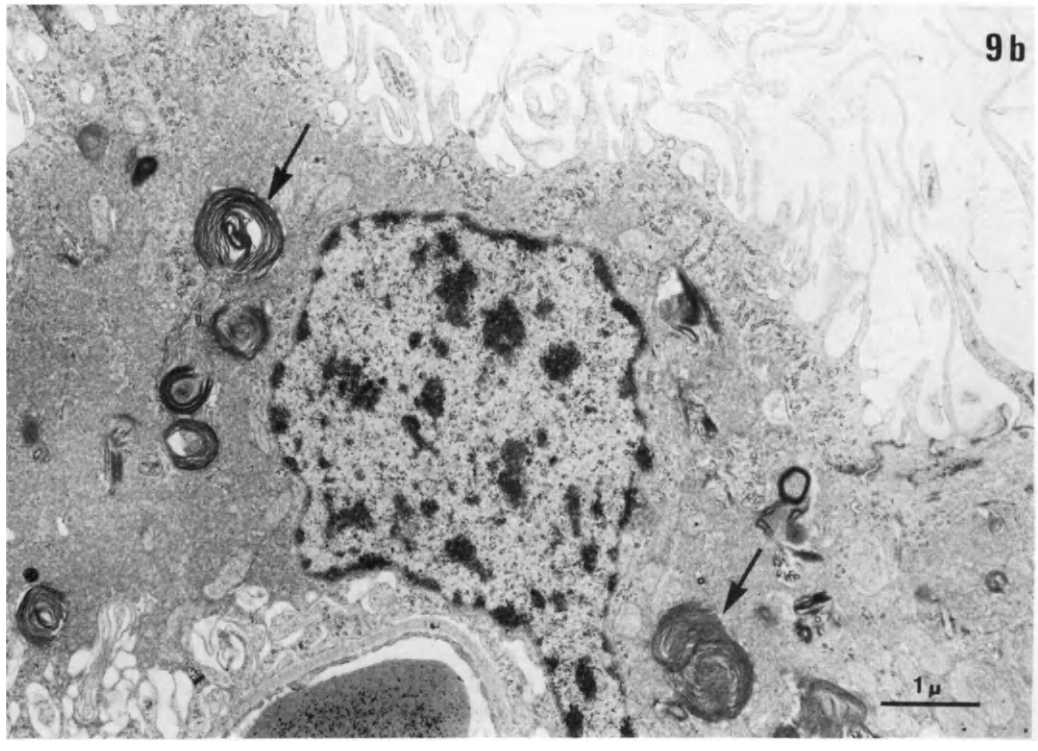
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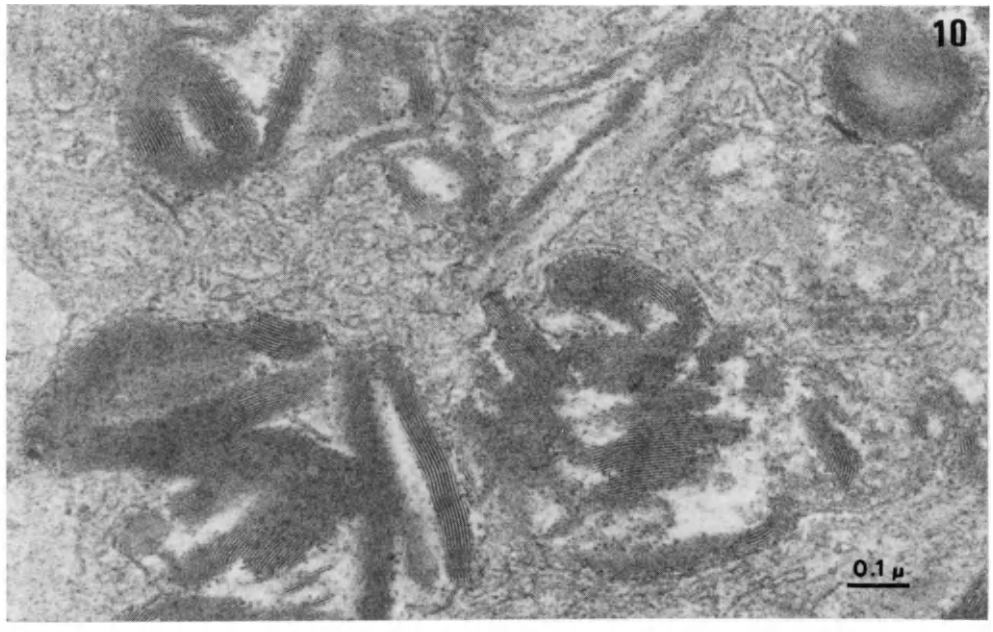
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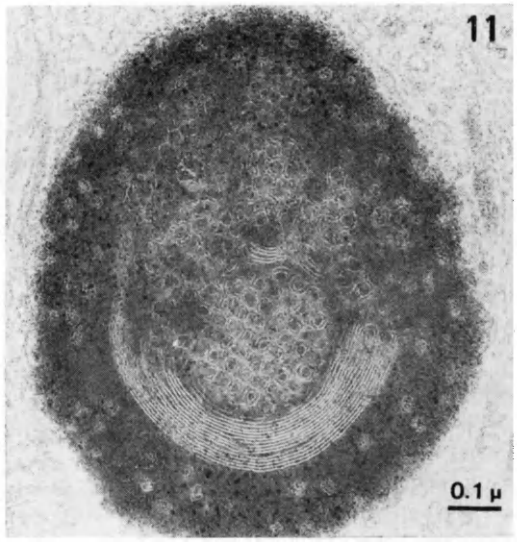
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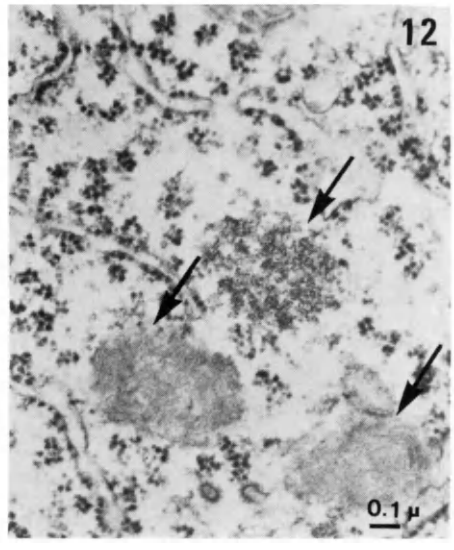

12
0.1 μ

Leukocyte peroxidase activity and clinical information on the English setters used in the ERG studies are presented in Table 1.

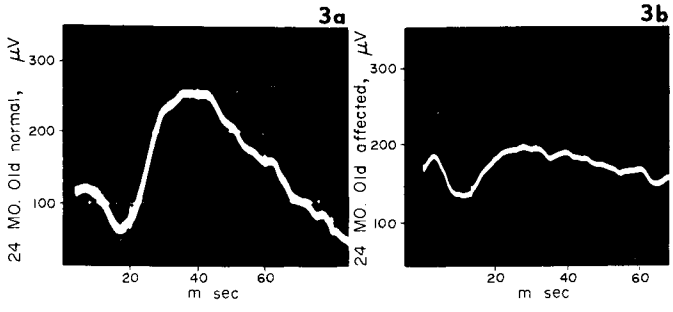
TABLE 1 Laboratory Studies Performed on CCL English Setters and Nonaffected Littermates

	Age	Sex	Disease Status	ERG Response (μV) a-wave	ERG Response (μV) b-wave	Soluble leukocyte peroxidase specific activity*
	6 mo.	female	CCL	30	80	94.3
young	6 mo.	female	CCL	60	140	204.0
	6 mo.	female	Unaffected	60(50-70)	140(130-150)	788.9
	8 mo.	male	CCL	40	120	85.5
	24 mo.	female	Unaffected	-	-	453.6
adult	24 mo.	male	Unaffected	80(60-100)	190(170-200)	304.1
	24 mo.	female	CCL	40(30-50)	70(60-80)	142.8
	24 mo.	male	CCL	-	-	218.2

Three littermates with CCL (6 mo.) had peroxidase activities ranging from 85.5 to 204.0 with a mean specific activity of 104.2 μ moles/mg protein.
*A normal, unaffected, 8 mo. old animal had an activity of 788.9 for comparison. Unaffected 24 month old dogs ranged in activity from 304.1 to 453.6 with a mean of 378.9 μmoles/mg protein. Two adult dogs with CCL had specific activities of 142.8 and 218.2 (mean = 180.5 μmoles/mg protein).

Electroretinographic recordings were obtained from six of the eight animals (Table 1). In the "young" group, the normal control underwent two ERG recording sessions. The average amplitude for the a and b-waves were 60 and 140 μV respectively. Two young affected littermates had depressed values on the tracing; with a-wave amplitudes of 30 and 40 μV, and b-wave amplitudes of 80 and 120 μV. However, a third affected littermate could not be distinguished from the unaffected control.

Tracings were recorded from one adult dog with CCL and one unaffected littermate on three different occasions. The average a-wave amplitude for the unaffected dog was 80 μV (range 60-100), and 190 μV (range 170-200) for the b-wave. (Fig. 3a).



In contrast, the adult dog with CCL had an "average" a-wave amplitude of 40 μV (range 30-50) and a b-wave "average" of 70 μV (range 60-80). Figure 3b shows a representative tracing from this animal. In this instance there was a 38% reduction in the a-wave relative to the control, and a 73% reduction in b-wave amplitude. Similar ERG studies (Table 2) were performed in another laboratory in 22 month old animals by Dr. G. Aguirre.

TABLE 2 Other ERG Studies

Age	Sex	Disease Status	ERG Response (μV) White	Red	Scotopically Balanced Red	Scotopically Balanced Blue
22	Female	Unaffected	110	71	97	102
22	male	CCL	63	43	63	50
22	male	CCL	49	27	57	45

After 20 minutes of dark adaptation, and a white light stimulus, a-wave amplitudes for the affected animals were only reduced by about 10%, but the b-wave amplitudes were decreased by 44 and 55% respectively. (Table 2). With red light, b-wave amplitudes were decreased by 40 and 62%; with scotopically balanced red light, amplitudes were 35 and 41% below normal, and with scotopically balanced blue light, amplitudes were decreased by 51 and 57% respectively.

All neurons of the retina contained abnormal inclusion bodies. Ganglion cells show the most striking changes and had large accumulations of pigment. Figure 4 shows a typical ganglion cell at low power (8200 x).

Although the cell appears to be well preserved, subcellular elements (mitochondria, golgi, RER) are displaced towards the cell periphery. Higher magnification (80,000 x) shows that each cytosome contains closely stacked membranes resembling "fingerprint" patterns and tubular profiles (Fig. 4a). These are identical in morphology to the inclusions described by Koppang in spinal cord neurons as early as 2 days of age (Fig. 4b-mag. 80,000 x).

The periodicity of the lamellae were 70-75 Å. Examples of other less frequent types of inclusions are shown in Figs. 5a, b and c. All inclusion bodies within ganglion cells were surrounded partially or completely by a unit membrane (mag. 58,300 x).

Cytosomes in the inner nuclear layer were different from those seen in ganglion cells. These inclusions, found in both amacrine (Fig. 6a) and bipolar cells (Fig. 6b - mag. 8200 x) were much denser than those seen in ganglion cells. The cytosomes in Fig. 6a and 6b consisted of tightly packed membrane collections, lying in a dark, finely granular material (Fig. 6c - mag. 91,300 x).

In the outer nuclear layer a third distinctive type of inclusion was observed. Accumulations were noted around the nucleus (asterisk) while other inclusions were prominent in the myoid region of the inner segments. Arrows indicate the inclusions in Fig. 7 - mag. 7700 x.

The predominant inclusion was membrane bound and contained many short, curved profiles which were faintly osmiophilic. A few inclusions contained lamellar structures similar to those seen in ganglion cells and cells of the inner nuclear layer. (Fig. 7a - mag. 55,900 x).

The majority of outer photoreceptor segments of affected dogs were intact and of normal appearance in both peripheral and central retina in the few dogs we have examined to date. Occasional disruption of discs was seen in adult dogs with CCL (Fig. 8 - mag. 8300 x). In more recent studies the gross anatomical appearance of the retina indicated that some severe pathogenic process was occurring (unpublished observations) in other adult dogs with CCL.

Retinal pigment epithelium contained phagosomes and the usual subcellular organelles in both young and adult dogs with CCL. This was true for the pigmented areas, (Fig. 9a) as well as for the nonpigmented tapetal region. Fig. 9b). In addition, the pigment epithelial cells contained deposits of abnormal material in both tapetal (Fig. 9a - mag. 15,200 x), and non-tapetal (Fig. 9b - mag. 16,800 x)

areas. The deposits in both micrographs are marked by arrows.

The density of inclusions per RPE cell was greater in older, more affected dogs. The commonest inclusion consisted of tightly packed membranes of dark and light lamellae 2.5 to 3.0 nm wide, arranged in linear or curved arrays, and as tubular structures (Fig. 10 - mag. 105,800 x).

These inclusions, not invested by a unit membrane, are obviously different from those accumulating in retinal neurons. Collections of similar lamellae were also seen inside melanin granules (Fig. 11 - mag. 85,500 x). These structures may represent a fusion of melanin and lysosomes to form structures termed melanolysosomes by Feeney (1978).

Koppang has previously described non-membrane bound inclusions, free within the cytoplasm of neurons, some of which contain the precursors of the unique cytosomes seen in retinal and pigment epithelial cells (Fig. 12 - mag. 49,710 x).

In a previous report, we have shown that soluble RPE peroxidase has optimal pH at 4.5 to 5.0 (Armstrong and colleagues, 1978), however additional enzyme can be released from the remaining cellular membranes by treatment with 1 M NaCl and 1% Triton X100. This insoluble enzyme is optimal at pH 7.4. Soluble and bound peroxidase activities in the retina and pigment epithelium are shown in Table 3 as a function of age.

TABLE 3 Changes in Peroxidase Activity and Intracellular Distribution as a Function of Age

Age	Diagnosis	Tissue	Soluble Peroxidase μ moles/mg protein	Bound Peroxidase μ moles/mg protein
6 mo.	Unaffected	Retina	0.2	0.3
10 mo.	Unaffected	Retina	3.5	0.5
22-24 mo.	Unaffected	Retina	3.7	14.4
6 mo.	CCL	Retina	0.3	0.3
13 mo.	CCL	Retina	1.0	13.8
22-24 mo.	CCL	Retina	1.8	1.2
6 mo.	Unaffected	RPE	15.0	3.0
10 mo.	Unaffected	RPE	60.0	135.0
22-24 mo.	Unaffected	RPE	147.0	72.0
6 mo.	CCL	RPE	231.0	6.0
10 mo.	CCL	RPE	105.0	48.0
22-24 mo.	CCL	RPE	36.0	18.0

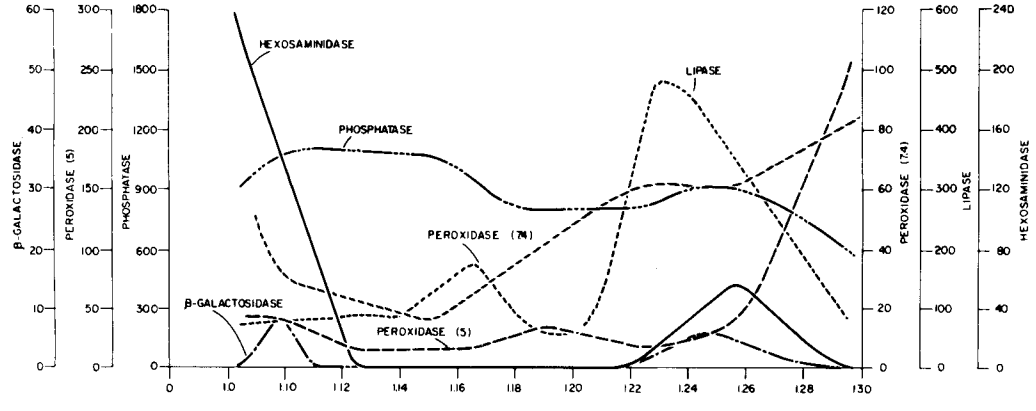
From 6 months until 2 years of age, soluble peroxidase from normal retina and pigment epithelium increase in a maturational fashion. In the unaffected animals, peroxidase increases 20 fold in the retina and 10 fold in the pigment epithelium. There is also a maturational increase in the retina from the CCL animal, but it is only a 6 fold increase. Thus, by 2 years of age, retinal peroxidase in the affected tissue is only 50% of the unaffected level. The age-related peroxidase curve in the affected pigment epithelium was quite different. Here, the enzyme level began at very high levels (15 times higher than control) and then dropped continuously throughout the life of the animals. By 24 months, the levels had decreased to just 24% of normal.

Bound or insoluble peroxidase levels in unaffected animals also increased in retinal tissues, however adult levels were 4 fold higher than corresponding soluble levels. Affected animals had a bell-shaped curve with peroxidase activity in ter-

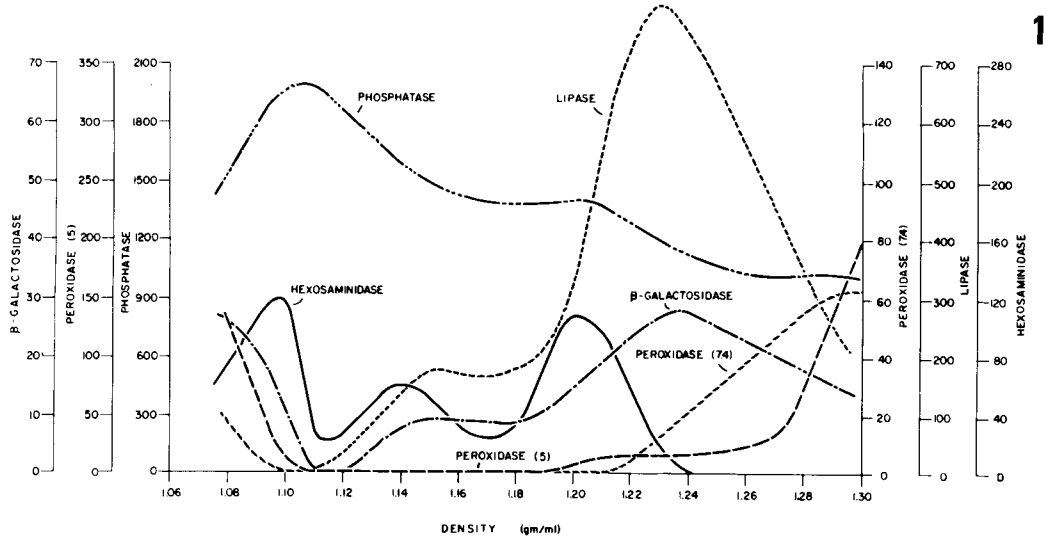
minal animals just 8% of normal. Interestingly, soluble and bound levels in these animals were roughly the same. In the unaffected pigment epithelium samples, the peroxidase exhibited a bell-shaped curve, with maximal activity occurring at about 1 year of age. This curve was in contrast to the enzyme levels found for affected pigment epithelial samples where peroxidase activities were much lower. Thus at 1 year, activity was 64% of normal, and by 2 years it had declined further to 75% below normal. The retinal and pigment epithelial peroxidase from whole tissue has also been measured on two siblings with the late infantile form of NCL.

Tissue	Sample	Diagnosis	Age(yr)	Peroxidase Activity (μ moles/min/mg)
Retina	Whole homogenate	Normal	10-20	53.0
		NCL	$4\,^{1}/_{2}$	0.0
		NCL	6	5.3
RPE	Whole homogenate	Normal	10-20	104.0
		NCL	$4\,^{1}/_{2}$	12.0
		NCL	6	6.4

As we have already demonstrated for the dog, RPE peroxidase is higher than retinal peroxidase in the human. In the tissues from both children with NCL, peroxidase was greatly reduced, and in one instance it was not measurable.



13a



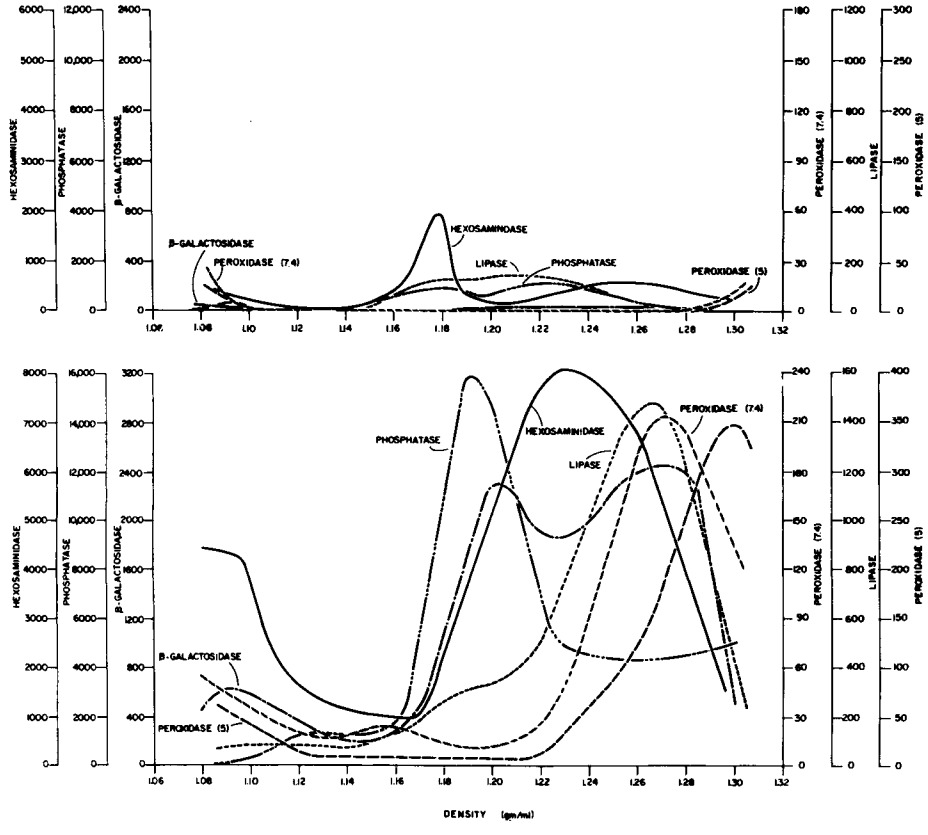
13b

Subcellular fractionation studies have previously been reported on the retina and pigment epithelium from young (10 months) and adult (22-23 months) dogs (Siakotos and colleagues, 1978). In this scheme, the supernatant fraction contains cytoplasmic enzymes and intact subcellular organelles, while the pellet fraction contained cell membrane fragments, melanosomes, and nuclei. Some early changes occurred in the subcellular fractions of tissues from the young affected animals which consisted of decreases in catalase and peroxidase, large increases in B-galactosidase, hexosaminidase, and acid phosphatase, and a slight increase in acid lipase activity at the various densities. Thus, a new particle was present even at this early age which was clearly demonstrable between the densities of 1.20 to 1.24 g/ml. (Siakotos and colleagues, 1978).

These changes were dramatic in the older affected animals in both the retina and the pigment epithelium. In the supernatant fraction from the adult affected pigment epithelium, the new particle sedimented around 1.22-1.24 g/ml, and contained high titers of hexosaminidase, acid phosphatase, and acid lipase. This subcellular particle did not contain peroxidase. In the pellet fraction, membranes sedimenting at 1.23 g/ml were again elevated (40%) in acid lipase activity, but markedly decreased (83%) in pH 7.4 peroxidase activity (Fig. 13b)

Catalase and peroxidase were decreased in the supernatant fractions from the retina and pigment epithelium, and increased slightly in B-galactosidase, acid phosphatase and acid lipase.

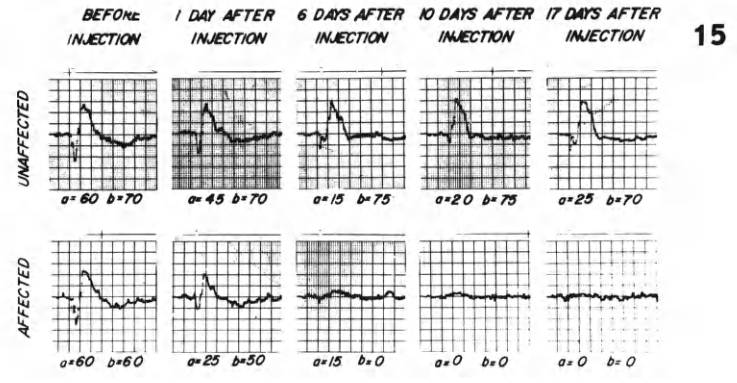
Enzyme activity was again markedly increased in the retinal pellet fraction and was



associated with a series of new membrane bands sedimenting between 1.18-1.20, 1.22-1.24, 1.26-1.28 and at 1.30 g/ml. (Fig. 14b).

These bands contained high levels of acid phosphatase (1.18-1.20), hexosaminidase and B-galactosidase (1.22-1.24), B-galactosidase, lipase, peroxidase at pH 7.4 (1.26-1.28), and a band containing the pH 5 peroxidase (1.30 g/ml).

We have recently explored the effect of certain compounds on the ERG, when injected directly with the vitreous of albino rabbit eyes. Details of this technique are being prepared for publication (Armstrong and colleagues). In these experiments, we inject into one eye of the rabbit, an aliquot of a whole retinal homogenate suspended in isotonic saline from an adult dog with CCL. Into the other eye we inject a similarly prepared sample from an unaffected littermate. The results are shown in Fig. 15.



Normal retinal homogenates decreased the a-wave somewhat during the 17-day experiment, but had no effect upon the b-wave. In marked contrast however, tissue from the affected dog showed an effect on both the a and b-wave amplitude after just 1 day. At 6 days post-injection, the a-wave was barely recordable but the b-wave was extinguished. By 10 days and thereafter, the ERG was isoelectric. This experiment has been repeated on a second affected adult dog with identical results.

DISCUSSION

Levels of leukocyte peroxidase were significantly reduced in dogs with CCL when compared to non-affected dogs of similar ages. These results are comparable to low peroxidase levels which have been recorded in other affected English setters. For example, dogs in a previous report had a mean specific activity of 132.6 (Siakotos and Armstrong, 1975) as compared to 104.2 (young) and 180.5 (adult) in the present study. On a percentage basis, this means that studies to date show a decrease in activity of between 60 and 76%. In addition, Patel and colleagues, (1974) found that leukocyte peroxidase was approximately 68% below normal. We chose to determine the leukocyte peroxidase in the present study only on the dogs where retinal morphology and electroretinography were simultaneously evaluated.

ERG tracings were obtained from 6 of the 8 animals tested in one series. The major abnormality was a reduction in the b-wave amplitude seen most prominently in the adult affected dogs. This decrease varied from as much as 57% to 63% below normal in young and old affected animals (average = 60%). There was no increase in latency of the a-wave, but occasional reductions anywhere up to 50% were seen in these animals. In a separate study in Dr. Aguirre's laboratory, the decrease in the b-wave amplitude of older affected dogs was similar but slightly less reduced than our results. Averaging the values for each of the 2 affected animals in his study,

and comparing them to an unaffected animal with a red light stimulus, there was a 51% reduction in the affected dogs. When scotopically balanced red and blue light was used (Aguirre and Rubin, 1977) it yielded a 38% and 53% decrease respectively. Thus, while there is not a total extinction of the ERG signal in these animals in most instances, even in adult animals, it would be difficult to ascertain to what degree retinal damage might have occurred had these animals lived longer than their shortened (24-26 months) life span.

When lipid peroxides are experimentally produced in the eye, their effect is greater on the b-wave than on the a-wave (Shvedova and colleagues, 1979). This is consistent with our observations that the a-wave is much less affected than the b-wave in a condition where lipid peroxidation is abnormal.

There are other reports of experimentally produced retinal degeneration in animals through stimulation of lipid peroxidation, but few have performed ERG recordings. In one study lipid peroxidation was induced by intraocular iron (Burger and Klintworth, 1974). Photoreceptors and pigment epithelium were especially susceptible to damage, and with the degree of pathology they showed, one would have certainly expected a decrease in the ERG. In another study, iron and ascorbate were used to induce the formation of lipid peroxides (Shvedova and colleagues, 1979). The electroretinogram of the frog retina in their study was decreased, and deleterious effect of oxidation were reversed by antioxidants. When animals are made vitamin E deficient, loss of this potent lipid soluble biological antioxidant results in severe degenerative structural changes to the outer segment - pigment epithelium complex (Robison, Kuwabara, and Bieri, 1979; Hayes, 1974; Farnsworth and Dratz, 1976). The pigment epithelium is likewise inherently sensitive to vitamin E deficiency, accumulating large amounts of fluorescent materials (Katz, Stone & Dratz, 1978). Such damage by dietary restriction of vitamin E, and selenium, produce decreased ERG signals (Stone and colleagues, 1978). The decrease in ERG correlated with a loss of docosahexanoic acid in the rod outer segments and in the pigment epithelium (Farnsworth, Stone and Dratz, 1979).

Because ceroid and/or other lipopigments are thought to result from an accumulation of intracellular peroxides, through free radical and lipid peroxidation, it seemed important to characterize and analyze the intracellular distribution of certain retinal and pigment epithelium enzymes from normal pooled dog eyes. Rod outer segments appear to be protected from light and oxygen damage by virtue of their component of α -tocopherol, (Dilley and McConnell, 1970) and superoxide dismutase, (Hall and Hall, 1975). Catalase has been demonstrated histochemically in the pigment epithelium by several investigators (Leuenberger and Novikoff, 1975; Robison and Kuwabara, 1975) and we have recently shown that biochemically, peroxidase is a major enzyme in this tissue (Armstrong and colleagues, 1979). The peroxidase enzyme has a broad pH optima peaking around 4.0-5.0 with a Km towards p-phenylenediamine of approximately 8.3×10^{-3} M and towards H_2O_2 of approximately 0.32×10^{-3} M. Pigment epithelial peroxidase is relatively stable at 15°C, for several months, and seems to be associated with subcellular fractions containing membrane fragments, melanolysosomes and nuclei (Armstrong and colleagues, 1978).

Enzyme activity in our study was roughly the same in melanotic (12.6 units/mg) and amelanotic (9.8 units/mg) tissue, so obviously melanin granules cannot be the sole source of peroxidase. We have however, demonstrated substantial peroxidase activity in purified melanin fractions which is high in young normal dogs, but decreases with age (Siakotos and colleagues, 1978). Peroxidase activity in the RPE was not demonstrable in the cytosol.

Although we had originally thought nuclei to be an unlikely source of peroxidase, a recent article has demonstrated peroxidase activity in the inner nuclear membrane of rat liver (Stubbs and Harris, 1978) but not in the plasma membrane where

it is found in the thyroid gland. Further work is needed to resolve the subcellular site of peroxidase in the retina and pigment epithelium.

We have had the opportunity now of examining at various ages, 12 English setters with CCL, and 9 unaffected littermates and determined levels of 4 key enzymes in the antioxidation cycle. Oxidases are continuously producing superoxide radicals, ($O_2^{\cdot-}$) which normally are degraded to H_2O_2 by the action of superoxide dismutase (Pryor, 1976). The hydrogen peroxide which is also toxic, is then normally degraded by peroxidases and catalase to water. If this part of the protective mechanism is altered, excess H_2O_2 can react with $O_2^{\cdot-}$ to produce the hydroxyl (OH·) free radical, one of the most damaging of all radicals especially to the lysosomal system (Fong, McCay and Poyer, 1973). Subcellular fractions contain their own hydrogen peroxide generating systems (Boveris, Oshino, and Chance, 1972). Microsomes produce the greatest amount, followed by a fraction containing peroxisomes, lysosomes, light mitochondria, and rough endoplasmic reticulum, and then heavy mitochondria.

Hydrogen peroxide produced within the cell can pass into peroxisomes to be degraded enzymatically by catalase, or it can be degraded by other cellular enzymes such as cytochrome c peroxidase, glutathione peroxidase and other peroxidases (i.e. PPD-peroxidase) each with its own specific biological cosubstrate hydrogen donor. Thus, peroxidases form a first line of defense against peroxide toxicity.

In studies to be reported later we found that PPD-mediated peroxidase, glutathione, peroxidase, catalase, and superoxide dismutase had quite different tissue distributions. Thus, the ROS only contains superoxide dismutase, while the RPE only contains PPD-mediated peroxidase, and a small component of superoxide dismutase. Peroxidase in the RPE is mostly soluble, but at least another 50% of this activity can be released from membranes or other osmotically stable organelles.

At 6 months of age, the soluble and bound peroxidase levels in the unaffected English setter retina were similar, but from 10 months onward, there was a relative decrease of soluble peroxidase in the affected animals. The changes in the RPE were different in that the soluble peroxidase in animals with CCL were extremely high at 6 months and thereafter declined steadily while the normal enzyme rose throughout life. Bound RPE peroxidase(s) showed a bell-shaped curve, but were at least 75% lower in overall activity. These decreasing enzyme changes in animals with CCL seem to correlate with the decrease in leukocyte peroxidase levels, and may be responsible for the accumulation of ceroid, and with observed abnormalities in the electroretinogram.

Carrying our studies further, we have examined in more detail, the subcellular distribution of organelles and membrane fragments on sucrose gradients from pooled dog eyes, and from as little as one English setter eye. Supernatant fractions from normal young dog retinas, normally have a subcellular particle, as yet unidentified, which sediments between 1.20 to 1.23 g/ml and contains predominantly acid phosphatase, some catalase, acid lipase, and pH 7.4 and pH 5.0 peroxidase. Affected young dogs have a particle sedimenting at the same density that shows less acid phosphatase, but a large increase in B-galactosidase, acid lipase, catalase but a decrease of pH 7.4 peroxidase. Peroxidase at pH 5 was virtually absent from this particle in affected dogs, and was generally lowered in all subcellular fractions. (Siakotos and colleagues, 1978).

Changes in this particle in the supernatant fraction from the CCL adult retina consisted of across the board increases in all enzymes except peroxidase, which was decreased. The greatest change occurred in the pellet fraction from the adult CCL retina where huge levels of enzyme were associated with new membranous materials at specific densities of 1.18 to 1.20, 1.22 to 1.24, 1.26 to 1.28 and at 1.30

g/ml. (Fig. 14, bottom photo). Of special note was the finding that peroxidase at pH 5 and at 7.4 were both located in the 1.26 to 1.28 and 1.30 g/ml fractions, perhaps somehow "trapped" within these fractions. Similar changes were observed in the RPE especially with respect to the very large increase of acid lipase in the 1.22 to 1.24 g/ml fraction.

It would appear therefore that a new particle is present in the retina of affected animals which is high in acid hydrolases, and that peroxidases are likewise incorporated into membranes or other particles from older animals sedimenting at slightly higher sucrose densities. Although the electron microscopy of the fractions is still in progress, it might be reasonable to assume that these particles, or membranes, are the abnormal ceroid inclusions so prominent in the affected tissues.

Finally in an attempt to resolve the controversy as to whether ceroid or some chemical species intimately related to, or produced by its action, could account for the observed dysfunction or loss (Koppang, 1973/74) of brain and retinal neurons as these animals progress in their disease. We have studied this in normal retinal tissues. In these experiments retinas were removed from unaffected and affected 24 month old littermates. The tissues from both groups of dogs were homogenized in normal saline and injected into the posterior chamber of rabbit eyes; each eye receiving one sample from an affected dog and one from the unaffected dog. Previous studies have established this technique and that of accurately and reproducibly recording the ERG in the rabbit (Armstrong and Hiramitsu, unpublished data). As one can see from the ERG recordings in Fig. 15, it is clear that something in the CCL retinal extract is extremely cytotoxic to the retina of the rabbit, and the electrical signal disappears approximately 1 week after injection. Such changes are not seen with the control injection. From the morphological studies presented here it is also clear that only ceroid can be considered as the abnormal particle present in the affected animals.

In summary, peroxidase, a key enzyme in regulating autoxidation is deficient in the retina and pigment epithelium, as well as in peripheral leukocytes in dogs with CCL. Some of this peroxidase enzyme may be "trapped" in the abnormal particles or membranes found by subcellular fractionation techniques, thus making it unavailable to function in it's normal intracellular mileau. Furthermore, this material, probably containing toxic metabolites, i.e.; peroxides, aldehydes, and/or free radicals, is certainly cytotoxic to the retina and may be responsible for the retinal degeneration observed in English setters with ceroid-lipofuscinosis.

Foulds, (1979) has recently proposed that pigmentary degeneration of the retina produced from any number of adverse conditions, affect rather specifically the outer segment-pigment epithelial interface. Our studies have shown that 1) the retina and pigment epithelium accumulate ceroid, 2) these tissues are deficient in peroxidase, 3) some outer segments are abnormal, 4) and these changes are associated with clinical blindness. The present findings can be explained by a common peroxidase deficiency, which allows free radicals and/or peroxides to damage cells of the retina and pigment epithelium resulting in impaired physiological function.

ACKNOWLEDGEMENTS

The work summarized in this paper is the culmination of 5 years effort, and is in part due to the assistance of many collaborators and friends. The authors wish to thank Drs. James Austin, Alan Laties, David Wenger, Gustavo Aguirre, Elaine Berman, Lynnette Feeney, Dale Van Wormer and T. Hiramitsu. Special thanks for technical assistance is extended to Ellen Connole, Sandy Dimmitt, George Santangelo, Debbie Kett, Cathryn Wilson, and Laurie Taylor. This project was supported in part by the National Retinitis Pigmentosa Foundation, the Children's Brain Disease Foundation,

the Denver Veterans Administration Hospital, NEI grant #EY 02627, NEI grant # EY 01806, BRFS grant RR-0537 awarded by the Biomedical Research Grant Program, Division of Research Resources, N.I.H., Fight for Sight Grant-in-Aid, Fight for Sight, Inc. , New York City, and the National Society to Prevent Blindness. The senior author is the recipient of a Research Career Development Award, #EY 00130. Permission to use some previously published work in this chapter was granted by Elsevier Sequoia, S.A. and the C. V. Mosby Co.

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Neurochemistry Vol. 1, pp. 427-440.
Pergamon Press Ltd. 1980. Printed in Great Britain.

CYCLIC GMP METABOLIC DEFECTS IN INHERITED DISORDERS OF rd MICE AND RCS RATS

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ABSTRACT

The inherited diseases of rd mice and RCS rats are compared. The rd disorder is characterized by the failure of rod visual cells to differentiate fully, by the accumulation of cyclic GMP, which results from a reduced level of phosphodiesterase activity, and by the rapid degeneration of visual cells, which is unaffected by light. The RCS rat disorder is characterized by the failure of pigment epithelium cells to phagocytize shed membranes of rod outer segments, which accumulate as debris, by a debris-associated reduction in cyclic GMP content, and by the slow degeneration of visual cells, which is accelerated by light. Both disorders result in blindness and both display abnormalities in cyclic GMP metabolism which occur before visual cells degenerate. Identification of a role for cyclic GMP in visual cell metabolism or function may suggest how either elevated levels of cyclic GMP in rd retinas or reduced levels of cyclic GMP in RCS retinas fit into the pattern of visual cell degeneration.

KEYWORDS

Retinal degeneration; inherited blindness; cyclic GMP; rd mice; RCS rats; cyclic nucleotide phosphodiesterase.

INTRODUCTION

An interest in inherited diseases which cause blindness has increased markedly over the past decade. The stimulus for growth has come from the scientific community as well as from lay groups of concerned individuals.

Morphological characterization of human inherited disease was presented by Gonin in 1903, but no animal models were available for study until Keeler (1924) identified inherited blindness in mice which is transmitted as an autosomal recessive characteristic (Sorsby and co-workers, 1954). Now, mainly through the dedication of individual scientists, a variety of inherited disorders have been identified. The inherited disorders of mice, rats and dogs have been investigated and characterized morphologically. The final stage of each disease is similar in that all visual cells of the retina are lost and the animal is blind. The disorders differ, however, in the time of onset of the disease and in the morphological changes

which characterize the pathology. For convenience, those disorders which become evident at the time of visual cell development and differentiation are designated as early-onset diseases (e.g. rd [retinal degeneration] mice and Irish setter dogs). Those occurring later in life are designated as late-onset diseases (e.g. RCS [Royal College of Surgeons] rats and miniature poodle dogs).

The availability of animal models is but one facet of progress in the last decade. Another is the explosive increase in knowledge about the retina and the rod visual cells, in particular. The majority of inherited disorders of the retina affect rod visual cells before the cones become involved. Therefore, the greater our understanding of the normal morphology, biochemistry and physiology of rod photoreceptors, the more potent our studies of retinal degeneration become. Even a decade ago, rhodopsin was only vaguely understood as a protein and cyclic GMP had only recently been discovered. Rod cells have now revealed themselves as highly sophisticated cellular entities with extreme specializations and biorhythms. For example, rod cells are active during the night; they shed the tips of their outer segments for the pigment epithelium to digest at dawn (LaVail, 1976) and re-form the lost receptor membranes during the day in preparation for another night's activity (Besharse, Hollyfield and Rayborn, 1977). In darkness, rod photoreceptors maintain, continuously, a dark current which flows from the inner segment to the rod outer segments (Hagins, 1972); light decreases this current, diminishing the synaptic output to second-order neurons of the retina. The beauty and complexity of the visual cell is staggering, and it is easy to conceive where flaws in the phototransducer mechanism or in the regulation of cellular metabolism could lead to visual cell degeneration.

Scientific advances and the availability of animal models are essential for understanding inherited disorders of the retina but, for success, there must be an active interest and participation in the quest for the cause and cure of blindness. In this age of action-oriented individuals, the ground-swell of increasing knowledge in visual science was felt within the population of individuals who were afflicted with inherited blindness. Organizations such as the Retinitis Pigmentosa Foundation were formed by concerned friends and relatives of affected patients. Their self-ascribed role was to raise funds for basic and clinical research and to raise the level of awareness of scientists concerning the urgency for applied knowledge. Mainly through their efforts, more scientists than ever before are working on inherited blindness or they wish to translate their basic findings into meaningful clues for deciphering the diseases.

Our investigations of retinal degeneration began with the rd mouse, continued to the RCS rat and, finally, extended to dog models as they became available. In this chapter, we will present a composite of the rd mouse and RCS rat disorders and refer the reader to the chapter by Dr. G. Chader and co-workers for a discussion of the Irish setter dog disease. The early-onset disorder of rd mice is different from that of the RCS rats and, where possible, the similarities and differences will be indentified.

INHERITED BLINDNESS IN rd MICE

The rd mutation causes the degeneration of visual cells of the mouse retina without noticeably affecting other cells of the retina or neurons of the central nervous system (LaVail and Sidman, 1974). Several strains of mice carry this mutation, e.g. C3H/HeJ, C57BL rd1e and CBA/J (Fuller and Wimer, 1966). The rd mutation appears to be distinct from that reported for Purkinje cell-deficient and reeler mice in which both the visual cells of the retina and neurons of the cerebellum degenerate (Mullen and LaVail, 1975). Photoreceptor cells of the rd retina appear to form normally during the prenatal and early postnatal period and to achieve

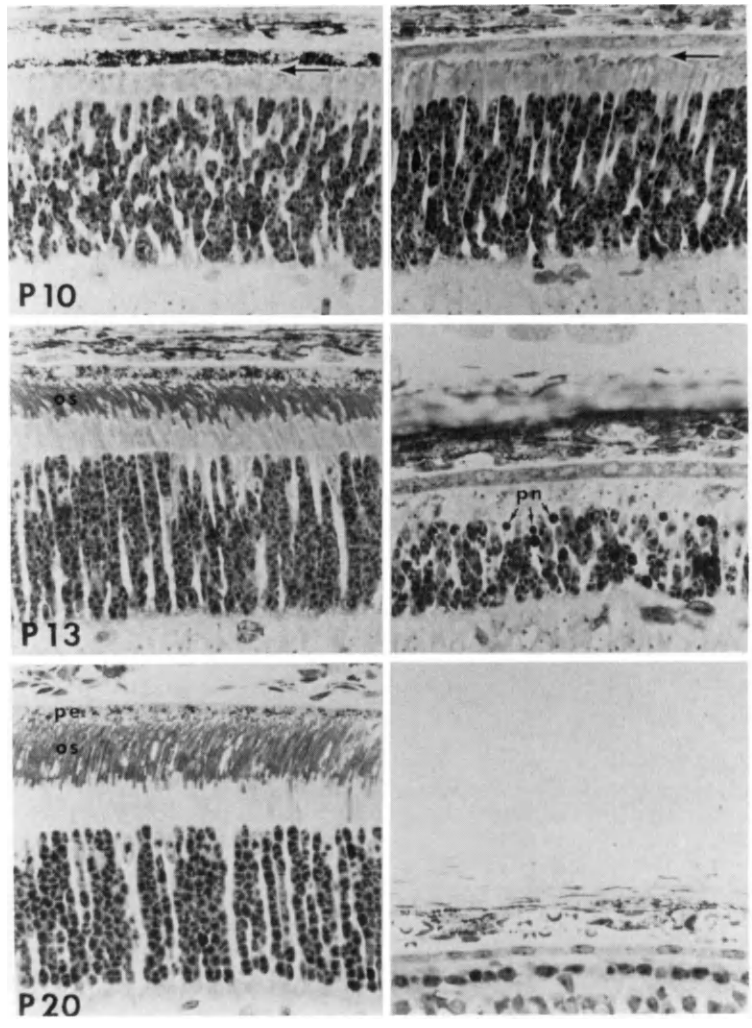

Fig. 1. Retinas from littermate pairs of C57BL/6J + + /rd1e and rd1e/rd1e mice at different ages. At 10 days of age, rod outer segments (arrows) have just begun to appear in the normal heterozygote (P10,left) and in the homozygous mutant (P10,right). At 13 days of age, in the normal retina (P13, left) the outer segments (os) have grown appreciably in length, whereas in the diseased retina (P13, right) outer segments are not evident and the outer nuclear layer has been reduced to four or five rows of photoreceptor nuclei, many of which are pyknotic (pn). At 20 days of age, rod outer segments (os) in the normal retina (P20, left) have just reached their adult length. In the mutant homozygote (P20, right) the outer nuclear layer has been reduced to a single row of pyknotic and hyperchomatic nuclei. The apparent difference in concentration of melanosomes in the pigment epithelium cells (pe) in the normal retinas (P10, left; P13, left; P20, left) is due to slight differences in section thickness (1μ to 2μ epoxy resin-embedded sections, toluidine blue, x600). Reprinted from Arch. Ophthal, May 1974, Vol. 91, pgs. 394-400.

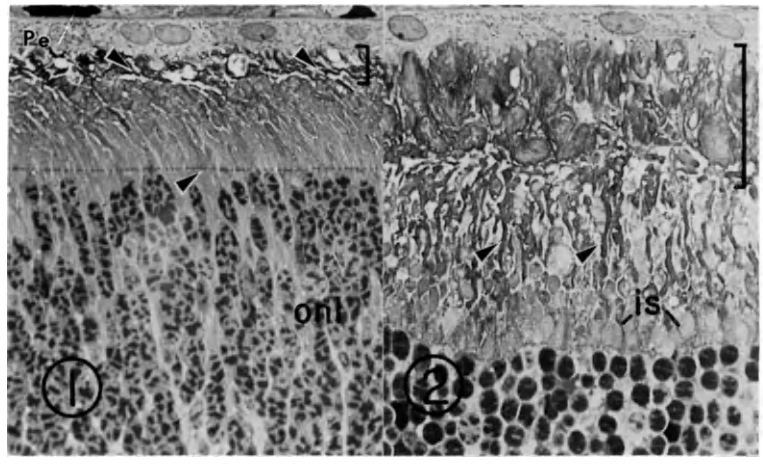

Fig. 3. Photoreceptor and pigment epithelium layers of RCS retina, showing progressive accumulation of membranous debris. 1) At 13 days, disorganized material (bracket) between rod outer segment tips (upper arrows) and pigment epithelium (pe) can first be observed with the light microscope. The outer limiting membrane (lower arrow) and outer nuclear layer (onl) can also be seen. 2) At 24 days, the layer of debris (bracket) has increased in thickness. Many outer segments (arrows) remain intact. Inner segments (is) are swollen and pyknotic nuclei are observed. Reprinted from the J. Cell Biol. 49, 664-682, 1971, by copyright permission of the Rockefeller University Press.

some degree of differentiation (Fig. 1) (LaVail and Sidman, 1974). The formation of outer segment membranes is perhaps less than normal, and the rhodopsin content is correspondingly lower (Carvaggio and Bonting, 1963). The synaptic terminal of rod visual cells begins to develop but degenerative changes in the cell appear to arrest the formation of a mature synaptic ribbon complex (Blanks, Adinolfi and Lolley, 1974). The pigment epithelium is morphologically and functionally normal in this disorder, suggesting that the rd gene is expressed in the visual cells (LaVail and Sidman, 1974).

The first sign of ultrastructural pathology in the rd photoreceptors has been observed on the 8th postnatal day as a swelling of mitochondria of the inner segments (Shiosi and Sonahara, 1969). Subsequently, the outer segment membranes become disoriented and the Golgi complex swells. Cellular death is apparent in the posterior retina by the 10th day. Degeneration begins centrally and spreads toward the ora serrata like a wave. By day 20, virtually all of the rod visual cells have degenerated (Noell, 1965). The rate of degeneration of the rod cells is not accelerated by light. The few cells that remain after 20 days appear to be cones (Carter-Dawson, LaVail and Sidman, 1978). Neither the rod or cone visual cells reach full maturity, and it has been proposed that visual cell death results from a defect in differentiation (Keeler, 1924; Noell, 1965; Lolley, 1973).

The most characteristic feature of this disease is its manifestation at a time during postnatal life when the visual cells have just differentiated to a degree which enables them to respond to light with excitation for the first time (Noell, 1965), as deduced from the electroretinogram (ERG). The ERG of the rd retina can be recorded first at about 9 postnatal days and, at 11 days, it is quite similar to that of control retinas. The ERG of normal mice changes rapidly in form and size during maturation, and the minimal intensity for eliciting a physiological response decreases as outer and inner segments grow to maturity, a stage reached at about 3 weeks. It is during the period of rapid visual cell growth (12-18 days) that the ERG of the affected mouse becomes increasingly abnormal (Noell, 1965). The a-wave of the ERG, which relates to photoreceptor function, is arrested first and a physiological response is extinct by 20-28 days concomitant with the death of the most disease-resistant cells near the ora serrata.

Evaluation of the rd retina by indirect ophthalmoscopy is difficult in the early stages of the disease since the rods develop and are in the process of degenerating by the time mice open their eyes at 12-13 postnatal days. The following are the most conspicuous features which were observed in the retina of a five-week old rd mouse, and they represent the final stages of the disease: "Pigment aberrations are widespread with areas of loss of pigment interspersed with dark brown pigment clumps. The retinal vessels are white and thread-like and the papilla is whiter than normal. A dark brown glial remnant of the hyaloid vessel overlies the disc" (Rubin, 1974).

An abnormality in cyclic GMP metabolism occurs about two days before the rd visual cells begin to degenerate (Schmidt and Lolley, 1973). The specifics of this defect are still unresolved but the abnormality results in the accumulation of cyclic GMP in the diseased visual cells (Fig. 2). Biochemical studies indicate that the enzyme guanylate cyclase, which synthesizes cyclic GMP, is present and active in the rd visual cells whereas the capacity for cyclic GMP hydrolysis is below normal (Farber and Lolley, 1976).

In retinas of normal mice, light causes a reduction in cyclic GMP content of rod photoreceptors (Orr and co-workers, 1976). The mechanism for this effect has been resolved partially. Light is absorbed by the visual pigment rhodopsin and the light-activated rhodopsin interacts with the phosphodiesterase (PDE) to produce its activated state (Keirns and co-workers, 1975; Krishna and co-workers, 1976).

Intermediate reactions or activation steps may occur between rhodopsin and the PDE (Bitensky and co-workers, 1978) but these are mainly speculative at the present time.

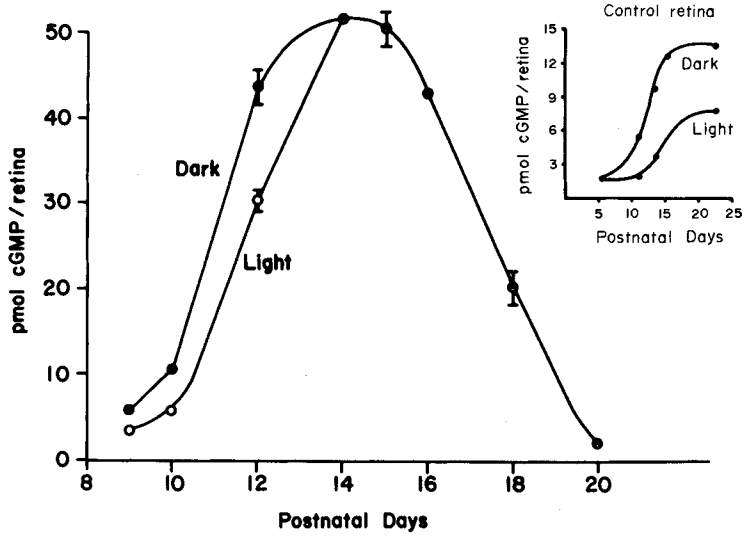

Fig. 2. Cyclic GMP content of developing retinas from dark/light-adapted rd mice. Inset: Corresponding values (mean ± SEM where n>6) of normal retinas from DBA/1J mice.

The rd retina was tested during its developmental period for the ability of light to decrease cyclic GMP levels (Fig. 2) (Farber and Lolley, 1977). It was observed that a small fraction of cyclic GMP in the rd retina could be hydrolyzed in response to light but the major portion of the cyclic GMP was unaffected by light. The light-induced reduction of cyclic GMP was observed in the rd retina only during the postnatal period when the visual cells had morphologically-intact outer segments. This indicates that rd visual cells have PDE activity which can be activated by light. The high content of cyclic GMP in the rd retina, following degeneration of the outer segments, indicates additionally that the light-insensitive cyclic GMP is localized in the inner segments, soma or synaptic terminals of the visual cells.

If the PDE is present in rd visual cells and the enzyme exhibits a normal K_m for cyclic GMP (Farber and Lolley, 1977), why does cyclic GMP accumulate in this disorder? Our studies suggest that the PDE enzyme which is present in rd visual cells is abnormally labile to biochemical procedures. Instability of the enzyme could occur from an error in the primary structure of the enzyme or it could result from an inability of the enzyme to bind properly to the receptor membranes. The latter speculation stems from our observation that solubilized PDE of rod outer segments is more susceptible to thermal denaturation than that which is bound to rod outer segment membranes.

The abnormality in cyclic GMP hydrolysis is restricted to visual cells of the rd retina. Body tissues of the rd mouse such as blood components, muscle, brain and skin have been evaluated for PDE activity, and all tissues appear normal (Table 1). The possible involvement of PDE in the visual transducer mechanism (Farber, Brown and Lolley, 1977) and the biochemical characteristics of the enzyme suggest that the PDE of rod visual cells is unique. Like rhodopsin, it is localized specifically in visual cells, and a genetic abnormality in its structure or activation mechanism would be expressed selectively in rod visual cells.

TABLE 1 PDE Activity in Tissues of Control and rd Retinas

Tissue samples	Control			rd	
	C57BL	DBA	rdle/++	C3H/HeJ	rdle/rdle
Plasma	2.0	3.3	1.8	3.0	2.5
Serum	0.05	--	--	0.02	--
Erythrocytes	0.42	0.50	0.42	0.25	0.72
Lymphocytes	85.1	92.0	90.0	68.0	92.0
Muscle	0.35	--	--	0.41	--
Skin	1.20	--	--	1.20	--
Hypothalamus	--	37.3	--	43.7	--
Cerebral Cortex	--	63.8	--	74.0	--

PDE Activity = nmol cGMP hydrolyzed/min/mg protein

The observations cited above imply that an accumulation of cyclic GMP in rod visual cells is an early and perhaps causative factor in the inherited disease of rd mice. A system for culturing eye rudiments of Xenopus laevis embryos, developed by Hollyfield, Mottow and Ward (1975), was suited for testing this hypothesis. In collaboration with Hollyfield and co-workers, we were able to show that drugs which inhibit PDE activity in normal cells could initiate photoreceptor degeneration (Lolley and co-workers, 1977).

The first signs of morphological pathology in the drug-induced degeneration of Xenopus photoreceptors were similar to those reported for the inherited diseases of rd mice, i.e. disorganization of rod outer segments and swelling of inner segment mitochondria. Following these changes, the cells became necrotic. Direct biochemical measurements of the rudiments showed that cyclic GMP accumulated in the rd visual cells and that cyclic GMP was released to the culture fluid when the cells degenerated.

The early-onset disease of rd mice can be characterized as one in which cyclic GMP accumulates before and during the period when visual cells degenerate. We still do not know how cyclic GMP is involved in the degenerative process. One idea is that cyclic GMP is associated with the visual transducer mechanism, regulating ion permeability of the receptor (Farber, Brown and Lolley, 1978). If this is the case, an accumulation of cyclic GMP might lead to the free passage of ions into the cell, leading to osmotic imbalance and eventual osmotic lysis of vital organelles. Another option which cannot be excluded is that cyclic GMP may regulate internal biochemical processes of the visual cell. If these processes involved membrane assembly or protein-translocation, an accumulation of cyclic GMP would disrupt the homeostatic balance within the visual cell and precipitate the degenerative process. Clarification of the role of cyclic GMP in rod visual cells will allow a choice between these alternatives and may set the stage perhaps for a rational treatment of the mouse disorder.

INHERITED BLINDNESS IN RCS RATS

The rat disease is morphologically and biochemically distinct from the mouse or Irish setter disorders. However, these diseases share some common features which cause them to be considered together in the study of inherited retinal degeneration. Like the mouse and Irish setter diseases, the RCS rat mutation is carried as an autosomal recessive characteristic (Bourne and Gruneberg, 1939), and the mutation leads eventually to the complete destruction of the visual cell population. The end-stage of these diseases is blindness but the processes which lead to photoreceptor cell degeneration differ between the RCS rat and the early-onset diseases of the mouse and Irish setter dog.

Evaluation of the RCS retina by indirect ophthalmoscopy has been reported for end-stages of the disease. The appearance is similar to that described for the rd mouse, except that extensive pigmentary changes are present in the rat retina after the visual cells have degenerated (Rubin, 1974).

The mutant gene of RCS rats is expressed in the pigment epithelium cells of the retina (Mullen and LaVail, 1976; Herron, Riegel and Mayers, 1969). The degenerative condition is typified by a period of normal photoreceptor cell differentiation, followed by an accumulation of lamellar debris in the extracellular space between the photoreceptor outer segments and the pigment epithelium (Fig. 3) (Bok and Hall, 1971). The defective gene appears to interrupt the process of photoreceptor renewal by disrupting phagocytosis of shed rod outer segment membranes by cells of the pigment epithelium.

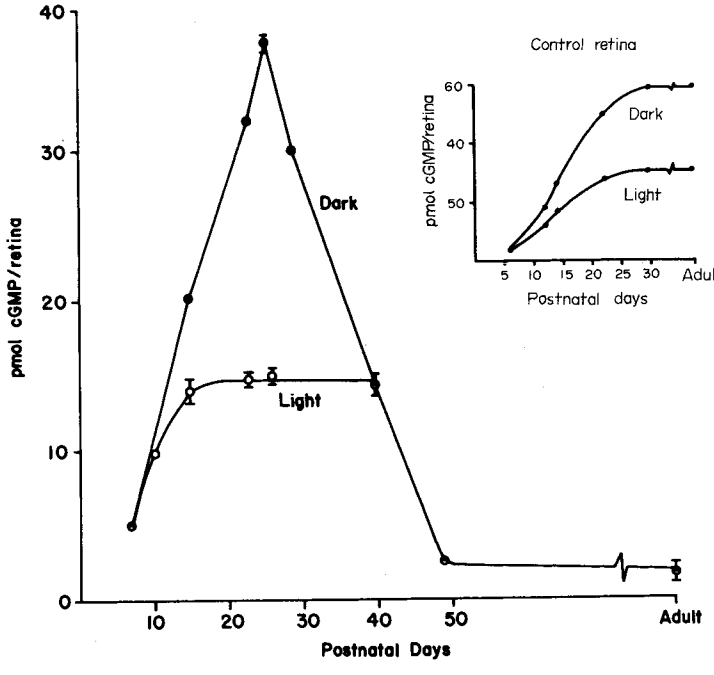

Fig. 4. Cyclic GMP content of developing retinas from dark/light-adapted RCS rats. Inset: Corresponding values (mean ± SEM where n>6) of normal retinas from Fischer CDJ rats.

Normally, vertebrate photoreceptor cells renew continually their outer segments by a balanced process of new disk synthesis and assembly, at the base of the outer segment (Young, 1967). The shed packets of rod outer segment membranes are subsequently phagocytized by the adjacent pigment epithelial cells to form phagosomes, which are degraded by hydrolytic enzymes contained within the lysosomes of the pigment epithelium (Young and Bok, 1969).

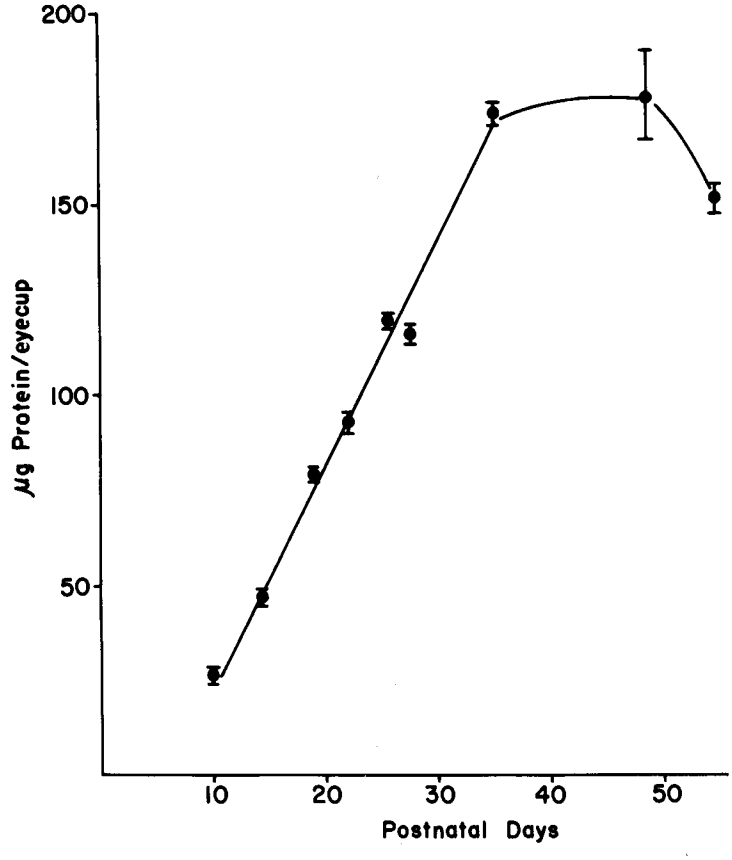

Fig. 5. Protein content of debris collected from developing eyes of RCS rats. After removal of the anterior segment of the eye, lens and retina, debris was collected by washing the interior of the eyecup with isotonic medium, using a micropipette. Microscopic examination of the debris revealed rod outer segment fragments, erythrocytes, macrophages and disorganized membranous material. Protein values (mean ± SEM where n>6) were measured by the method of Lowry.

In RCS rats, the synthesis of rod outer segment membranes and their transport and assemblage into disk membranes appear to be essentially normal (Bok and Hall, 1971). The shedding mechanism also is intact, and it follows a diurnal rhythm like that of normal rat visual cells (Goldman and O'Brien, 1978). Since most of the debris is derived from rod outer segments (LaVail, Sidman and O'Neil, 1972), RCS retinas show a corresponding accumulation of rhodopsin (Dowling and Sidman, 1962). The inability of RCS pigment epithelium cells to phagocytize the membranes of the rod outer segments as they are shed is apparently the primary abnormality of the RCS disease. The functional impairment and degeneration of visual cells is a secondary aspect of this disease.

The physiological response of the visual cells of the RCS retina follows an essentially normal development, with the ERG just slightly reduced by 21 postnatal days (Noell, 1965). However, in the subsequent 10 days, an abnormality is clearly evident, mainly in the form of a decreased and slower a-wave in response to a strong light flash. Concomitantly, there is a fall in the overall amplitude of the response, particularly to weak stimuli in the dark-adapted state. The ERG decreases progressively during the following weeks, and it disappears completely after 3 months. The disruption of function appears to relate to the appearance of histological abnormalities in the visual cells.

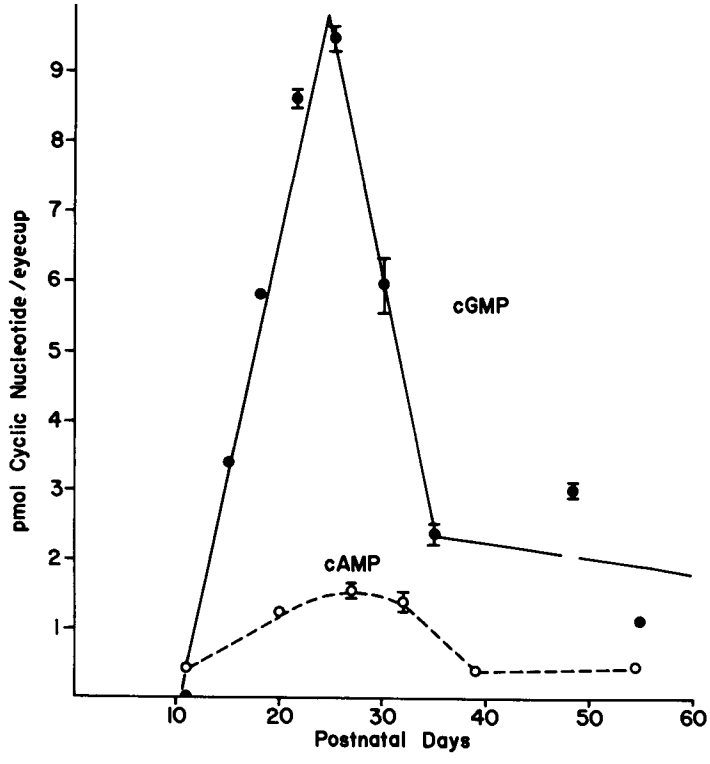

Fig. 6. Cyclic nucleotide content of debris from developing eyes of RCS rats. Debris (see legend of Fig. 5) was extracted with 0.1 N HCl, boiled for 5 min, centrifuged and assayed for cyclic GMP and cyclic AMP by the respective binding protein methods. Values/eyecup (mean ± SEM where n>6) can be expressed per mg protein using data from Fig. 5.

Why do the visual cells die if the genetic abnormality is in the pigment epithelium? At present, we do not know, but two options are being considered. First, the pigment epithelium may fail to support the visual cells in some vital aspect of their metabolism or function. Secondly, components may form in the debris which are toxic to the visual cells but not to the pigment epithelium. While neither alternative can be excluded, experimental evidence suggests that cyclic GMP metabolism may be disturbed by the accumulation of debris (Lolley and Farber, 1975).

The synthesis and hydrolysis of cyclic GMP in RCS visual cells appears to be normal during the first 12-14 days of postnatal life, when the retina is free of debris (Lolley and Farber, 1976). As debris begins to accumulate, the hydrolysis of cyclic GMP is affected more strongly than is cyclic GMP synthesis. In vitro, the phosphodiesterase of rod visual cells appears to acquire a greater affinity for cyclic GMP, increasing its rate of hydrolysis. In vivo, the content of cyclic GMP in RCS retina, representing primarily that in rod visual cells, is decreased from normal dark- or light-adapted controls as debris begins to accumulate (Fig. 4).

The content of cyclic GMP in the RCS visual cells is greater in dark-adapted than in light-adapted retinas, indicating that the bleaching of rhodopsin can activate the phosphodiesterase of rod visual cells and stimulate the hydrolysis of cyclic GMP. Light/dark differences are observed during the postnatal period when photoreceptor outer segments are present (9-35 days). Throughout this time period, the cyclic GMP content of dark- or light-adapted RCS retinas is below that of the respective control. The light-adapted retina contains about 50% less cyclic GMP than a comparably illuminated normal retina and the dark-adapted RCS retina only slightly more than the normal light-adapted retina. The subnormal levels of cyclic GMP in light-adapted RCS retinas might be related to the light-accelerated visual cell degeneration which occurs in this disorder (LaVail and Batelle, 1975). The role that cyclic GMP plays in the degenerating of RCS photoreceptors still remains unclear. Nevertheless, a depression of cyclic GMP levels in RCS photoreceptors is the earliest biochemical defect that has been identified, and it occurs after debris begins to accumulate and before visual cells start to degenerate.

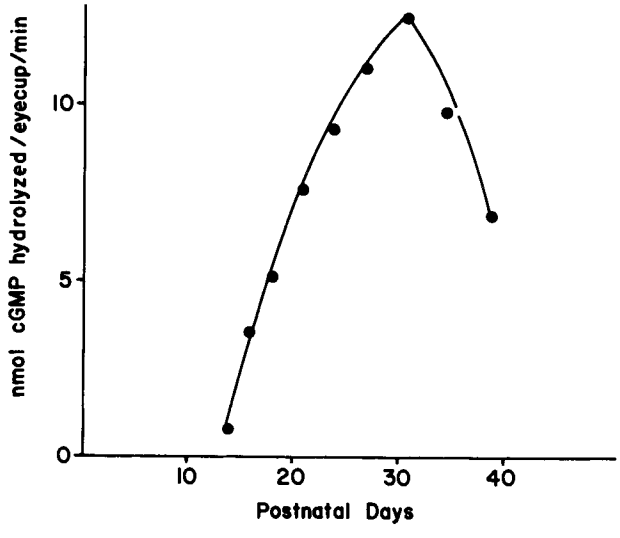

Fig. 7. Cyclic nucleotide PDE activity in debris from developing eyes of RCS rats. Debris (see legend of Fig. 5) was assayed using cyclic GMP (3 mM) as substrate. Values/eyecup can be expressed per mg protein using data from Fig. 5.

The major component of the debris is shed rod outer segments, and little is known of the composition or characteristics of shed rod outer segment membranes. Initial studies in our laboratory suggest that the debris of RCS retina is similar to that of intact rod outer segments. The debris, which accumulates in RCS eyes, increases almost linearly between 10 and 35 days (Fig. 5). The debris contains much more cyclic GMP than cyclic AMP (Fig. 6). The debris also contains appreciably cyclic nucleotide phosphodiesterase activity (Fig. 7.). The PDE of the debris has an unusually high apparent K_m for cyclic GMP. The debris material probably deserves further study because it might contain a substance which interferes with the normal metabolism of RCS visual cells.

The inherited disease of RCS rats is more complicated than that of rd mice or Irish setter dogs in that at least two cell types are involved. The mutation is expressed in the pigment epithelium cells which sluggishly phagocytize the shed outer segments of the visual cells. The continuous production of shed outer segment membranes leads to the accumulation of debris material which seemingly causes the visual cells to degenerate. Cyclic GMP metabolism is disrupted when debris begins to accumulate, and the resulting low levels of cyclic GMP may be associated with the degenerative process.

CONCLUDING REMARKS

The scientist, like the gambler, must be oriented toward the future because the jackpot is always expected in the next series of experiments. However, even with progress, we seem to remain one series of experiments short of unraveling the mystery of retinal degeneration. This is not to discount the great strides that have been made in understanding the normal activities of rod visual cells or the genetics, morphology and fragmentary biochemistry of diseased rod photoreceptors. Much is still to be learned and, hopefully, the study of animal models of inherited blindness will lead eventually to an understanding and cure for the human condition.

It is reasonable to ask which animal model of inherited blindness is most likely to provide insight into the human conditions such as retinitis pigmentosa. At present, a meaningful choice is impossible since we are ignorant of the human disease processes. The causes of human blindness are probably numerous since the mode of genetic transmission of retinitis pigmentosa is autosomal recessive, autosomal dominant or sex-linked (Jay and Bird, 1973; Francois, 1961). Therefore, it seems necessary to consider any animal model as appropriate for the study of inherited blindness because each animal disease may give a clue to specific mechanisms that are capable of initiating visual cell degeneration.

For individuals who are devoted to deciphering specific problems, however, an immediate goal can be the identification of what cyclic GMP is doing in rod visual cells of rd mice and Irish setter dogs (Aguirre and co-workers, 1978). Is it part of the rod visual transducer mechanism which control ion permeability? If so, do elevated levels of cyclic GMP produce an osmotic imbalance within the cell which leads to cellular lysis? Is cyclic GMP part of the light-dark adaptation process or is it an internal messenger for metabolic regulation? Identification of the role of cyclic GMP in visual cell metabolism or function will be an important step toward understanding particularly the early-onset form of inherited retinal degeneration.

ACKNOWLEDGEMENT

We thank Elisabeth Racz for developing the technique for isolating debris and Louise V. Eaton and Inga Anderson for thoughtful assistance in preparation of this chapter. We also acknowledge support from the National Eye Institute (EY00395), the National Science Foundation (BNS 76-81471) and the Medical Research Service of the Veterans Administration.

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Neurochemistry Vol. 1, pp. 441-458.
Pergamon Press Ltd. 1980. Printed in Great Britain.

CYCLIC GMP PHOSPHODIESTERASE ACTIVATOR: INVOLVEMENT IN A HEREDITARY RETINAL DEGENERATION

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ABSTRACT

Cyclic GMP and its enzyme of metabolism, Phosphodiesterase, (PDE) appear to be involved in the normal processes of vision. An abnormality in the development of cyclic GMP PDE in the retina of the newborn Irish Setter dog also appears to be involved in at least one form of inherited retinal degeneration. Specifically, the enzyme in affected retinas fails to switch from a Calmodulin-Dependent to a Calmodulin-Independent form. This failure, along with low levels of Calmodulin could account for the high cyclic GMP content and photoreceptor loss characteristic of early-onset retinal degeneration.

KEYWORDS

Cyclic GMP, phosphodiesterase, Calmodulin, retinal degeneration, Irish Setter dog, differentiation.

1. DOG MODELS FOR RETINAL DEGENERATION

In the human, Retinitis Pigmentosa (RP) is a family of degenerative diseases of the retina (Berson, 1975). Although each of these diseases may have special characteristics and a distinctive time course, they all lead to severely impaired retinal function and blindness. Study of RP in the human has been made difficult by factors of multiple etiology as well as lack of suitable clinical material. Thus, although a good deal is known electrophysiologically (Berson and co-workers, 1968; Ripps and co-workers, 1978) and morphologically (Kolb and Gouras, 1974; Szamier and Berson, 1977) about human RP, little is known physiologically or biochemically about the disease. Most importantly, nothing is known about the basic processes in the retina whose malfunctioning lead to RP.

Animal models have thus been used extensively in an attempt to circumvent some of these problems. For example, several rodent models for retinal degeneration have been described. Of these, interest has focused on the RCS rat and C3H mouse models. Much of the morphological and biochemical information obtained over the last few years has been summarized elsewhere in this volume. There are also several dog models for RP that are known although these have been less extensively studied.

The canine retina is quite similar to that of the human except for the specialized macular area and thus serves as the best readily available model for studying normal and abnormal retinal function. Development in the canine retina is also similar to that in the human although specific events occur at somewhat different times in the two species. It is useful, however, that the bulk of retinal maturation occurs within the first 2-3 weeks after birth rather than during the fetal period when tissues are relatively inaccessible for biochemical analysis.

Several dog species have now been described where the clinical picture exhibits many features similar to those of human RP patients. In general, the hereditary retinal diseases in the dog fall into 2 main categories:

Central Progressive Retinal Atrophy (CPRA). In this condition, the retina develops normally and functions normally. After this period (which varies somewhat in different species), degenerative changes are observed in the pigment epithelium (PE) with pigment clumping and cell atrophy observed (Parry, 1954). The PE thus is thought to be the site of the primary lesion in this disease. Photoreceptor cell death and death of other cells in the central retina follows leading to loss of central vision although peripheral vision may remain for some time. Examples of this disease are found in Border Collies and Labrador Retrievers.

General Progressive Retinal Atrophy (GPRA). Changes in this entity are more general than in CPRA and are thought to be due to a primary lesion in the neural retina rather than the pigment epithelium (Parry, 1953). It thus better mimics the clinical picture of human RP than does CPRA. The onset of the disease differs in the several species which exhibit GPRA. In the Miniature Poodle, the disease is apparent only after several years of life (Aguirre and Rubin, 1972) whereas in the Irish Setter, ophthalmoscopic changes are evident well within the first 4-6 postnatal months (Parry and coworkers, 1955). Electroretinographic changes are also evident soon after birth in the Setter (Aguirre and Rubin, 1975). This is analogous to the human RP condition where Berson (1976) found that the ERG of several patients who ultimately developed RP was not normal even before the onset of the clinical symptoms.

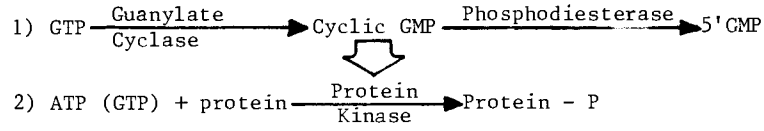
The disease in the Irish Setter is inherited as an autosomal recessive trait. The early onset of the disease leads to night blindness within 6-8 weeks and subsequent total blindness. Electrophysiologically, neither the rods nor the cones demonstrate a normal response, even during the early developmental period, i.e. by 3 weeks, indicating that the photoreceptors never reach final functional maturity (Aguirre and Rubin, 1975). Morphologically, retinal photoreceptors also never reach the final length achieved in the normal retina. Thus, the disease in the Setter is best classified as a "dysplasia" (abnormal development) rather than a degeneration.

2. CYCLIC NUCLEOTIDES AND VISION

In retinal photoreception, a photic stimulus is converted into a neural response (Fig. 1). Two features are of some importance in the process: 1) translation of the photic stimulus into a chemical signal and 2) amplification of that signal. It has been suggested that calcium acts as the chemical mediator in visual transduction (Hagins, 1972). More recently, evidence points to a collateral or possibly even primary role for cyclic GMP in the process.

The enzymes of cyclic GMP synthesis (Pannbacker, 1973; Bensinger and coworkers, 1974) and metabolism (Pannbacker, 1972; Chader and coworkers, 1974) are in extremely high concentration in the photoreceptor unit. A cyclic GMP-dependent protein kinase system has also been described in isolated photoreceptor preparations.

(Farber and coworkers, 1979) as outlined below:



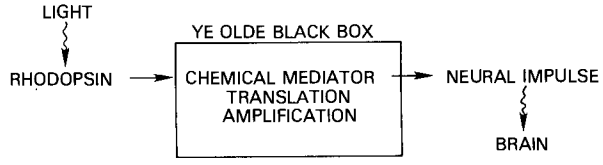


Fig. 1. General Scheme of Photoreception

A dramatic, light-induced drop in cyclic GMP but not cyclic AMP content of photoreceptor units has been observed (Fletcher and Chader, 1976; Woodruff and coworkers, 1977) as seen in Table 1.

TABLE 1. Effect of Light on Cyclic Nucleotide Concentration in Rod Outer Segments (ROS) of the Frog Retina

A-ROS prepared from retinas of frogs dark- or light-adapted in vivo for 16 or 2 hrs respectively

Condition	Cyclic Nucleotide Concentration (pmol/mg protein)	
	Cyclic GMP	Cyclic AMP
dark-adapted	29.3 ± 4.3	1.8 ± 0.8
light-adapted	3.1 ± 1.2	3.4 ± 1.9

B-ROS prepared from dark-adapted frogs. ROS then incubated for 3 min at 23°C in dark or after bleaching

Condition	Cyclic Nucleotide Concentration (pmol/mg protein)	
	Cyclic GMP	Cyclic AMP
dark-adapted	23.4 ± 4.1	2.2 ± 0.4
bleached	3.2 ± 1.6	1.9 ± 1.2

(Data from Fletcher and Chader, 1976)

This is ostensibly due to a small but rapid decrease in guanylate cyclase activity in bleached vs dark-adapted ROS (Krishna and coworkers, 1976) and a dramatic light-stimulated increase in phosphodiesterase (PDE) activity (Miki and coworkers, 1973; Chader and coworkers, 1974) in the presence of a nucleoside triphosphate (Table 2).

TABLE 2. Effect of Light and Nucleoside Triphosphate on Cyclic GMP Phosphodiesterase Activity in Isolated Bovine Rod Outer Segments

Addition	Condition	PDE Activity (nmol/mg)	Fold-Increase in Activity*
None	Dark	85	1.1
None	Light	80	1.0
ATP	Light	680	8.7
GTP		660	8.2
ITP		510	6.5
UTP		460	5.9
CTP		150	1.9

*Value for light-adapted ROS with No Addition was arbitrarily set at 1.0. (Data from Krishna and coworkers, 1976).

A light-stimulated GTPase activity has been described in outer segment preparations that may play a role in the control of PDE activity (Wheeler and Bitensky, 1977). Moreover, a high GTP content has recently been shown to be present in outer segments (de Azeredo and coworkers, 1979). From these data, a reasonable mechanism for the control of PDE activity and cyclic GMP concentration in the photoreceptor can be postulated (Fig. 2A).

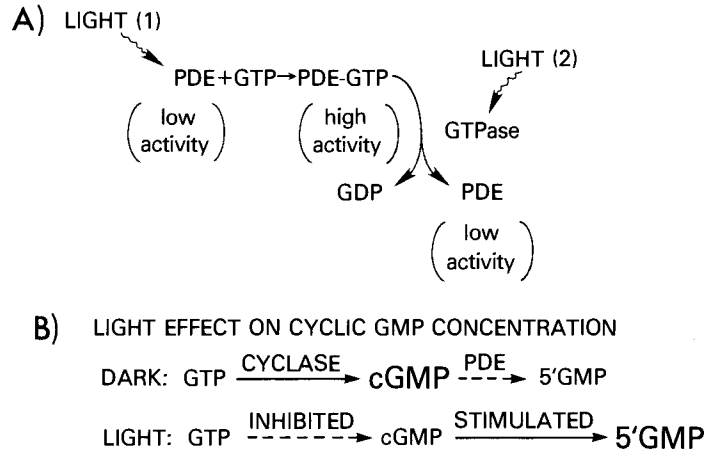


Fig. 2. Possible Control of A)Phosphodiesterase Activity and B)Cyclic GMP Concentration in the Photoreceptor Rod Outer Segment.

First, it can be postulated that PDE exists in a low activity state in dark-adapted outer segments. Upon bleaching, the resultant conformational shifts allow for interaction between GTP and PDE where the nucleoside triphosphate may act as an allosteric modifier in a manner similar to that described by Rodbell and his collaborators for activation of adenylate cyclase by GTP in fat cell membranes (Rodbell and coworkers, 1975). The extremely high specific activity of light-activated PDE and its rapid kinetics (Yee and Liebman, 1978) are consistent with such a role for PDE in the visual process. In a second step, light-activated GTPase may then reduce the GTP concentration, effectively returning PDE activity to basal (i.e. dark-adapted) activity. Such a scheme would allow for a high concentration of cyclic GMP in the dark due to high guanylate cyclase and low PDE activities (Fig. 2B). In the light, the small inhibition of the cyclase and large PDE activation lead to a rapid decrease in cyclic GMP concentration. This could directly influence membrane events or possibly modulate protein kinase activity of the ROS as recently postulated by Farber and coworkers, (1979) and control membrane permeability, potential, etc. through phosphorylation.

In an important series of experiments, Miller and Nichol (Nichol and Miller, 1978; Miller and Nichol, 1979) have recently demonstrated that cyclic GMP causes depolarization of the rod plasma membrane to the Na^+ equilibrium potential and that hyperpolarization is dependent on cyclic GMP hydrolysis by PDE. It thus may be that Na^+ permeability is maintained by a high cyclic GMP concentration in dark-adapted photoreceptors (Fig. 3) while a light-induced drop in cyclic GMP concentration results in blockage of Na^+ channels and hyperpolarization. Derangement in this system of light-activated PDE and ensuing events of Na^+ transport and electrical activity may play a significant role in at least some forms of retinal degeneration as discussed below.

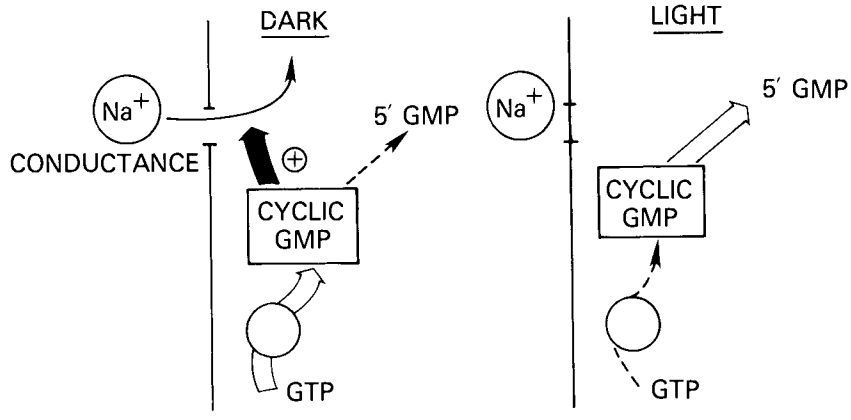


Fig. 3. Possible Effect of Cyclic GMP on Electrophysiological Events in the Photoreceptor Outer Segment. In the DARK, cyclic GMP is at a high level and sodium (Na^+) conductance is maintained. In the LIGHT, Cyclic GMP levels decrease and the sodium channels close.

3. MORPHOLOGICAL STUDIES ON THE IRISH SETTER

Much of the development of the retina in dogs as in rodents and many other species occurs in the early postnatal period. The second week after birth thus is critical in retinal development since the photoreceptors begin to differentiate at this time (Fig. 4). At 10 days of age, much of the inner retina is well differentiated but photoreceptor outer segments are in the earliest growth stages. As seen in Fig. 4A, the external limiting membrane (ELM) is separated from the pigment epithelium (PE) by inner segments (IS) and only small amounts of outer segment disc membranes (short arrows) are observed.

By 13 days in normal animals, (Fig. 4B), photoreceptor outer segments have begun to elongate and contain well-organized stacks of outer segment lamellar discs. Some of these discs seem to have been engulfed (arrow) by the pigment epithelium (PE). In the 13 day old affected Setter (Fig. 4C), small photoreceptor inner segments (IS) project through the external limiting membrane (ELM). A minimal amount of outer segment material is seen; some is apposed to the PE apex (arrow) and some seems to be located within the PE cytoplasm (double arrow).

By 8 weeks of age, normal retinal development is virtually complete but substantial retinal degeneration has taken place in affected dogs (Fig. 5). In the tapetal zone of 10 week old control retinas (Fig. 5A) photoreceptor inner segments (IS) and outer segments (OS) are elongated and about the non-pigmented pigment epithelium (PE). The sections from the affected retinas (8 weeks, Fig. 5B; 10 weeks, Fig. 5C) are taken from the nontapetal zone where the PE is variably pigmented. The photoreceptor layers of the diseased retinas are greatly reduced in width and density. The inner segments are diminutive and there is only minimal outer segment material (white arrowheads) especially in the 10 week old animal. The extensive loss of rod photoreceptors (Fig. 5C) causes increased prominence of cones (black arrows) and reduction in the width of the outer nuclear layer (ONL). This is most striking in peripheral areas of the retina as seen in the 8 week specimen (Fig. 5B).

Thus, morphological abnormalities in affected retinas are already apparent in the second postnatal week, at the earliest stages of photoreceptor development. By 2 months of age, affected retinas have lost the bulk of their rod outer segment membranes as well as many of the rod photoreceptor cells themselves. One can conclude from these results that the disease is not one of classical degeneration of mature structures but rather an early abnormality of development and differentiation.

4. BIOCHEMICAL STUDIES ON THE IRISH SETTER

Disc Gel Electrophoresis Studies

In any biochemical study of a hereditary disease, it is important to establish if the genetic lesion is of a general or a specific nature. In other words, is there a general defect in synthesis/degradation of cellular components (eg. proteins) or is the defect more delimited, possibly to one enzyme? Schmidt and coworkers (1979) have shown greatly reduced rhodopsin synthesis in 5-8 week old animals, a time of marked photoreceptor degeneration as shown above. We have looked at general protein synthesis in dogs at 1-2 weeks of age using disc gel electrophoresis in an ef-

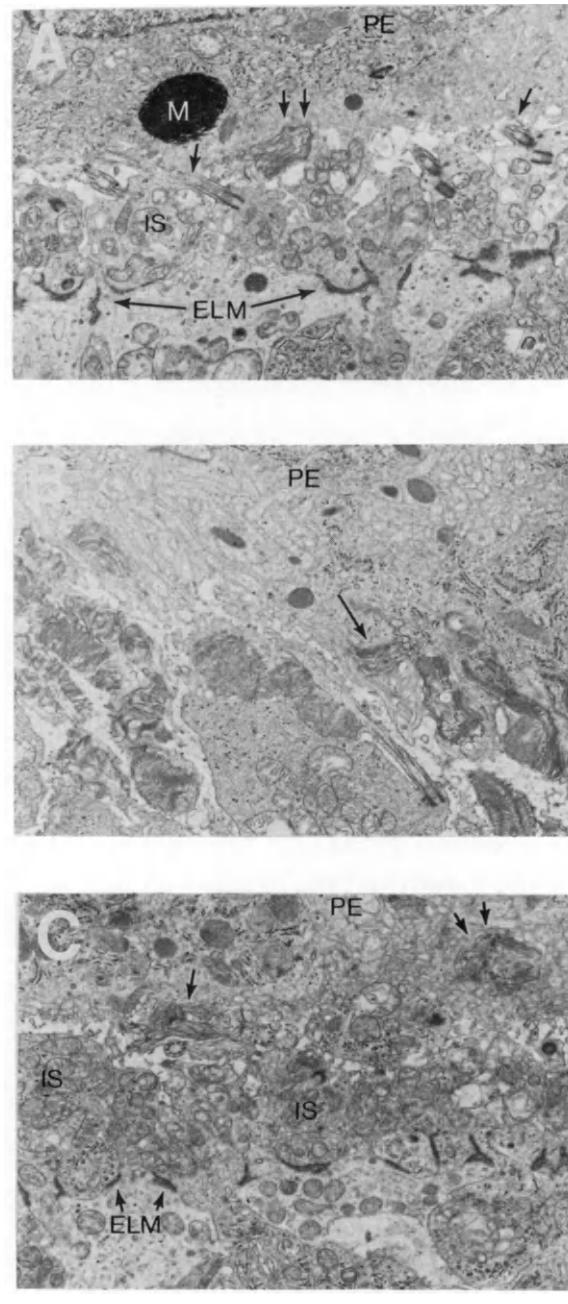


Fig. 4. Electron micrographs of photoreceptor layer from A) 10 day Normal, B) 13 day Normal and C) 13 day Affected Irish Setters.

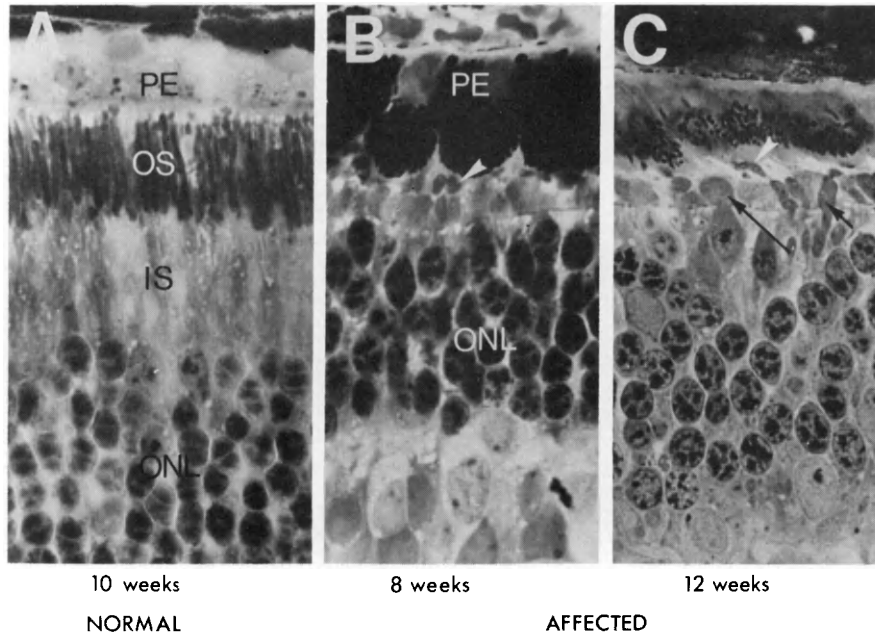


Fig. 5. Light Micrographs of Areas of the Retina and Pigment Epithelium (PE) from A) 10 week Normal, B) 8 week Affected and C) 12 week Affected Irish Setters. (from Aguirre and coworkers, 1978).

fort to examine this point when the photoreceptor units first begin to develop and concomitantly, to degenerate. In these experiments, affected and control retinas were incubated for 2 hrs. with ^{3}H-leucine or ^{14}C-leucine respectively. After washing and homogenization, the 100,000 xg pellets were collected and solubilized in sodium dodecyl sulfate. Aliquots of ^{3}H- and ^{14}C-labeled protein from retinas of comparable ages were mixed and applied to disc gels. After electrophoresis, the gels were stained, scanned or photographed, cut into 1.5 mm slices and counted (Fig. 6B). Specific deficiencies in the synthesis of membrane protein by any retinal cell type would be expected to produce discrepancies in the $^{3}H/^{14}C$ ratio of the affected proteins (Fig. 6A). At no developmental age studied (5-16 postnatal days) was there any evidence of a specific deficit in the synthesis of any protein. We conclude that membrane protein synthesis in the photoreceptor is normal during the period when outer segment development is initiated. Synthesis of soluble protein is yet to be examined.

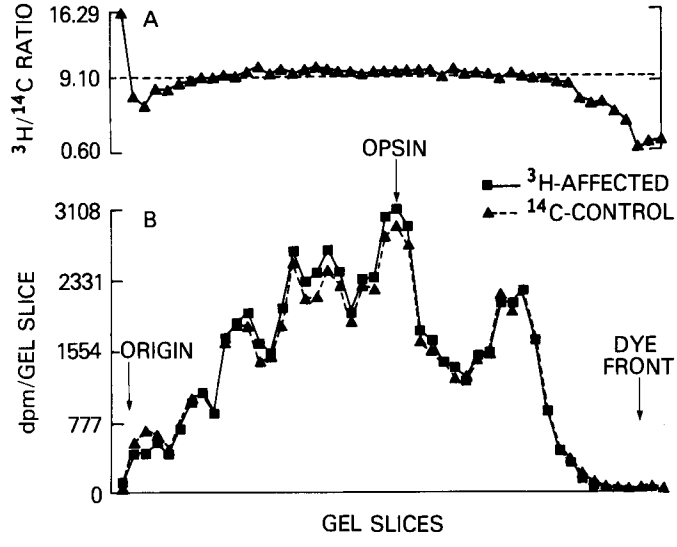


Fig. 6. Disc Gel Electrophoresis of Newly Synthesized Photoreceptor Membrane Protein from 13 day Affected and 12.5 day Control Setters. (manuscript in preparation)

Phosphodiesterase Studies

Phosphodiesterase was originally thought to play only a passive role in the metabolism of cyclic nucleotides in mammalian tissues. Now, it is known that PDE is present in multiple forms differing in substrate specificity (eg. cyclic AMP vs cyclic GMP), kinetic characteristics (i.e. several K_m values) and subcellular localization (Appleman and Terasaki, 1975). Chung (1967) and Kakiuchi (Kakiuchi and Yamazaki (1970) and their groups have found that some forms of PDE are depedent on the presence of a Protein Activator which increases PDE activity in conjunction with calcium. This Activator, also called "Calmodulin" is a small, heat-stable

protein that has been isolated and characterized from several tissues notably brain (Liu and coworkers, 1974). Other types of PDE are not activated by Calmodulin and are called "Activator-Independent". Figure 7 illustrates the mechanism by which Calmodulin can activate PDE. Initially, Calmodulin forms a complex with calcium. This is followed by binding to PDE to form a ternary complex and conversion of the enzyme to an active form.

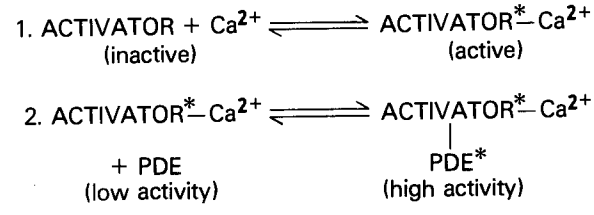


Fig. 7. Activation of Phosphodiesterase (PDE) by Protein Activator (Calmodulin)

In the bovine retina, both cyclic AMP- and cyclic GMP-specific types of PDE are present with differing kinetic characteristics (Chader and coworkers, 1974). Cyclic GMP PDE is preferentially compartmentalized in photoreceptor outer segments (Pannbacker and coworkers, 1972; Chader and coworkers, 1974). In isolated adult bovine ROS, the cyclic GMP PDE is Activator-Independent although substantial amounts of cyclic AMP PDE Activator are present in all retinal subcellular fractions (Liu and Schwartz, 1978). Incubation of ROS with EGTA (a specific Ca^{2+} chelator), increasing concentrations of Ca^{2+} or the ionophore X-537A had no effect on cyclic GMP PDE activity (Table 3).

In the Irish Setter, Aguirre and coworkers (1978) found large amounts of cyclic GMP in retinas of 8-18 week old dogs in comparison to that in heterozygous control animals. Values averaged 10-fold higher in affected animals (103 pmol/mg protein) than in age-matched unaffected animals (12 pmol/mg protein). Cyclic AMP levels were similar in affected and control retinas at this time. Also no changes in cyclic nucleotide content were observed in other affected dog tissues such as liver, visual cortex or the pigment epithelium-choroid unit. At this age however, degeneration of rod photoreceptors is virtually complete, with considerable degeneration observed in the inner segment and outer nuclear layers (Aguirre and coworkers, 1978). As with protein synthesis studies, it was important to determine if this increase in cyclic GMP occurred early in the development of the disease or was merely a late event, secondary to the massive cellular changes observed after about 2 months of age. An ontogenic survey was thus conducted to determine if differences in cyclic GMP concentration were detectable in the early postnatal period. As can be seen in Table 4, the cyclic GMP content in control retinas is low within the first 1-2 weeks after birth (<1 pmol/mg protein) and then rises slowly to about 12 pmol/mg protein by 2 months of age.

In contrast, cyclic GMP levels are higher in affected retinas even by 9-12 days, a time when photoreceptor outer segments are in their earliest stages of formation.

It may be of interest that the retinal sample from the 12 day old animal which showed higher cyclic GMP (i.e. 18.5 pmol/mg) was from a well-developed animal whose eyes were newly open while the lower value (2.9 pmol/mg was from a less-well developed littermate whose eyes were yet unopened. By three weeks of age, cyclic GMP levels in affected retinas are 10-20 fold above those of the unaffected controls and remain high through at least 18 weeks of age when very little ROS material remains.

TABLE 3 Effect of EGTA, CA^{2+} and X-537A on PDE Activity

Addition (Molarity)	Phosphodiesterase Activity (% of control)	
	Cyclic GMP	Cyclic AMP
None	100	100
EGTA 10^{-6}	118	111
10^{-4}	106	107
Ca^{+2} 10^{-6}	119	113
10^{-4}	113	145
$5x10^{-3}$	94	138
X-537A $2x10^{-5}$	104	121
X-537A $2x10^{-5}$ Ca^{+2} 10^{-4}	113	96

Values of cyclic AMP and cyclic GMP PDE activity were arbitrarily set at 100% in each case.

The high cyclic GMP concentration appears to directly reflect a defect in cyclic GMP PDE activity. Retinal homogenates from control animals at 10 weeks of age demonstrate two distinct kinetic types of cyclic GMP PDE activity, K_m-A at $1.2x10^{-4}$M and K_m-B at $2.0x10^{-5}$M but the affected retina loses the K_m-A PDE activity. This loss at 8-12 weeks could be due 1) to an early loss in PDE activity prior to photoreceptor degeneration or 2) compartmentalization of the high K_m enzyme in ROS and loss of activity concomitant with photoreceptor loss. To examine these possibilities, we studied PDE activity at early postnatal times. We also studied the possible role of the Protein Activator and Ca^{2+} in controling PDE activity in the dog retina. For this purpose, control (heterozygous, unaffected) and affected dogs were deeply anesthetised with pentobarbital, their eyes enucleated and the retinas quickly dissected on ice and frozen in liquid nitrogen. Animals were kindly supplied by the National Retinitis Pigmentosa Foundation, Baltimore, MD. PDE activity was determined in the dog retinal homogenates using 1μM ^{3}H-cyclic AMP or ^{3}H-cyclic GMP as previously reported (Liu and coworkers, 1979). Activation by the Protein Activator was determined by adding 10 μg of purified bovine Calmodulin to the incubation. The role of calcium was determined by addition of 250 μM EGTA to the incubation. Endogenous Activator in dog retina was determined by adding boiled retinal extracts to purified, bovine brain PDE (Liu and coworkers, 1979).

By the second week after birth (day 9), cyclic GMP PDE activity was already lower in the affected retinas than in control retinas (Table 5). Cyclic AMP PDE activity was identical in affected and control retinas at this time. Cyclic GMP PDE activity rose 7-fold by the time of photoreceptor maturation in the normal retina at 7 weeks with little effect on cyclic AMP PDE activity, supplying strong circumstantial evidence that the cyclic GMP but not cyclic AMP PDE activity is predominantly

TABLE 4 Cyclic GMP Content in Normal and Control Retinas of the Irish Setter

Age	Cyclic GMP Content (pmol/mg protein)	
(day)	control	affected
9	0.6,0.7	0.2, 2.7
12	-	2.9, 18.5
13	0.8,0.7	-
17	1.8,2.2	-
18	-	22
22	-	115
25	4.0	-
30	-	76
34	4.9	-
38	-	76
47	-	62
(weeks)		
8	12	99
12	14	119
18	11	92

TABLE 5 Cyclic GMP Phosphodiesterase Activity and Protein Activator Concentration in Control and Affected Retinas

Animal Age (day)	Animal Type	PDE Activity (pmol/mg/min)		Protein Activator (units/mg)
		Cyclic AMP	Cyclic GMP	
9	control	100	310	125
9	affected	100	245	100
31	control	105	885	55
34	affected	105	325	25
48	control	145	2,275	95
48	affected	150	540	55

compartmentalized in the photoreceptor outer segment. The increase in cyclic GMP PDE activity in affected retinas was only 2-fold. Endogenous Protein Activator for cyclic GMP PDE was lower in affected retinas than in control tissue at each age studied. The drop in Calmodulin concentration between 9 and 31 days may be due to natural modulation over the ontogenic period. No marked differences were observed however with the Activator using ^{3}H-cyclic AMP as substrate.

The effect of exogenous brain Activator on cyclic GMP PDE activity is seen in Fig. 8. EGTA was also studied since it is an effective chelator of Ca^{2+}. Brain Activator increased PDE activity and EGTA decreased activity in both affected and con-

trol retina of the 9 day old dog (Fig. 8 A, B). This indicated that the PDE present at this stage of development (i.e. early photoreceptor budding) was the Calmodulin-dependent type. By 5-7 weeks however, neither Calmodulin nor EGTA had any marked effect on PDE activity in the control retina (Fig. 8 C, E) although effects were yet apparent in affected retinas (Fig. 8 D, F). This indicated that there was a switch in PDE type (Activator-Dependent to -Independent) in the control retinas while PDE activity in the affected retinas remained Activator-Dependent.

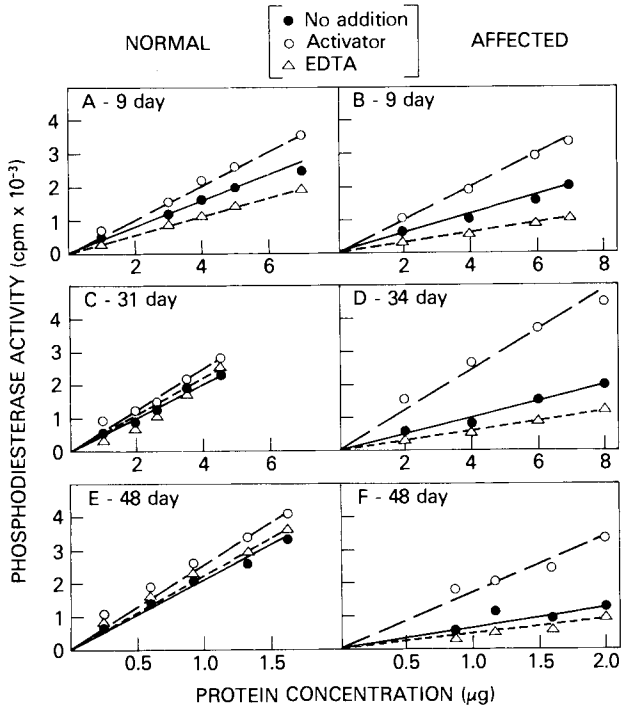


Fig. 8. Cyclic GMP PDE Activity in Retinas of Different Ages. (from Liu and coworkers, 1979)

Our results thus indicate that there is a normal "switch" in PDE type in control retinas from an Activator-Dependent to Activator-Independent form as found in the adult bovine retina. Such developmental changes from "fetal" to "adult" enzyme forms are well known (Villee, 1966; Greengard and Thorndike, 1974). Recent evidence suggests that PDE activity also changes during differentiation in some tissues with regard to its sensitivity to regulation by the Ca^{2+}-dependent Protein Activator (Singer and coworkers, 1978). Our data indicate therefore a sequence of events as shown in Fig. 9:

1. There is not a problem with general membrane protein synthesis at the early times of photoreceptor development (and concomitant degeneration in affected animals).
2. A normal switch in PDE type does fail to occur however in early photoreceptor development in affected retinas. This, and the lowered level of Calmodulin in these retinas lead to greatly increased cyclic GMP content.
3. Morphological degeneration follows.

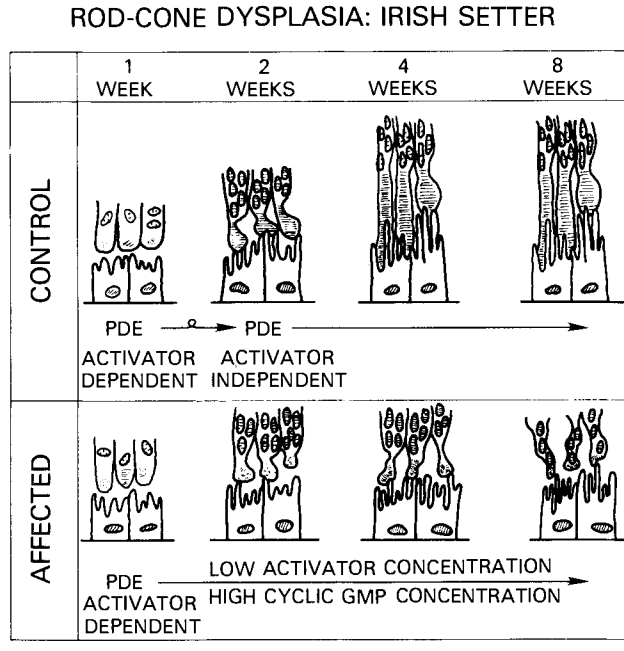


Fig. 9. Schematic Outline of Morphological and Biochemical Events in the Development of Outer Segments in Retinas of Control and Affected Irish Setters. (from Liu and coworkers, 1979).

5. QUESTIONS AND FUTURE WORK

Several problems yet remain to be worked out before the theoretical sequence of events depicted in Fig. 9 can be said to occur in vivo. In the present study, for example, we have used homogenates of the entire retina but make the assumption that the bulk of the cyclic GMP PDE activity measured as well as the Calmodulin activity determined is compartmentalized in the photoreceptor outer segments. There is evidence for this from several laboratories (Chader and coworkers, 1974; Orr and coworkers, 1976; Farber and Lolley, 1977) at least in the case of cyclic GMP and cyclic GMP PDE compartmentalization, although only analysis of quickly frozen microdissected retinal layers will answer this point definitively. Another question concerns the persistently high cyclic GMP content in the affected retina long after most of the outer segment membranes have disappeared. (eg 8-12 weeks). This would indicate either high cyclic GMP content in other retinal or pigment epithelial cell processes or incredibly high cyclic GMP levels in the small amount of outer segment material remaining.

A third question concerns the site of the actual defect in cellular metabolism which leads to the disease. Is there a transcriptional or translational defect? It is possible that the defect occurs directly at the gene level coding for defective enzyme protein activities. One might postulate basic problems in the photoreceptor DNA as in Xeroderma Pigmentosum where a DNA-repair defect has been pinpointed (Robbins, 1974). Alternatively, developmental anomalies in m-RNA synthesis/processing or nuclear proteins (eg. Non-Histone Chromosomal Proteins) could be defective. Post-translational events could also be present at the ribosomal level

resulting in incorrect protein sequences or persistence of "fetal" vs "adult" protein forms.

The mechanism by which a high cyclic GMP content could cause photoreceptor degeneration is also not known. The PDE inhibitor Isobutylmethylxanthine however has been shown to cause increased cyclic GMP levels in the eye rudiment of Xenopus laevis embryos along with photoreceptor cell degeneration (Lolley and coworkers, 1977). Also if cyclic GMP does function as a chemical messenger in the visual process, the basic electrophysiological state of the photoreceptor cell would be altered as depicted in Fig. 3. Abnormally high cyclic nucleotide could simulate an extreme state of dark adaptation with continuous depolarization due to nucleotide effects on Na^+ flux. Shinohara and Piatigorsky (1977) have shown that the synthesis of specific proteins is influenced by changes in the intracellular Na^+/K^+ ratio in cultured chick embryo lens. Carrasco and Smith (1976) have previously shown that Na^+ and K^+ can effectively control translation by discrimination between different types of m-RNA. It is similarly possible in the developing photoreceptor cell that an abnormality in the Na^+/K^+ ratio brought about by the high cyclic GMP level leads to general cell dysfunction and death. Alternatively, the Na^+/K^+ ratio could effectively control expression of the PDE types in the developing photoreceptor cell as it does with crystallin synthesis in the developing lens. It is relevant to note that cataract formation is a common feature in almost all types of retinal degeneration yet described.

Thus, the presently described defect in Phosphodiesterase may not be the basic "primary" hereditary lesion. The scheme outlined in Fig. 9 should only be considered to be a working hypothesis which attempts to combine most of the facts known about canine early-onset retinal degeneration into a theory that can be tested. Simplistically, from the PDE data alone (Aguirre and coworkers, 1978; Liu and coworkers, 1979) one would assume that an Activator-dependent cyclic GMP PDE is present in inner retinal layers prior to ROS development and that a ROS-specific, Activator-independent PDE enzyme fails to develop in affected retinas. One would then conclude that the Activator-dependent PDE observed by Liu and coworkers (1979) would be due to remaining Activator-dependent enzyme in the inner retinal layers. This scheme, however, does not take into account the decrease rather than increase in Calmodulin levels that occurs in older affected retinas where only inner retinal layers remain. Thus the "least-common-denominator" scheme which accounts for 1) decreased PDE activity 2) continuing Activator-dependency in retinas of older affected dogs and 3) lowered Calmodulin levels in older affected retinas is that the type of cyclic GMP PDE fails to switch as depicted in Fig. 9. Although the bulk of the cyclic GMP PDE activity is compartmentalized in retinal outer segments as described above, the Activator-specific PDE may very well be present not in ROS but rather in inner retinal layers. Farber and Lolley (1975) have described a low K_m cyclic GMP PDE in inner retinal layers of the RCS rat, a species which also exhibits retinal degeneration albeit of a different type from the dog. Substantial amounts of light-sensitive cyclic GMP are also present in the inner nuclear layer of the frog retina (de Azeredo and coworkers, 1979) demonstrating that at least two distinct PDE compartments must be present in frog as well as RCS rat retinas. One should also keep in mind that the drop in Calmodulin observed might not significantly affect cyclic GMP PDE activity since normally large excesses of Calmodulin are present in tissues (eg. brain). It is not yet known if such is the case in retina. Moreover, Calmodulin is now known to modulate many enzyme systems including adenylate cyclase and ATPase. It may yet be found that the major effect of Calmodulin in the retina is on other than phosphodiesterase.

In spite of these unresolved problems and questions of "primary vs. "secondary" hereditary lesions, the Activator-related PDE defect is present very early in the

history of the canine disease. Moreover, it appears that the low retinal PDE activity and resultant high cyclic GMP levels probably bear primary responsibility for outer segment degeneration and photoreceptor cell death. With this knowledge and access to purified Calmodulin we now have an opportunity to possibly increase retinal PDE activity (eg. intraocular injection of Activator) and perhaps to slow or even halt the progress of the disease.

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Neurochemistry Vol. 1, pp. 459-463.
Pergamon Press Ltd. 1980. Printed in Great Britain.

GYRATE ATROPHY OF THE RETINA AND CHOROID IS AN INBORN ERROR OF METABOLISM OF ORNITHINE AMINOTRANSFERASE

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ABSTRACT

Gyrate atrophy of the retina and choroid is an autosomal recessive progressive retinal degeneration caused by an inborn error of metabolism of ornithine aminotransferase activity. Some cases are metabolically treatable. Carrier testing and prenatal diagnosis by amniocentesis are possible.

KEYWORDS

Gyrate atrophy, retina, choroid, ornithine, ornithine aminotransferase, pyridoxine, inborn error of metabolism.

INTRODUCTION

Gyrate atrophy of the retina and choroid is an autosomal recessive condition characterized by a progressive peripheral retinal degeneration with scalloped margins, increased ornithine in blood and urine, and an inborn error of ornithine aminotransferase activity.

CLINICAL DISEASE

Gyrate (Latin gyratus - turned round) was originally used to describe this disease because the margin of the retinal atrophy distinctively curves as circular segments. As such, this is one of the few hereditary retinal degenerations, known collectively as retinitis pigmentosa, that can be ophthalmoscopically defined. The ability to make a specific descriptive diagnosis has facilitated clinical investigation. The disease was originally described by Jacobsohn (1888), Cutter (1895), and Fuchs (1896). The name, "Fuchs's Gyrate Atrophy" is sometimes applied to this disease. Usher (1935) found 26 cases in the literature for his review. Kurstjens (1965) added 10 new cases to 35 reported cases for his thorough thesis on gyrate atrophy. The disease has been reported in many parts of the world (Collier, 1962; Akiya, 1977; Cappelli, 1964; Gillespie, 1964; Hilsdorf, 1967; Horodenski and co-workers, 1973; Rieger, 1972; Caballer, 1975; Roveda and co-workers, 1970; Schaeffer and Tennes, 1970; Wu and Yang, 1960) but the greatest concentration is probably in Finland (Takki, 1974a) where pedigree analysis and consanguinity documentation prove autosomal recessive inheritance.

The disease typically presents in youth with complaints of nightblindness. Clinical examination shows peripheral retinal degeneration with scalloped margins. The visual field is constricted. Most patients are myopic. Electroretinograms are extinguished or show reduced B waves; electro-oculograms show reduced light/dark ratio. Fluorescein angiography demonstrates a sharply defined edge of atrophy with

a functioning choriocapillaris at the edge. The area posterior to the atrophy shows many retinal pigment epithelium transmission defects with fluorescein. The abnormal electro-oculogram, fundus appearance, and fluorescein angiography point to the retinal pigment epithelium as the site of the primary pathologic change. The complicated cataracts seen are probably a secondary change. Filamentous vitreous opacities are noted in most cases. Subluxated lenses have been noted in two cases (Collier, 1962; Akiua, 1977).

Although the retinal degeneration is the most significant effect of the disease, systemic findings have been subsequently described. Tubular aggregates have been noted on ultrastructural examination of skeletal muscle (McCulloch and Marliss, 1975). Sipilä and co-workers (1979) reported that atrophy and tubular aggregates specifically affect type 2 muscle fibers. Abnormal liver mitochondria have been noted (McCulloch, 1978). Seizures and abnormal electroencephalograms in 2 patients, one of whom had mental retardation, have been noted (Takki, 1974b). These seizures may have been coincidental or the expression of another recessive disease in consangiuneous families since other gyrate atrophy patients have not had seizures.

METABOLIC FINDINGS

Simell and Takki (1973) made a major advance with the discovery of increased urine and plasma ornithine. The hyperornithinemia was confirmed (McCulloch and Marliss, 1975; Berson, Schmidt, and Rabin, 1976). This placed gyrate atrophy of the retina and choroid in the disease category of inborn error of metabolism. Investigation of the primary enzyme deficiency in gyrate atrophy progressed with the speculation that ornithine aminotransferase was deficient (Takki, 1974; Berson, schmidt, and Rabin, 1976). Ornithine aminotransferase is also known as ornithine ketoacid transaminase and L-ornithine:2-oxoacid aminotransferase (EC 2.6.1.13). An in vivo metabolic infusion study suggested ornithine aminotransferase deficiency in gyrate atrophy (Arshinoff and co-workers, 1977).

Ornithine aminotransferase is a mitochondrial matrix enzyme which catalyzes the interaction of L-ornithine and α-ketoglutarate to produce glutamic-γ-semialdehyde and glutamate (Strecker, 1965; Peraino, Bunville, and Tahmisian, 1969). Spontaneous cyclization converts glutamic-γ-semialdehyde to Δ^1-pyrroline-5-carboxylate, a proline precursor.

Several investigators have demonstrated ornithine aminotransferase deficiency in cultured cells from gyrate atrophy of the retina patients. Trijbels and co-workers (1977) used a radiochemical assay to demonstrate total ornithine aminotransferase deficiency in cultured skin fibroblasts from a gyrate atrophy patient. They noted ornithine aminotransferase inhibition by the radioactive substrate used in their assay. Kennaway, Weleber, and Buist (1977) used a colorimetric assay to demonstrate reduced ornithine aminotransferase activity in cultured skin fibroblasts from gyrate atrophy patients and intermediate ornithine aminotransferase in fibroblasts from obligate heterozygotes for gyrate atrophy. The low sensitivity of the colorimetric assay requires large amounts of cultured cells to provide sufficient material for ornithine aminotransferase measurement. Berson, Schmidt, and Shih (1978) also used a colorimetric assay to demonstrate absence of ornithine aminotransferase activity in cultured fibroblasts from gyrate atrophy patients and intermediate activity in obligate heterozygotes. Valle, Kaiser-Kupfer, and Del Valle (1977) demonstrated deficient ornithine aminotransferase activity in stimulated lymphocyte cultures from a gyrate atrophy patient using a radioisotopic assay.

The author has demonstrated deficient ornithine aminotransferase activity in cultured fibroblasts from a gyrate atrophy patient and intermediate activity in a heterozygote using a radioisotopic assay (O'Donnell, Sandman, Martin, 1977). Mixing homogenate from normal cells with homogenate from cells of a gyrate atrophy

patient produced the expected diluted activity of ornithine aminotransferase from the normal cells indicating that there was not an inhibitor in the diseased cells. In addition, a block in the conversion of ^{14}C-ornithine to ^{14}C-proline in cultured fibroblasts from a gyrate atrophy patient has been demonstrated (O'Donnell, Sandman, Martin, 1978a). The block was not present in an obligate heterozygote. This is the expected metabolic block with ornithine aminotransferase deficiency. A subsequent improved assay using high pressure liquid chromatography to separate and quantitate the reaction product has facilitated the diagnosis of ornithine aminotransferase deficiency in the small amounts of material in cultured cells (O'Donnell, Sandman, Martin, 1978b). The pathophysiology of gyrate atrophy of the retina and choroid is not understood. Although clinical evidence suggests that the retinal pigment epithelium is primarily affected, no pathology has been reported. The biochemical block at ornithine aminotransferase could cause disease by toxic accumulation of precursor ornithine or by deficit of some product of the enzyme reaction such as proline.

MANAGEMENT AND TREATMENT

Ornithine aminotransferase uses pyridoxine (vitamin B_6) as a cofactor. Some inborn errors of metabolism such as homocystinuria caused by deficient activity of a pyridoxine requiring enzyme have been successfully treated with massive doses of pyridoxine (Seashore, Durant, Rosenberg, 1972; Mudd and co-workers, 1970). The rationale for this increase in enzyme activity with massive doses of cofactor is that the mutation in the enzyme causes a decreased affinity for the cofactor which can be overcome by increasing the cofactor concentration. Pyridoxine lowered the plasma ornithine in one case of ornithine aminotransferase deficiency (Berson, Schmidt, Shih, 1978; Shih and co-workers, 1978). Other cases did not respond to pyridoxine. This may represent different mutations in the allele coding for ornithine aminotransferase. It is not yet known whether pyridoxine will stabilize the eye disease in addition to lowering the ornithine, but therapeutic trials are indicated.

Another experimental treatment is lysine loading. Dibasic amino acids share a common transport system in the kidney tubule. The hypolysinemia seen with hyperornithinemia in this disease has been explained as resulting from urinary lysine loss because the high ornithine concentration competes for reabsorption. The rationale for lysine loading is that high lysine concentration will compete for ornithine reabsorption leading to increased urine loss of ornithine. One group has reported decreased plasma ornithine with this treatment (Giordano and co-workers, 1978), but another group has not found lowered ornithine (Yatziv, Statter, Merin, 1979).

Since heterozygous carriers have one-half of normal enzyme activity, biochemical detection of carriers should be possible. Since ornithine aminotransferase has been measured in cultured amniotic fluid cells with a colorimetric method (Shih and Schulman, 1970) and with the high pressure liquid chromatography method (O'Donnell, Sipilä, Vannas, 1979), prenatal diagnosis by amniocentesis should be possible in matings where both parents are carriers. Information about carrier testing, prenatal diagnosis, and metabolic treatment of some cases can be conveyed to families in genetic counseling sessions to enable informed choice of the available alternatives.

Gyrate atrophy of the retina and choroid is a specific disease in the broad category of retinitis pigmentosa. The discovery of the inborn error of metabolism of ornithine aminotransferase activity has led to a better understanding of the disease and to better management and treatment. Other autosomal recessive types of hereditary retinal degeneration may be caused by as yet undetermined inborn errors of metabolism.

ACKNOWLEDGEMENT

Supported by a Basil O'Connor Award from the National Foundation-March of Dimes and by National Eye Institute Grant EY-02706.

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Neurochemistry Vol. 1, pp. 465-475.
Pergamon Press Ltd. 1980, Printed in Great Britain.

EFFECTS OF EXOGENOUS GANGLIOSIDES ON THE PATTERN OF RESPIRATORY ENZYMES IN EXPERIMENTAL DIABETIC RETINOPATHY IN THE RAT

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ABSTRACT

The prevalence of LDH1 in the retina let us think that this tissue, in normal conditions, is particularly resistant to anoxia; yet the datum relative to the variations, of LDH1 pattern in the degenerating retina with decrease of LDH1, makes more probable the hypothesis of a variable sensitivity of the enzyme in the normal metabolic conditions. This consideration, which can also be extended to the other enzymatic systems, justifies our research line.

a) In the retina of hyperglycemic rat by Alloxan, LDH1 presents a progressive decrease of its activity up to reach values overlapping those observed in the degenerating retina; in rats pretreated with a gangliosides mixture this change does not occur;
b) also the degenerating-like increase of iso-enzyme 4 is undone by pretreatment with gangliosides;
c) The ATPase activities of hyperglycemic rat retina are also depressed in pretreated rats with gangliosides;
While the relation between ATPase (Na^+ - K^+) dependent activities and Mg^{++} dependent activity remains depressed until 10th day of hyperglycemia, on the contrary, in the rats protected with gangliosides this relation becomes normal in 4th day;
d) The variations of MDH activity in the rat retina in experimental hyperglycemia do not appear statistically significant.

KEYWORDS

Diabetic retinopathy, glucose ratio, LDH, MDH, ATPase, gangliosides.

INTRODUCTION

Though Warburg established as early 1927 that retina has a greater rate of anaerobic metabolism than any other tissue, a number of reports on the glycolysis in the retina under different experimental conditions became available only since 1959 (Graymore, 1959; Walters, 1959; Bonavita and co-workers, 1963, 1967; Graymore, 1970; Ponte and co-workers, 1974; Piccoli and co-workers, 1978).

A topic has been represented by investigations on normal and dystrophic retina, i.e. inherited retinal degeneration in the rat, during development. Morphological studies of Lucas and co-workers (1955) established that in the affected retina the maturation proceeds normally up to the fourteenth day or so after birth, and that only at this stage, when the rods are developing, at their maximal rate, the distal segments appear to break down and form amorphous masses of eosinophilic debris. The first occurrence of metabolic changes is not so well defined. Only Walters (1959) and Brotherton (1962) have found that the rate of anaerobic glycolysis is lower in affected than in control retinae even as young as 12 days of age, before the full differentiation of visual cells.

During the past fifteen years it became easier to understand this aspect of the metabolism of the retina, after a number of investigations on enzymes involved in glucose metabolism (Bonavita and co-workers, 1963, 1965, 1967; Keen and Chiouverakis, 1965; Graymore, 1970; Piccoli and co-workers, 1978).

We would make an attempt to summarize briefly the available data. The first point to emerge is that both anaerobic glycolysis and respiration are approximately doubled during maturation. It is generally agreed that this is connected with the differentiation of visual cells. Lactate dehydrogenase activity, in normal retina, increases during post-natal maturation, while the enzyme of degenerating retina decrease progressively after the third week of age. The enzyme from normal retina does not exhibit any significant post-natal change in isoenzymatic composition; by contrast the enzyme of affected retina exhibits a striking change of its multiple composition in animals even younger than 10 days. Hexokinase specific activity in the normal and degenerating retina increases after birth, however in the distrophic tissue the early increase is followed by a slow decline which occurs after the third week of life. The same pattern was described in the case of phosphofructokinase and ATPase, but for this enzyme the $Na^+ - K^+$ activated form shows a less evident increment in the dystrophic tissue (Bonavita and co-workers, 1963, 1967). These observations can induce to suggest the existence of an alteration of oxygen-linked metabolism in degenerating retina, that can be considered a referring point for other studies on metabolic alteration of the retina.

In the present investigation some enzymatic activities have been studied in the retina of rats submitted to alloxan hyperglycaemia. Alloxan is known to be a sulphydril poison, characterized by its selective action on the beta-cells of the Islets of Langerhans. By virtue of this property it has become a common tool in investigation where a diabetic condition must be simulated. The enzymes studied were: ATPase, malate dehydrogenase and lactate dehydrogenase.

Furthermore recent studies on the functional significance of sialo-glyco-macromolecules in nervous tissue, suggested a study on the effect of exogenous gangliosides on the experimental diabetic retinopathy. It is well known, for instance, how gangliosides are localized at cell surfaces, particularly at the synaptic terminals, both in the nervous tissue (Morgan and co-workers, 1976) and in the retina (Dreyfus and co-workers, 1976); how these macromolecules are associated with receptor functions (Brunngraber, 1969); how they are involved in metabolic responses to environmental stimuli (Rahamann, 1976) and in de- and regeneration (Ceccarelli and co-workers, 1976). More recently it has been shown, for gangliosides, a clear definite pharmacological effect on peripheral neuropathies (Dantona and co-workers, 1978; Lonati Grillo, 1978).

EXPERIMENTAL METHODS

Adult male albino rats of the Wistar strain were utilized for all the experiments. Chronic hyperglycaemia was obtained by one i.p. injection of 150 mg/kg of alloxan and a blood level of glucose of 300mg/100 ml was reached within 12 h. For treat-

ment with gangliosides, two different experimental sets were utilized: i) 50 mg/kg pro die of a mixture of brain cortex gangliosides containing 32% GM_1, 38% GD_{1a}, 17% GD_{1b}, 13% GT_1 were injected i.p. for ten days before administration of alloxan, i.e. pretreatment; ii) 50 mg/kg pro die of the same mixture were injected i.p. through the entire experimental time, started at the same day alloxan was administered. Gangliosides did not affect blood glucose level in the controls as well as in the alloxan-treated rats.

Extraction of Enzymes

Soon after killing by decapitation, the cornea, iris and lens were gently removed, the eyeball was immersed in 0.25 M sucrose at 4°C, and the retina was separated from pigment epithelium and choroid. Soon after the removal, 8-12 retinae were homogenized in 1.5 ml of glass distilled water. The homogenate was centrifuged at 22000 g for 20 minutes in a Lourdes refrigerated centrifuge. Experimental values reported below represent the average of at least three determinations on various pools of retinae.

Assay Procedures

ATPase activity was measured in the presence of MG^{++} or Mg^{++} plus Na^{+}-K^{+}, under the experimental conditions suggested by Skou (1957). The enzyme specific activity is expressed as micromoles of P/min/mg of protein at 37°C. Starch grain electrophoresis of malate dehydrogenase (MDH) was carried out at pH 6.25 as described by Grimm and Doherty (1961), obtaining two MDH fractions. Rates of malate dehydrogenation were determined under the same experimental conditions described by Bonavita and co-workers (1965). One MDH unit was defined as the amount of enzyme giving a decrease of optical density at 340 mμ equal to 0.001/min. Starch gel electrophoresis of lactate dehydrogenase (LDH) was carried out, at pH 8.6, as suggested by Plagemann and co-workers (1960), obtaining four LDH fractions. LDH activity was determined under the same experimental conditions as described by Bonavita and co-workers (1963). One LDH unit was defined as the amount of the enzyme which gives an optical density decrease at 340 mμ of 0.001/min.

Nitrogen Determination

The protein content of retina extracts was determined by a submicrospectrophotometric method (Ballentine, 1957).

Chemicals

L-malic acid, nicotinamide adenine dinucleoside (NAD), NADH, Tris-ATP, diphosphopyridine nucleotide and its reduced form, were products from Sigma Chemical Co. (St. Louis, Mo.), L+lactic acid and sodium pyruvate were obtained from Fluka (Basel, Switzerland), Starch-hydrolyzed from Connaught Medical Research Laboratories (Toronto, Canada) was used for electrophoresis. Gangliosides mixture was a gift from Fidia (Abano Terme, Italy). All other compounds were reagent grade.

RESULTS

ATPase and MDH Activity in the Retina of Hyperglycemic Rats

Both Na^{+}-K^{+} activated ATPase and Mg^{++} activated ATPase present a sharp decrease of activity in the retina of rats submitted to alloxan hyperglycaemia. From Fig. 1 it appears that 48 h after administration of alloxan Na^{+}-K^{+}ATPase specific activity shows a decrease as high as 30%. Such a decrease is still measurable at the 20th day.

EXPERIMENTAL

ANIMALS: male albino rats (Wistar strain) weighing 180-200 g

HYPERGLYCEMIA INDUCTION: Alloxan i.p. (150 mg/Kg, once)

BLOOD GLUCOSE: 300 mg%ml 12 h after Alloxan injection (normal level: 150 mg/100 ml)

TREATMENT: Gangliosides (50 mg/Kg/die) i.p. during the experimental period

PRETREATMENT: Gangliosides (50 mg/Kg/die) i.p. for 10 days before the alloxan injection

ENZYMES ASSAYED: ATPase; MDH (and related subfractions); LDH (and related subfractions)

Fig. 1. Na^{+}-K^{+} ATPase specific activity. c= control; a= alloxan treatment; g.p.= ganglioside pretreatment.

In the case of Mg^{++} ATPase the decrease seems to be of the same extent but somehow delayed (4th day) Fig. 2. When rats are pretreated with gangliosides the activities of both the forms of ATPase reach again their normal values between the 10th and 20th day of hyperglycaemia, while in the untreated animals retina ATPase appears still subnormal Fig. 1, 2. A parallel change is shown in the case of MDH specific activity Fig. 3. At the 20th day after the injection of alloxan the enzyme activity is still lower than control while the pretreatments with gangliosides seem to be able to induce a full recovery of MDH at the 4th day of hyperglycaemia. When MDH isoenzymes are investigated a more complex picture must be considered Fig. 4.

Hyperglycaemia, in fact, seems to induce a precocious and long lasting increase of the so called supernatant (S) fraction, and a parallel decrease of the so called mitochondrial (M) subfraction, so that a severe change in ratio between the two enzyme fractions can be measured. Pretreatment with gangliosides reduces both the increase of S-fraction and the decrease of M-fraction.

MDH Isoenzymes Activity in the Retina of Hyperglycemic Rats

When LDH from adult normal rat is submitted to the electrophoretic migration on starch gel at pH 8.6, four enzyme forms are separated. If the isoenzymatic pattern is compared to that of the brain or peripheral nerve, the lack of a fifth, most negatively charged, component seems to be the major difference. When LDH of retina from hyperglycemic rats is submitted to electrophoresis, four isoenzymes are separated too. The percentage distribution of the isoenzymatic composition, by con-

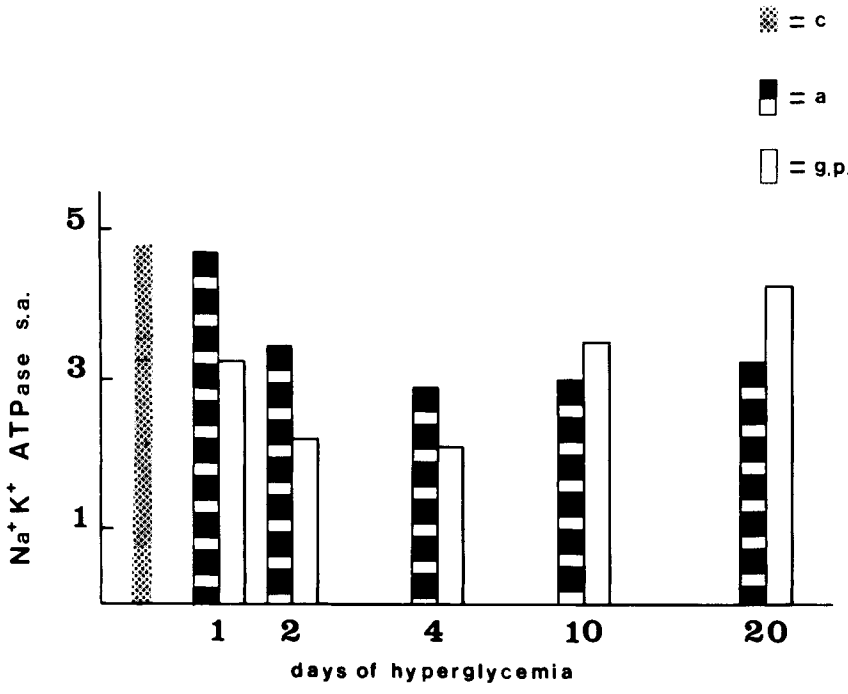

Fig. 2. Mg^{++} ATPase specific activity (for details, see Fig. 1)

trast, appears to be significantly altered if compared to controls. Fig. 5 and Fig. 6 illustrate the gradual evolution of the observed changes of two isoenzymes (i.e. LDH_1 and LDH_4 and it is worth noting: i) the first appearance of a shift in the isoenzyme per cent activity is found earlier than 4 days after the injection of alloxan; ii) the shift in isoenzyme per cent activity is clearly less extended when gangliosides are administered to the rats, either as pretreatment or as a treatment; iii) the level of LDH_1 as well as that of LDH_4 in the affected animals shows a shift parallel to that observed in the dystrophic retina.

DISCUSSION

The data concerning enzymes activity of the retina from hyperglycemic rats, do not deserve a long comment. Na^+-K^+ ATPase activity has been considered as the enzyme system participating in the maintenance of ionic equilibrium at membranes (Skou, 1957; McIlwain, 1966). The cleavage of the terminal -P bound of ATP releases the energy required for K^+ to enter the Na^{++} to leave the cellular membrane after depolarization. Bonting and co-workers (1964) have applied this general theory to the visual cells. If it is true that light generates a current which flows from the photoreceptors cell body to the outer segment, it follows that a movement of ions occurs through the receptor membrane upon the light excitation, just as it occurs in the nerve. The ATPase system would then operate in the repolarization to pump out Na^+ and move K^+ back. The diabetic retina lacking a normal electroretinogram, has appeared a system which might reveal significant changes of the ATPase system.

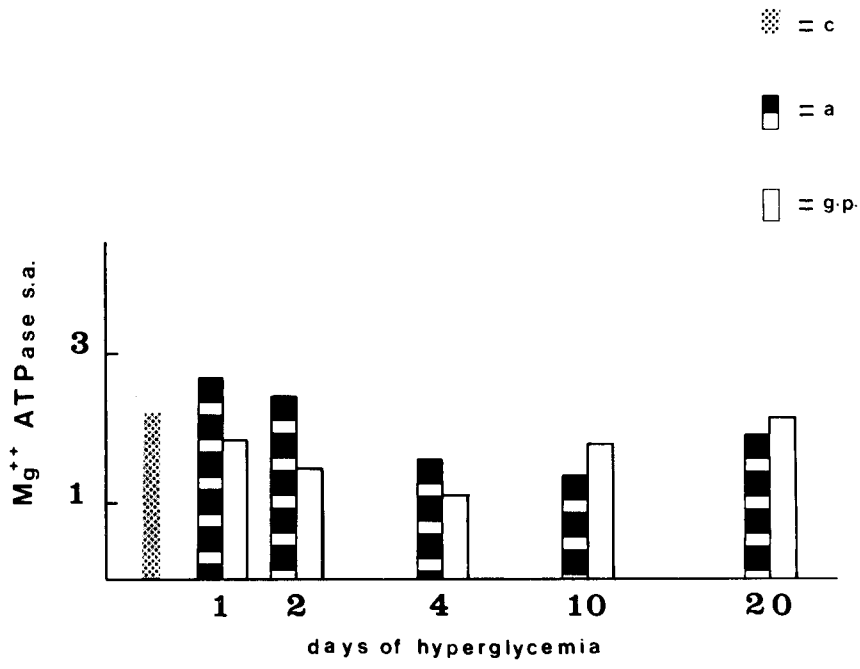

Fig. 3. Malate dehydrogenase specific activity (for details, see Fig. 1).

Experimental findings are in keeping with the hypothesis. One might argue whether the decrease of enzyme activity and the ERG alteration are supposed to appear at the same moment or not. Clearly, an alteration of the ERG requires a longer period of hyperglycaemia to be detected than the enzyme change. If ATPase is an allosteric enzyme, as suggested by Squires (1965), a simple conformational alteration of the catalytic protein at the allosteric site for Na^+ and K^+ ions would be responsible for the observed early changes in ATPase activity. A long lasting subnormality of the enzyme could be responsible for the ERG alteration.

The physiological significance of the described changes of MDH and related subfractions does not appear at first sight. With reference to functional relationship between the two MDH forms, the presence of the "mitochondrial" and the "supernatant" enzyme in both cellular compartments (mitochondria and cytoplasm) must be emphasized . In fact, it seems to be against a high efficiency of the hypothetical cycle involving malate and oxalacetate as shuttles between mitochondria and cytoplasm (Kaplan, 1961). This cycle assumes, in fact, that each molecular species of MDH is present in one cellular compartment and that H-form catalyzes preferentially the dehydrogenation of malate, while the soluble enzyme is geared for the reduction of oxalacetate (Kaplan, 1961). According to Kaplan (1961), soluble NADH would reduce oxalacetate to malate, which would penetrate into mitochondria and be reoxidized to oxalacetate; oxalacetate would, in turn, reenter the soluble compartment and be reduced by a second cytoplasm molecule of NADH, thus making mitochondrial NADH available to respiratory enzymes with a coupling of its oxidation to the formation of ATP (Bonavita and co-workers, 1967). The observed changes of M- and S- fractions of MDH in the retina of hyperglycemic rat seem to justify to raise a doubt on the cycle involving malate and oxalacetate as shuttles between two cellular compartments.

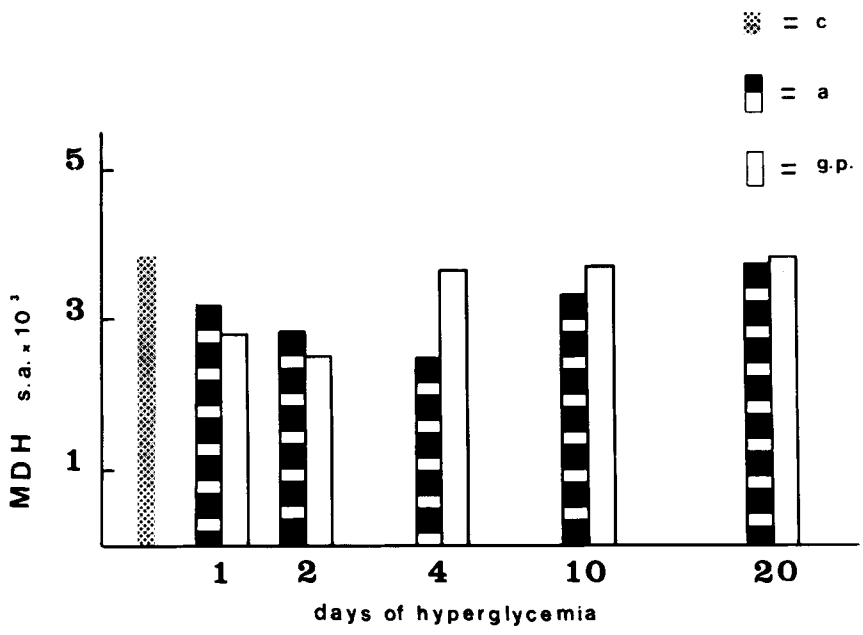

Fig. 4. Per cent activity of MDH subfractions. m.c= mitochondrial fraction control; s.c.= supernatant fraction control; s.a= supernatant fraction: alloxan; s.g.= supernatant fraction: gangliosides; ma= mitochondrial fraction; alloxan; m.g. = mitochondrial fraction: gangliosides.

Observing the electrophoretic data of LDH, two main questions can be formulated: i) which is the primary condition responsible for the shift in the isoenzymatic composition of LDH? ii) is this alteration responsible for change in glycolysis as it can be observed in degenerating retina? (Bonavita and co-workers, 1963). In connection for the first question it can be supposed hyperglycaemia itself might rapidly slow down the synthesis of LDH_1 and this appears in keeping with the observed early decrease in the specific activity of the retina LDH which occurs in hyperglycemic rats (Piccoli and Guarnieri, 1979). In so far as the second question is concerned, the early occurrence of change in LDH composition is suggestive of a casual relationship between the changes and the described alteration in the rate of glycolysis (Graymore, 1979). The sequence of metabolic events may be visualized as follows: the most negatively charged isoenzyme undergoes a strong percentage increase in the affected retina. Since this isoenzyme is inhibited by excess pyruvate, it can be assumed that the usual concentration of pyruvate formed through glycolysis would inhibit this fraction geared for activity in the presence of low amounts of pyruvate. As a consequence, pyruvate would accumulate and slow down glycolysis. A first conclusion can be drawn that the retina from the hyperglycemic rat has a metabolic organization parallel to that observed in the inherited retinal degeneration of the rat.

Among our findings the most intriguing point may be considered the effect shown by gangliosides on retina enzymes activity. The physiological role of gangliosides is

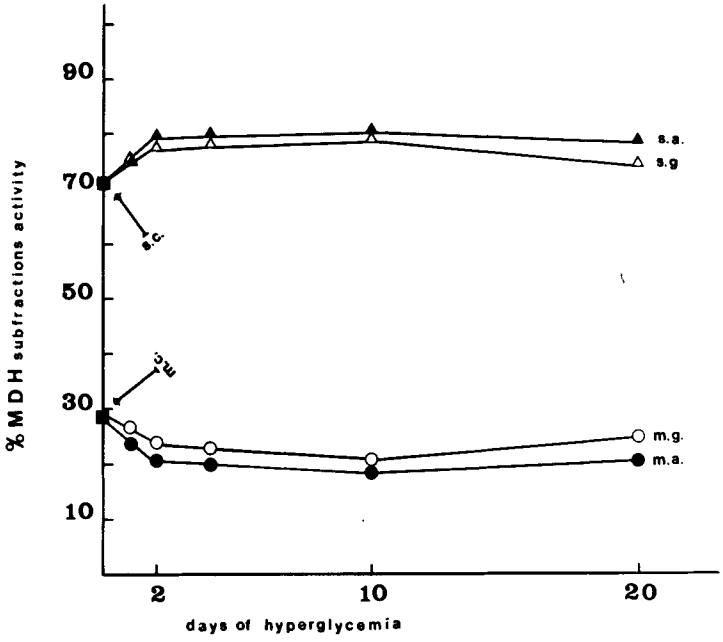

Fig. 5. LDH_1 per cent activity. N.R.= per cent activity in normal retina; N.R.= LDH_1 per cent activity in dystrophic retina; a = alloxan treatment; g = ganglioside treatment; g.p. = gangliosides pretreatment.

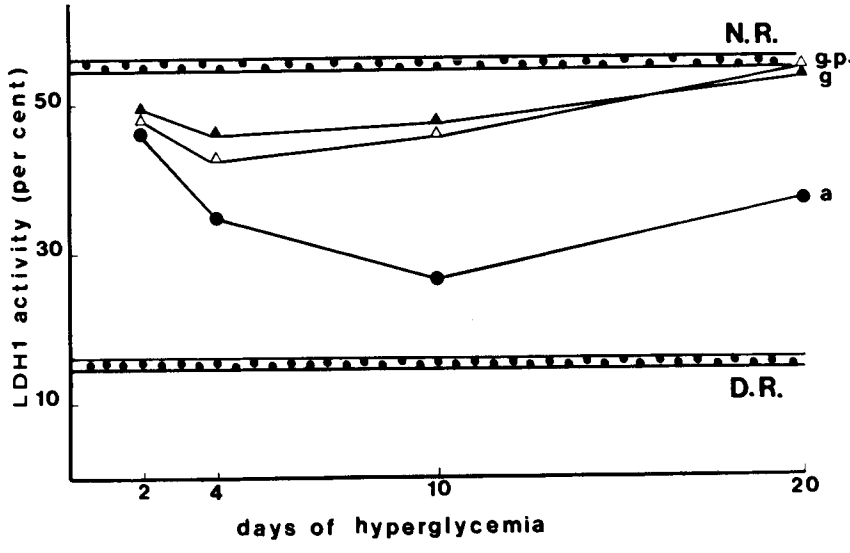

Fig. 6. LDH_4 per cent activity (for details, see Fig. 5).

still under investigation, although recent studies have established their role in many cell membrane functions. Gangliosides interact with cell cultures increasing cholera toxin binding sites (Cuatrecasas, 1973); with synaptosomes inducing dopamine release (Cumar and co-workers, 1978) and increasing high affinity choline uptake (Schulze and Rommelspacher, 1978); activate deoxycholate solubilized ATPase activity (Caputto and co-workers, 1977) as well as Na^+- K^+ ATPase from rat brain (Aporti, 1979). Furthermore it has been recently reported that exogenous GM_1 interact with neuronal membranes with a relatively high affinity (Toffano and co-workers, 1979).

As far as it concerns retinal gangliosides, in 1971 Hess and co-workers reported the presence of only two gangliosides in frog photoreceptors outer segments, and in 1972 Holm and co-workers identified the structure of the major gangliosides of human, bovine and rabbit retina. They found that the three GD_{1a}, GD_{1b} and GT_1 had a pattern of fatty acids very similar to that of cerebral gangliosides (Holm and Hansson, 1974). Recently, beta-glycosidase activity in streptozotocin-diabetic rat tissues have been investigated (Fushimi and Tarui, 1976) and the results show that the impairment in glyco-compounds metabolism play a major role in the pathogenesis of diabetic microangiopathy; moreover, Kawamura and co-workers (1976) showed that the level of sialic acid-bound macromolecules decreased in the membrane of liver and red cells in diabetic animals.

The functional role of these compounds, and mainly their localization at cell surfaces, particularly at synaptic terminals (Morgan and co-workers, 1976) as well as their involvement in metabolic responses to environmental stimuli (Dreyfus and co-workers, 1973; Rahamann and co-workers, 1976; Aporti, 1979) should be emphasized in an attempt to interpretate the described action of gangliosides on ATPase, MDH and LDH of the retina from hyperglycemic rat. It is not possible, at the present time, to discriminate between a structural and/or a metabolic role of gangliosides in our experimental conditions, nevertheless, our results, concerning with the earliest moments of the metabolic disorders induced by alloxan, seem to suggest a preminent metabolic action of exogenous gangliosides, at least when the early stages of diabetic retinopathy are considered.

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Neurochemistry Vol. 1, pp. 477-485.
Pergamon Press Ltd. 1980. Printed in Great Britain.

BIOCHEMISTRY OF RETINAL DEGENERATION IN RATS AND MICE: A SHORT REVIEW

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ABSTRACT

A review of the biochemistry of retinal degeneration in mice and rats is presented, recording research over the past 20 years. The finding particularly relevant to the aetiology of the condition is an anomaly in cyclic GMP metabolism in the photoreceptors of affected retinae. In mice, the consequence of this is an increase in cyclic GMP content in photoreceptor cells, whilst in the rat, a decrease occurs. In both species, there is failure in proper development of the photoreceptor cells. It is suggested that the inherited dysfunction in cyclic nucleotide metabolism is connected with lysosomal enzyme release also seen in this condition, and that the sequence produces cellular degeneration.

KEYWORDS

Retinal degeneration, biochemistry, rats, mice, cyclic nucleotides, lysosomal enzymes.

INTRODUCTION

Inherited, pigmentary retinal degeneration occurs in a number of animal species, as well as in man. Direct experimental studies on human material are difficult for obvious reasons, so that researchers over the past 20-30 years have concentrated on animal models of this condition. Inbred strains of rats and mice have provided the majority of such models and in these species, the condition is inherited as an autosomal recessive characteristic.

Condition In The Rat

Most of the experimental studies have been carried out on the pink-eyed 'RCS' or 'Campbell' rat strain, descended from the rats originally described by Bourne, Campbell and Tansley (1938). The lesion in this rat is characterised by normal retinal development until about 12 days after birth, when the outer segments of the photoreceptors show ultrastructural changes. Later an accumulation of a large amount of disorganised extracellular lamellar material, containing rhodopsin, occupies the space between the outer retina and the pigment epithelium (PE). (Bok

and Hall, 1971; Dowling and Sidman, 1962). This material consists of debris from shed, effete rod outer segments (ROS) which have failed to be phagocytosed by the PE in the normal manner. Heron, Reigel and Rubin (1971) and LaVail, Sidman and O'Neil (1972), showed conclusively that in the affected rat, the rates of shedding and digestion of the ROS were unequal, due to the loss by the PE of the ability to phagocytose. In the affected rat, the deposition of rhodopsin-containing debris occurs before morphological changes have begun, i.e., as early as 15-25 days after birth. After accumulation of this debris, degenerating nuclei become visible and complete degeneration of the rod cell layer occurs. Important observations made by Dowling and Sidman (1962), LaVail and Batelle, (1975), and Dewar, Barron and Yates, (1975), proved that degeneration of the photoreceptors is accelerated by the action of light on the retina, whilst light deprivation slows it down, so establishing a connection between the action of light on the retina and photoreceptor degeneration.

Biochemical Changes

The affected RCS rat shows anomalies in rate of protein metabolism in both retina and PE, in addition to the failure in phagocytosis of the PE (LaVail, Sidman and O'Neil, 1972; Reading and Sorsby, 1964). It would appear that a consequence of the accumulation of rhodopsin-containing debris is the presence of an excessive concentration of retinol in the PE, occurring before the onset of cellular degeneration (Reading, 1966). Concerning the nature of the accumulated rhodopsin-containing debris in the rat, Chaitin and Williams (1977) found no difference in rates of bleaching of rhodopsin extracted from RCS and normal rats, leading them to question the idea that affected retina may contain a 'labile' type of visual pigment (Reading, 1970). However Perlman (1978) recently compared *in vivo* the photosensitivity of regenerative rhodopsin from RCS and normal rats. The affected (RCS) retina showed a higher and significant photosensitivity of visual pigment than unaffected retina, resulting in more retinol release with the same illumination. This confirms the earlier measurement of increased retinol levels in the RCS rat eye reported by Reading (1966). Perlman (1978) suggests that the rods in affected retina differ fundamentally from normal rods accounting for the increased photosensitivity.

Lysosomal Activation

The localized accumulation of retinol in the PE suggested a breakdown of lysosomal membranes with the consequent release of the constituent acid proteases and hydrolases which could account for the cellular breakdown and disappearance of the debris (Reading, 1970). It was already known that retinol breaks down lysosomes in cartilage and liver and *in vitro* and *in vivo* experiments with rat and bovine retina and PE (Dewar, Barron and Reading, 1975; Vento and Cacciopo, 1973) confirmed the labilizing action of retinol in eye tissues by increasing release of lysosomal enzymes. The PE is well endowed with lysosomes on account of its phagocytic function and Burden and her colleagues (1971) confirmed the instability of lysosomes in affected rat PE as early as 1-2 weeks after birth, becoming more unstable with increase in age. Affected eyes had a higher free and total acid protease activity than normal.

In consequence of the observed accumulation of retinol and lysosomal fragility in the affected rat retina and PE, attempts were made to counteract the labilizing action of retinol on the retina by the use of anti-inflammatory agents. Encouraging results were obtained *in vitro* and *in vivo* with normal retina (Dewar, Barron and Reading, 1975). This led to studies with the inbred RCS affected rat. Three drugs were tested, acetylsalicyclic acid, cortisone and 4-acetamidophenyl-2-

acetoxybenzoate (Benorylate). The drugs were administered daily by i.p., injection from 10 days to 8 weeks of age (Dewar, Barron and Reading, 1977). A small improvement in delaying the progress of the lesion was obtained as judged by a 10% reduction in rate of loss of retinal DNA, compared with untreated, affected animals, using acetylsalicylate with slightly better results using Benorylate. Cortisol acetate had no effect. Further attempts along these lines have not been made.

Condition In The Mouse

Degeneration in the affected mouse retina has been described in detail at the light microscopic (Sorsby and others, 1954) and electron microscopic levels (Blanks, Adinolfi and Lolley, 1974; Caley, Johnson and Liebelt, 1972). In the normal mouse retina, the ROS proceed from a primitive cilium to the formation of membranous material which is re-orientated into sacs, finally building up into a definite regularly layered structure. In the affected mouse, changes occur at the re-orientation stage and the regularly layered structure never appears (Lasansky and De Robertis, 1960). Ultrastructural changes can be discerned prior to 10 days of age and by the 11th post-natal day, rods of affected mice are shorter than normal, whilst by the 21st day, they have almost completely degenerated.

Biochemical Changes Common To Mouse and Rat

A specific enzyme deficiency associated with inherited retinal degeneration was first observed in the affected C3H mouse by Schmidt and Lolley (1973), who found an abnormality in metabolism of guanosine 3'5'-monophosphate (cyclic GMP) occuring before visual cell degeneration. This was associated with a deficiency in cyclic nucleotide phosphodiesterase (PDE) activity at an early stage of the lesion. A similar defect has been found in the RCS and Hunter rate, both homozygous for the blindness allele (Dewar, Barron and Richmond, 1975) and in the Irish Setter dog (Aguirre and others, 1978). In the three species, kinetic analysis of the retinal PDE has shown that in the normal retina, immediately after birth, a low K_m (high affinity) PDE is present and after 6 days of age a second PDE of high K_m (low affinity) appears. There is morphological differentiation between the two PDE, the low K_m enzyme being confined to the inner retinal layers, and the high K_m enzyme to the outer photoreceptor layer. The net result of the 'missing' high K_m enzyme is a reduction in total PDE activity in the affected retina and owing to morphological differentiation of both enzyme and cyclic nucleotides, an increase in cyclic GMP in the photoreceptor cells. Aguirre and colleagues (1978) suggested that such a defect in cyclic GMP metabolism is characteristic of early onset retinal degenerative diseases in animals and possibly in humans.

The significance of the enzymatic anomaly appears to lie in the fact that the correct functioning of cyclic GMP metabolism is a prerequisite for proper differentiation and development of the photoreceptor cells. In addition, cyclic GMP plays a part in photoreceptor function. Bonting and Daemen, (1976) and Fletcher and Chader (1976) have shown that following the bleaching of rhodopsin by light, there is an associated activation of cyclic GMP phosphodiesterase (GMP-PDE) in the ROS, so that cyclic GMP breakdown is accelerated. It would appear that the levels of cyclic nucleotides, especially cyclic GMP may regulate sensitivity of photoreceptor membrane to varying photon fluxes, permitting function over a wide range of light intensities. Phosphorylation of the ROS disc membrane protein, associated with Ca^{2+} movement is also involved.

Differences Between Rat And Mouse Model

There are some important differences between the mouse and rat condition. Firstly, in the affected rat, the mutation is expressed primarily in the PE whereas in the mouse the primary lesion occurs in the photoreceptor cells (Mullen and LaVail, 1976). Progress of the lesion in the rat is associated with accumulation of masses of lipoprotein, multi-lamellar debris between the retina and PE which does not occurr in the mutant mouse strains studied. In the mouse, there is no evidence for a failure in phagocytic function in the PE as there is in the rat (Farber and Lolley, 1973; LaVail, 1973).

Another marked difference between the affected mouse and rat, lies in the effect of light on the progress of degeneration. In the affected mouse exposure of the retina to light does not affect the rate of progress of the lesion (Farber and Lolley, 1977) whilst exposure of the affected rat eye to light, speeds up photoreceptor degeneration (Dowling and Sidman, 1962; LaVail and Batelle, 1975). Finally, differences in cyclic GMP content occur. Dark or light-adapted rat (RCS) retina shows a reduced cyclic GMP content compared with controls, whilst cyclic GMP content of mutant mouse visual cells increases with age, in respect to controls. The reduction in cyclic GMP in RCS rat retina occurs before the onset of visual cell degeneration. Farber and Lolley (1977), suggested that this difference reflects differences in aetiology in the mouse and rat.

Other Species

Although the condition in the dog was not included in the brief to write this review, recent evidence on inherited retinal degeneration in the dog should be mentioned, since it involves an abnormality in cyclic GMP metabolism. Liu and colleagues (1979) have shown in the retinae of Irish Setters affected with inherited rod-cone dysplasia, a greatly increased cyclic GMP content. They reported that the basic defects are a decrease in a Ca^{2+}-dependent protein activator for PDE, concomitant with a failure to 'switch' PDE-type at a crucial stage in development of ROS (between 9 and 31 days of age). In normal retinae, following differentiation of the photoreceptors, the PDE 'switch' involves the enzyme changing from being dependent on the Ca^{2+}-activator-protein complex to non-dependence. The concentration of activator protein in the affected retina is inadequate to maintain PDE activity.

Consequences Of Cyclic GMP Abnormality

The most important common factor at the moment, in the animal condition and especially in rats and mice, seems to be abnormality in cyclic GMP metabolism, with intracellular accumulation of cyclic GMP due to the anomaly in cyclic GMP-PDE. To carry the argument further, it is clear that abnormal levels of cyclic nucleotides occur in all the animal species examined and this could be the key factor in photoreceptor degeneration. In support of this, Lolley and his colleagues (1977), induced photoreceptor degeneration in normal frog retina in culture, closely mimicking the inherited condition in animals, by the use of PDE inhibitors such as isobutylmethylxanthine, causing an accumulation of cyclic GMP in the visual cells. Differences between mouse and rat could be associated with differences in accumulation of ROS debris. Lolley and Farber (1975), claimed an activation of cyclic GMP-PDE by a factor present in the ROS debris in the rat.

Lysosomal breakdown in the rat retina is well established, (Burden and colleagues, 1971) but seems to have been overlooked in recent years yet could be a consequence

of changes in content or proportion of cyclic AMP and cyclic GMP. Evidence implicating the cyclic nucleotides in control of lysosomal membrane stability is accepted. Ignarro, (1975) concluded that the stabilizing effect of catecholamines, prostaglandins, theophylline etc., on lysosomal membranes is mediated through the cyclic nucleotides. In general, the effects of cyclic GMP on cellular structures are opposite to those of cyclic AMP, (Goldberg, O'Dea and Haddox, 1973). β-adrenergic stimulation elevates cyclic AMP levels and reduces lysosomal breakdown, (Ignarro and George, 1974), whilst increased intracellular cyclic GMP occurs during discharge of lysosomal enzymes from human neutrophils during muscarinic receptor stimulation (Ignarro, 1975).

Extracellular Ca^{2+} was required in Ignarro's (1975) experiments both for the release of lysosomal enzymes and increase in cyclic GMP in neutrophils. This could explain the exacerbating effect of light on degeneration in the rat retina, since Ca^{2+} are released from binding sites in the photoreceptor disc membrane during the visual process (Bonting and Daemen, 1976).

Other Experimental Evidence

In a study of the morphology of the retinal PE in the vitamin-A deficient rat, Yang, Hollenberg and Wyse (1978) found ultrastructural degenerative changes in PE and ROS and, significantly, an accumulation of concentric aggregates between ROS and PE which they concluded were the result of abnormal formation of outer segment membranes. They have a close resemblance to the disorganised lamellar material seen in the RCS rat. It is difficult to interpret these findings with respect to the inherited condition since the vitamin-A deficient PE still retained its phagocytic activity intact. There is no evidence of vitamin-A deficiency in RCS rats or mutant mice since these animals grow and produce viable litters normally. However, it is important that cycling light conditions are necessary to produce photoreceptor degeneration in the vitamin-A deficient rats; if animals are left in the dark no breakdown occurs. In this feature the vitamin-deficient rats resemble RCS rats. The evidence points to an anomaly in the recycling of vitamin-A in reduction and re-oxidation to form the rhodopsin chromophore in both RCS and vitamin-A deficient animals. Possibly this explains the excessive accumulation of retinol in the PE (Reading, 1966) and that the primary feature of degeneration is the accumulation of retinol.

Conclusion

There is a case to reconsider the importance of the involvement of lysosomal breakdown in retinal degeneration in the rat and mouse model as part of the sequence of events leading to the eventual loss of visual cells. Such considerations should involve attempts to halt or retard the progress of the lesion by the use of membrane stabilising agents or activators of PDE. Experimental evidence gained in this way may have a bearing on therapy in the human retinal degenerative conditions. However, interpretation of results with the animal models in terms of the human condition must be exercised with great caution, since the mode of inheritance in all the models is relatively simple, whereas in the human, inherited retinal degeneration is a multiple gene defect.

APPENDIX: CATALOGUE OF MOUSE AND RAT STRAINS USED FOR STUDIES ON INHERITED RETINAL DEGENERATION

In the mouse and rat, retinal degeneration has been studied on specifically bred strains in which the lesion is inherited in one way, viz., as an autosomal reces-

sive characteristic.

The Mouse Model

C3H and ICR Swiss Mice. The C3H strain carries the mutation for retinal degeneration and all sub-lines tested are affected (Noell, 1958). The mutant gene is also carried in the prolific ICR/Ha Swiss Mice, and this strain is deliberately non-inbred, 13% being homozygous for the mutant gene, the others either heterozygous or homozygous for the wild-type (dominant) allele, so that phenotypically, the major proportion do not show retinal degeneration. No delayed or partial manifestation of the disease has been detected in breeding experiments. The C3H mouse has two simple gene mutations, showing similar timings in expression, one for retinal degeneration and the other for lack of β-glucuronidase. The two have a close linkage on the same chromosome, the loci being about five cross-over units apart and are in linkage Group III. They do not control the same physiological process.

"Rodless" Mice This abnormality results in retinas completely or partially devoid of visual cells (Keeler, 1927). The condition is under the strong influence of modifying genes. Progeny of outcrosses to normal strains contained mice in which the visual cells were partially absent. Unfortunately, the "rodless" strain is extinct, since it obviously carried a mode of inheritance enabling partial manifestations of the disease.

C57BL/6J Mice LaVail and Sidman, (1974) developed this stock from an inbred mouse strain C57BL which already had a number of pigmentation mutants. They wished to generate mutant and control mice which were: (a) littermates and (b) distinguished by linked marker genes prior to expression of the blindness mutation. From several breeding schemes, they found that relevant genetic determinants for light ear (le) and blindness (rd) were only one locus apart. The light ear formed an excellent marker for retinal dystrophy and this strain provides advantages - there are affected and normal (sighted) mice in the same litter which can be segregated by ear colour.

Tetraparental Mice Wegmann and his colleagues (1971) produced chimaeric mice by combining two eight-cell stage embryos into one (tetraparental), from a strain homozygous for the rd gene (C3H/HeJ) and a strain carrying the wild-type allele at this locus C57BL/10SRJ. Retinas with 'patchy' areas of degeneration interspersed with normal were produced. In addition, chimaeras from albino mice, homozygous for the rd mutant (rd/rd) and normal, pigmented mice, homozygous for the wild allele RD/RD, showed a mosaic of pigmentation and retinal degeneration, with no correspondence between the two, adding support to the concept of the mutant gene in the mouse being manifest in the visual cells themselves.

The Rat Model

The most commonly used rat has been the Campbell or RCS strain (Bourne, Campbell and Tansley, 1938). These are piebald agouti-rats, with pink eyes (no pigmentation), in which the lesion appears about 21 days after birth.

Lucas, Attfield and Davey, (1955) described the pathological changes in the developing lesion. An outstanding characteristic of the condition in this rat is the accumulation of amorphous masses of eosinophilic debris between the outer retina, and the pigment epithelium (Dowling and Sidman, 1962).

Outcrosses from inbred strains (Congenic strains). Lucas, Attfield and Davey, (1955) out-crossed male RCS rats (pink eyed) to normal (sighted) black-eyed female PVG rats. They found no linkage of the gene for retinal dystrophy with that for pink eye and proved that inheritance was autosomal recessive. The heterozygous carriers of the retinal dystrophy mutant did not show any difference in development of the lesion.

For many years, the RCS rat was the only available rat model and the normal rat mainly used for comparison was the black-eyed PVG rat since many albino strains are susceptible to spontaneous retinal degeneration. Biochemical differences could always be ascribed to genetic characteristics associated with loci completely unrelated to the dystrophic gene. This prompted Yates and colleagues (1974) in Edinburgh to develop a strain of black-eyed dystrophic rats for experimental use. This strain developed healthily and did not show lens opacities as do a small proportion of the RCS rats. The onset of the retinal lesion is delayed by about one week.

In a similar programme, LaVail, Sidman and Gerhardt, (1975) later reported development of congenic strains of RCS rats with retinal dystrophy. All strains are homozygous for the blindness allele. Neither of these programmes produced strains with segregating litter mates of affected and sighted animals, since the paucity of genetic classification of characteristics in laboratory rat strains compared to the mouse does not allow selection of 'marker' genes from closely associated loci.

Chimaeric Rats Good evidence that the primary lesion in the rat lies in the pigment epithelium was provided by rat chimaeras produced by Mullen and LaVail (1976). This is in contrast to the mouse where it is confined to the retina. Embryos from pink-eyed RCS rats were fused by embryos from sighted, pigmented rats. In the chimaeras, beneath patches of mutant pigment epithelium (no melanin), abnormal photoreceptor cells were found, plus the extracellular debris. Visual cells in contact with pigmented pigment epithelium appeared normal.

Inbred Wag/Rij Rats Lai and colleagues (1975) recently described hereditary bilateral retinal degeneration affecting all adult rats in an inbred colony in the Netherlands of Wag/Rij rats. The disorder is characterised by early onset but slow progressive course.

This is important, since work on RCS or congenic strains show degeneration commencing earlier. Another marked difference from the RCS rat is that degeneration beings in the photoreceptor body and only secondarily affects rod outer segments. Accumulation of extra lamellar debris is not seen. The degeneration in the Wag/Rij strain would appear to be the better model for human retinitis pigmentosa.

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Neurochemistry Vol. 1, pp. 487-500.
Pergamon Press Ltd. 1980. Printed in Great Britain.

THE RETINA AS A BIOCHEMICAL MODEL OF CENTRAL NERVOUS SYSTEM REGENERATION

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ABSTRACT

The visual system of primitive vertebrates has long served as a useful model for the resynthesis of damaged neurons. The prior optic nerve crush imparts a marked tendency on the part of cultured retinal explants to extend neurites. Neurites grown in culture from retinal ganglion cells of the explant have been characterized by lectin-binding and immunohistochemical techniques. Biochemical studies on the retina following optic nerve crush reveal altered RNA and protein metabolism. Results indicate that tubulin mRNA is activated and that new tubulin synthesis is enhanced following the crush. An early and dramatic increase in the rate of nucleotide phosphokinase activity is also seen. The retinal tissue culture data, together with in vivo studies in normal and newly regrown nerve give additional insight into the nature of the regenerative process.

KEYWORDS

Fiber guidance; nerve growth; recognition; specificity; tissue culture; lectin binding; acetylcholine receptor; nucleotide metabolism.

INTRODUCTION

It is probably fair to say that much of our interest in the retina stems from the fact that its ganglion cells send visual information to the brain which can then be processed in a way that is of biological significance to the organism. Important gains have been made in understanding the molecular basis of sensory transduction in the retina, but we can at present say little regarding how its connection to the brain arises during development or how its reconnection following injury is mediated in those species that have this ability. In addition to the intellectual challenge of the problem, there is potentially great practical value in further understanding in what ways those species that cannot support CNS regeneration (including man) differ from those that can. For all of these reasons, the biochemical events that mediate the response of the visual system of lower vertebrates to injury and that lead to recovery of function warrant scrutiny. No attempt will be made here to review exhaustively the vast neurobiological literature that serves as background for this topic. Rather, current findings will be discussed in the

context of viable hypotheses. In vivo studies, primarily electrophysiological and anatomical in nature, will be compared and contrasted with in vitro studies, primarily at the molecular level. Relevant properties of the ganglion cell growth cone are presented and illustrated with current experimental findings from our laboratory.

DEVELOPMENT AND REGENERATION

There are both differences and similarities between de novo development of the visual pathway and regeneration of the adult optic nerve following injury. In both instances, ganglion cells must recognize the correct addresses to which presynaptic terminals must ultimately be assigned. How the ganglion cell fibers are guided to the tectum, however, may be quite different in the two conditions. Within the developing brain, cell replication, migration and axon extension take place concurrently. This is not the case in regeneration, where the pre-existing scaffolding may play an important role in the guidance of regrowing fibers. Significant neuroanatomical and electrophysiological contributions have come from many laboratories (e.g., Weiss, 1966; Sperry, 1966; Gaze, 1970; Jacobson, 1978). In initial experiments, the course of regeneration following axotomy in teleosts and amphibians was studied. Later, various surgical manipulations of both eye and brain were employed to ask more complex questions of the in vivo preparations. The past decade has been marked by extensions and refinements of the basic studies, largely occasioned by the availability of new histochemical tools, such as ^{3}H-labeled proline for anterograde tracing of axonally transported proteins by radioautography (Neale, Neale and Agranoff, 1972; Jones and Hautman, 1978) and of horseradish peroxidase (HRP) (LaVail, 1975; Bunt, Lund and Lund, 1974) primarily for retrograde tracing. It has been demonstrated by combinations of morphological (including histochemical), electrophysiological and behavioral studies, that in the goldfish, regeneration of fibers from an eye from which one half of the retina has been removed results in innervation of only that part of the tectum that would ordinarily have been innervated by the hemi-retina (Jacobson, 1978; Edds and coworkers, 1979). With the passage of time, the optic nerve fibers extend to cover the entire tectum with the partial visual field. Similarly, if an entire retina regenerates to a partially ablated tectum, there is an orderly compression with the net result that the retinal projection is represented on the remaining tectum. One may thus use experimental evidence to conclude that either the retina, the tectum, or the visual system as a single entity exhibit properties of respecification during regeneration. What can be said with some certainty is that the rules for formation and maintenance of connections are dynamic and complex and that there appears to be much inherent redundancy in the mechanisms of specification, since the regenerating subject can overcome a variety of imposed obstacles. While much has been learned from interventive experiments, as this approach becomes more remote from the physiological mechanism of specification, it becomes less likely to shed additional light on its basis.

A somewhat different approach toward understanding the ways in which retinotectal connection and reconnection occur is the consideration of an important hallmark of the growing axon - the growth cone. Both in vivo and in vitro, the growing axon extends itself in a rather smooth translational fashion, while at its extending tip, hair-like filopodia actively thrash about in a seemingly random fashion, sampling its microenvironment in a surround that encompasses several axon diameters. The growth cone may play roles in guidance of fibers to the target region as well as in the process of selective respecification, once initial contact has been made. What is the mechanism by which the growth cone accomplishes its purpose? Does it "taste" or does it "smell" its way to its target? By this we mean, does it sense macromolecular clues at short ranges (e.g., recognition sites on membranes), or does it sense a spatial gradient of a diffusible substance that is operative at great distances? For example, nerve growth factor can be shown to stimulate a vectorial

growth pattern in vitro (Campenot, 1977). Experimental in vivo evidence can be marshalled for either possibility. In favor of the short range argument is the observation that regenerating fibers are guided along tracts of degenerating ones, even if they are inappropriate. An example is found in the regenerating teleost visual system. Axons of the goldfish retina normally cross to reach the contralateral tectum. If one eye of the goldfish is removed, and the other is crushed intraorbitally, so that both tecta have been denervated, a majority of regrowing fibers from the remaining eye (traced by ^{3}H-proline radioautography) regrow to the contralateral optic tectum as expected. However, a significant number of fibers, upon reaching the chiasm, appear to sense degenerating fibers of the enucleated eye, and leave the chiasm along the ipsilateral optic tract to innervate the ipsilateral tectum (Sharma, 1973; Springer and co-workers, 1977). If an optic nerve is crushed and the opposite eye is not disturbed, the ipsilateral tract is not formed. It is reported that following unilateral optic nerve crush in the frog, some regenerating optic nerve fibers will enter the chiasm, cross and grow rostrally, to follow fibers of the opposite eye into the retina (Bohn and Stelzner, 1979). In this instance we must imagine that the regrowing fibers recognize optic nerve by local clues and enter the existing tract, albeit in a reverse and seemingly not useful direction. It has been shown in a number of ways that outgrowing fibers from ectopically placed eyes in developing amphibians will find their way to the tectum (Sharma, 1972; Constantine-Paton and Capranica, 1976). Evidence in this case is seemingly in support of the existence of long-range gradients, but even here there may be elements of short-range recognition: the ectopic optic nerves have been claimed to enter the Rohon-Beard tracts of the spinal cord (Katz and Lasek, 1978; Giorgi and VanderLoos, 1978). If confirmed, the result would suggest that the visual efferent fibers detect a sensory tract "conduit". A recent observation bears directly on this issue. Singer has shown that growing spinal cord fibers in developing Xenopus are preceded by channels in the ependyma (Singer, Nordlander and Egar, 1979). This finding together with those of other laboratories, lends credence to Singer's "blueprint" hypothesis which proposes that the guidance apparatus is the result of neither afferent fiber nor target influences but rather of interposed nonneuronal elements. It remains possible that the channels are the result of an inductive mechanism generated from the advancing growth cone.

The nature of the preexisting trace pathways required for Singer's hypothesis is presently unknown, but its existence may be related to recent studies in which Jacobson traced cell lineage in the developing Xenopus embryo. Injection of HRP into a single cell at the blastomere stage serves as a "fate stain", (Jacobson and Hirose, 1978) since daughter cells bear the same marker. An analogous technique is somatic crossing-over currently being used successfully in studies on Drosophila development (Kauffman, Shymko and Trabent, 1978). The results have altered classical concepts of neurogenesis and may in fact lead to changes in how we view the neuroanatomical organization of the brain. Examination of histological sections of the retina of a tadpole that had been injected with HRP at the two-cell stage reveals the presence of the marker in one half of the embryo's cells. On the labeled side, the brain and most of the eye are also marked. An exception is a small unlabeled ventromedial portion of the retina and diencephalon. Correspondingly, only these structures are labeled on the otherwise unlabeled side. The result indicates that during development, at least two neuroblasts have exchanged position across the midline and that their progeny have given rise to a segment of retinal cells via the optic stalk. Jacobson suggests that the translocation of these "founder" cells results in the establishment of an "incipient chiasma". We can infer from the results that these contralateral progeny send axons back to the brain via trace markers and thus guide neighboring ipsilateral retinal cell axons to the opposite tectum.

Growing nerve fibers tend to form bundles. In Daphnia (Lopesti, Macagno and Levinthal, 1973), the invertebrate ommatidium generates a lead fiber during development

that in turn guides the surrounding axons to the brain. This finding bears resemblance to recent studies in goldfish brain. As the retina continues to grow during adult life, new cells are added at the periphery (Johns, 1977). The new ganglion cell fibers grow toward the optic disc in a spoke-like fashion, unite and travel to the optic tectum in packets (Rusoff and Easter, 1979). Upon reaching the tectum, they then "disembark" and proceed to their various tectal addresses in accordance with the existing two-dimensional map, which may be roughly described as a superimposition of the retinal nasotemporal gradient upon the tectal rostrocaudal axis on the opposite side. The timed basis of optic nerve fascicle organization then reduces the importance of maintenance of neighboring retinal ganglion cell relationships by their axons within the optic nerve in arriving at the final retinotectal map. The result does not preclude the existence of a retinotectal map based on chemical gradients. Nearest neighbor relationships within a given retinal annulus that gives rise to an optic nerve fascicle are in fact preserved. The finding suggests however that the Cartesian x-y coordinate system so favored in speculations regarding retinotectal specification should now be replaced by a system of polar coordinates in which rho, the distance of a ganglion cell from the optic disc, is correlated with the cell's age, while omega expresses its sector.

Developments in cell biology provide insights that begin to point to the kinds of molecular codes and signals that might be required. In terms of their chemical nature, many biochemists favor involvement of glycoproteins and glycolipids, which together constitute the external glycocalyx of the cell (Roseman, 1974). What is the basis of this prejudice? It probably derives partly from our knowledge concerning immunological recognition, a phenomenon in which precise selection mechanisms operate within an immensely diverse population of molecules, and in which carbohydrates figure prominently. In the plant world, symbiotic plants and bacteria seek one another out by means of lectins, proteins whose function is to bind surface carbohydrate moieties. Evidence for the existence of lectins in animal cells has been put forth (Nowak, Haywood and Barondes, 1974). Inferential support comes from *in vivo* experiments in which it can be shown radioautographically that newly synthesized glycoprotein is inserted in the region of the synapse (Bennett and co-workers, 1973), or at the growth cone (Tessler, Autilio-Gambetti, and Gambetti, 1977). Why carbohydrates should be particularly suited for recognition functions may derive from the large variety of possible glycosidic bonds between two sugars. In addition to two or three available functional groups per residue (compared with but one per amino acid in proteins), there is also the possibility of an alpha or beta linkage, branching, etc. Furthermore, while protein synthesis is stringently prescribed in the nucleus, carbohydrate modification of proteins is post-translational, and both glycoproteins and glycolipids could be modified by ectoenzymes, permitting the mediation of intercellular interactions at a distance from the nucleus. These and other arguments support the involvement of carbohydrate-mediated recognition codes in development, but at present, the issue must be regarded as speculative.

IN VITRO STUDIES

Single cells, dissociated from undifferential retina, can reaggregate into vesicles with stratified layers that bear resemblance to the retinal laminae seen *in vivo* (Sheffield and Moscona, 1970). A similar comparison has been proposed between layers seen in reaggregates of cerebellar cells and those seen *in vivo*. Cerebellar cells obtained from a genetically ataxic mouse strain (reeler) fail to exhibit the ability to form the layered appearance (DeLong and Sidman, 1970). These model systems suggest that there may be a role for self-assembly at the cellular level in development. Retinal elements other than ganglion cells possess recognition functions, and dissociated retinal cells do in fact exhibit evidence of temporal and spatial coding. For example, aggregation of retinal cells is blocked by diffusible factors obtained from retinal extracts, the age of which correlates with the age of

retinal cells optimally stimulated to aggregate (Merrell, Gottlieb and Glaser, 1975). Dissociated retinal cells thus can provide useful information that may enhance our eventual understanding of retinotectal coding. A convenient source of cells for such studies is the chick retina since it yields large numbers of viable cells following mild disruptive procedures. After labeling, for example by brief incubation with $^{32}P_i$, probe cells obtained from retinal sectors can be used in binding studies with coated beads or fibers, with monolayer cultures, or with full thicknesses of optic tectum (Marchase, Voxbeck and Roth, 1976). Such experiments have in common that they assume that the dissociated retinal probe cells will express recognition properties that relate to specification in vivo. One could imagine a rather stringent set of rules which would preclude use of this approach for studying retinotectal recognition. It might be, for example, that only fully differentiated ganglion cells recognize tectal sites, that receptors are present only at the growing tip of the axon and perhaps only at a specific time. Furthermore, expression of the recognition site might require the presence of an array of appropriate fibers of neighboring cells. That the rules of retinotectal specificity are more relaxed than outlined above is evident from experimental findings. Binding of cells from various retinal sectors to predicted tectal sites has been reported (Barbera, 1975) and interpreted to reflect a gradient of glycosyl transferases in the retina forming the basis of a chemoaffinity mechanism (Marchase, Vosbeck and Roth, 1976). The importance of these findings to in vivo development awaits extension of the initial observations. It remains uncertain whether dissociated cells can demonstrate properties that reflect recognition or guidance mechanisms. A model which begins to bridge the gap between the cell and the organism is the tissue explant, since some developmental history and structure are preserved in an in vitro system. The seemingly straightforward experiment of putting a piece of retina and tectum together in a culture dish, however, turns out to be an experimentally difficult task. It was noted some time ago by Weiss (1934) that neurites growing out from explants tend to grow into one another regardless of the source the so-called "two-center effect". Metabolic gradients, mechanical stresses within the three-dimensional substratum, etc., may explain the basis of this artifact (Dunn, 1971). Were a retinal explant to invade a tectal explant in vitro in a manner that retained properties of specificity it could be a problem to demonstrate clearly that it had. Up to the present, it has been difficult to label an explant unambiguously in a way that would permit tracing of its fibers within a target explant. The lack of success of this direct approach may also reflect the fact that conditions for optimizing neurite outgrowth in vitro are quite unphysiological and probably unsuitable for the evocation of physiological recognition mechanisms. For example, according to Singer's blueprint hypothesis, neurites are guided by ependymal cells, yet in vitro, we generally grow neurites out on an unstructured substratum - unfamiliar territory. The explant preparation has nevertheless permitted us to characterize some of the properties of the outgrowing neurite, since we can examine its surface and its growing tip in the absence of the contiguous supporting cells present in the optic nerve.

THE EXPLANTED GOLDFISH RETINA

While explants and dissociated primary cell culture are routinely performed with embryonic tissues, our laboratory has been examining outgrowth from explants of adult goldfish retina. Like adult tissues from higher vertebrates, explants from the goldfish retina do not ordinarily support outgrowth, but if a conditioning lesion of the optic nerve is made 1-2 weeks before explantation, we observe excellent outgrowth of neurites. We have demonstrated elsewhere (Johns, Heacock and Agranoff, 1978) that this outgrowth derives from ganglion cells, and that early neurites, seen after 1-2 days in vitro, appear on the edge of the explanted fragment closest to the optic disc of the retina from which they had come (Johns, Yoon and Agranoff, 1978) (Fig. 1).

A marked tendency for neurites from our explants to form fascicles and for the fascicles to grow out onto the substratum in a clockwise fashion is seen (Fig. 2) (Heacock and Agranoff, 1977).

This result has been interpreted to indicate that the outgrowing fibers have a helical nature. Spiralling of fibers can be seen within fascicles (Fig. 3), but it is questionable whether it is characteristic of single neurites grown out from ganglion cells.

Spiralled fascicles may have significance in vivo; the lead fiber and its complement of fibers together form a more coherent cable than the individual neurites, and the spirality assures that mechanical flexion of the nerve will not unravel single fibers. In chick spinal ganglia, fasciculation appears to be mediated by a neuronal protein, the so-called "cell adhesion molecule" (CAM) (Rutishauser, Gall and Edelman, 1978) and it has been proposed that fasciculation may play a role in cell recognition or in fiber guidance. Fasciculation has also been implicated in ganglion cell axon guidance in the mouse retina (Goldberg and Frank, 1979).

Explant culture has also permitted examination of the neurite membrane in the absence of oligodendroglia and other cellular elements. Light microscopic and ultramicroscopic studies with lectins have indicated that alpha-D-mannose, D-galactose and N-acetyl-D-glucosamine are prominent in the neurite membrane surface. It is possible that the galactose is in a glycolipid, while the N-acetyl-D-glucosamine is more likely to be part of a glycoprotein (Feldman, Heacock and Agranoff, 1978).

We have also studied the presence of proteins characteristic of excitable membranes. Antibodies to electroplax Na^+-K^+ ATPase and to acetylcholine receptor (AChR) were available (courtesy of Drs. R.W. Albers, NIH, and S. Fuchs, Weizmann Institute, respectively), and immunohistochemical studies in our laboratory have indicated that goldfish brain can react with each of the rabbit antisera. Immunohistochemical studies both in vivo and in explant culture indicate the presence of the enzyme (Schwartz and co-workers, 1979a) and receptor (Schwartz and co-workers, 1979b) in optic nerve fibers. An HRP-linked indirect method was used to localize the AChR, which appears to be present in optic nerve fibers (Fig. 4).

The presence of ATPase in the nerve membrane was anticipated (Wood and co-workers, 1977), while the presence of AChR was not, since the latter is generally thought to exist in areas of synaptic specialization on the postsynaptic surface. However, AChR has in fact been found in axonal membranes of invertebrates (Marquis, Hilt and Mautner, 1977). The question of its possible role in conduction will have to be considered further. The finding also underlines that care be exercised in interpretations of experiments purported to elucidate the nature of the neurotransmitters of the goldfish retinal ganglion cell (Schechter and co-workers, 1979; Oswald and Freeman, 1977).

The explant has also served for studies on membrane addition during neurite extension. We used a lectin-anti-lectin complex to mark the neurite membrane in a fashion which prevents diffusion of the marker, yet does not block outgrowth. Neurites were tagged with Concanavalin A (Con A) followed by rabbit anti- Con A, then permitted 24 h of additional growth. The preparation was then reacted with fluorescent goat anti-rabbit IgG. By these means we have been able to establish that new membrane originates at the growth cone (Feldman and co-workers, 1979) (Figure 5).

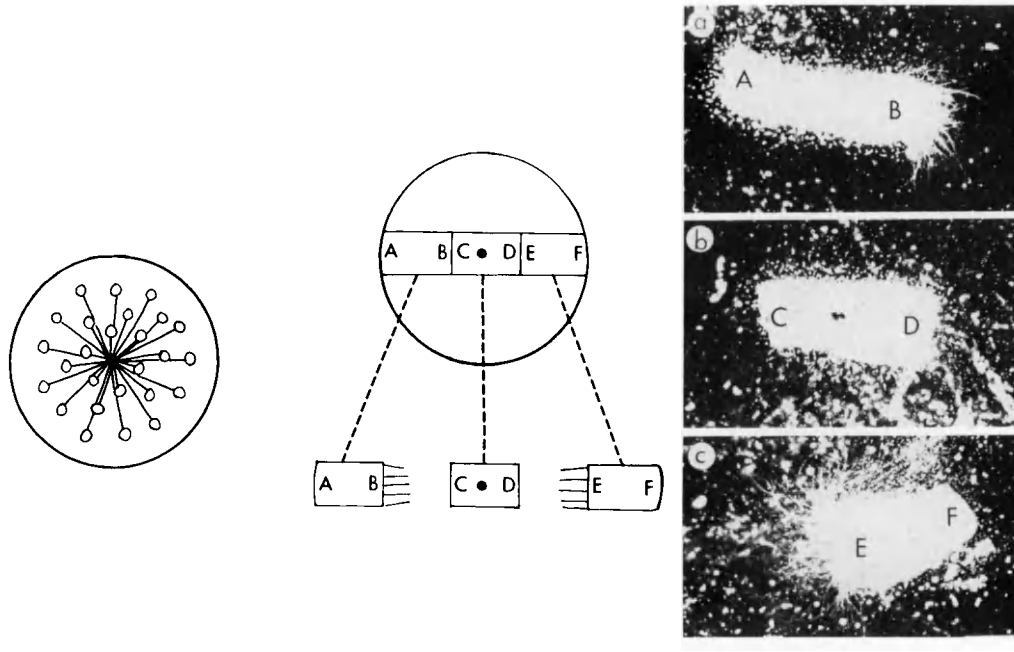

Fig. 1. Directed growth of goldfish retinal neurites. Diagram on left shows radial pattern of optic fibers in the retina. In the center, the retina is shown as it was prepared for explantation. A strip of retina that included the optic disc was divided into three approximately equal pieces and then explanted. If the neurites maintained their centripetal orientation (towards the disc), they would grow out from the ends labeled B and E. The results of such an experiment are illustrated on the right. From Johns, Yoon and Agranoff, 1978.

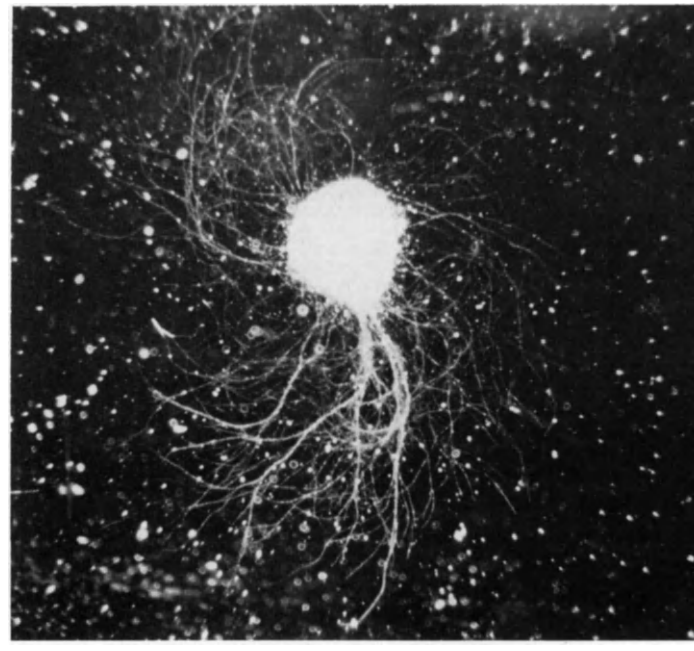

Fig. 2. Clockwise growth of retinal neurites. Dark-field photomicrograph of goldfish retinal explant on a polylysine-coated substratum.

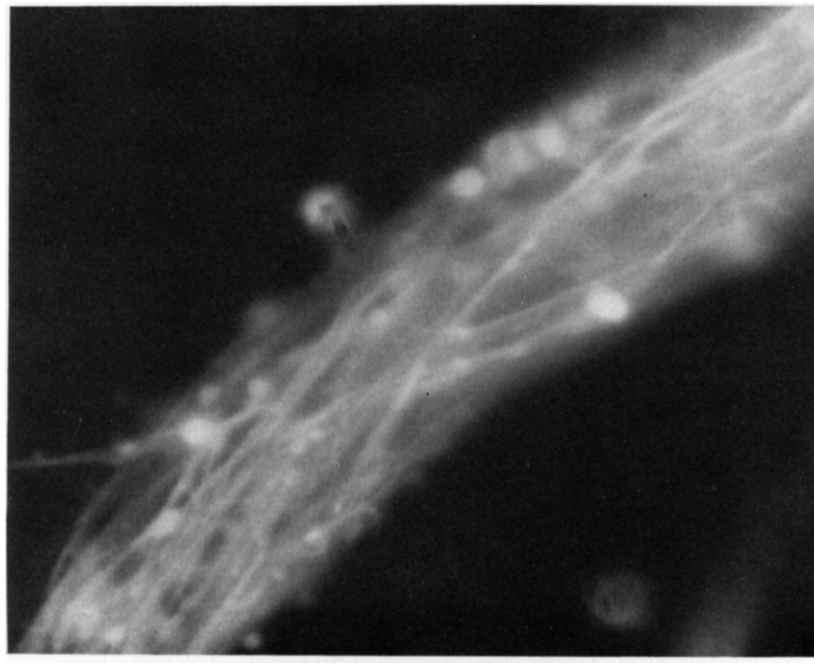

Fig. 3. Spiralling of fibers within a neurite fascicle. Fluorescence photomicrograph of a neurite fascicle stained with the lipophilic dye, dioctadecylindocarbocyanine.

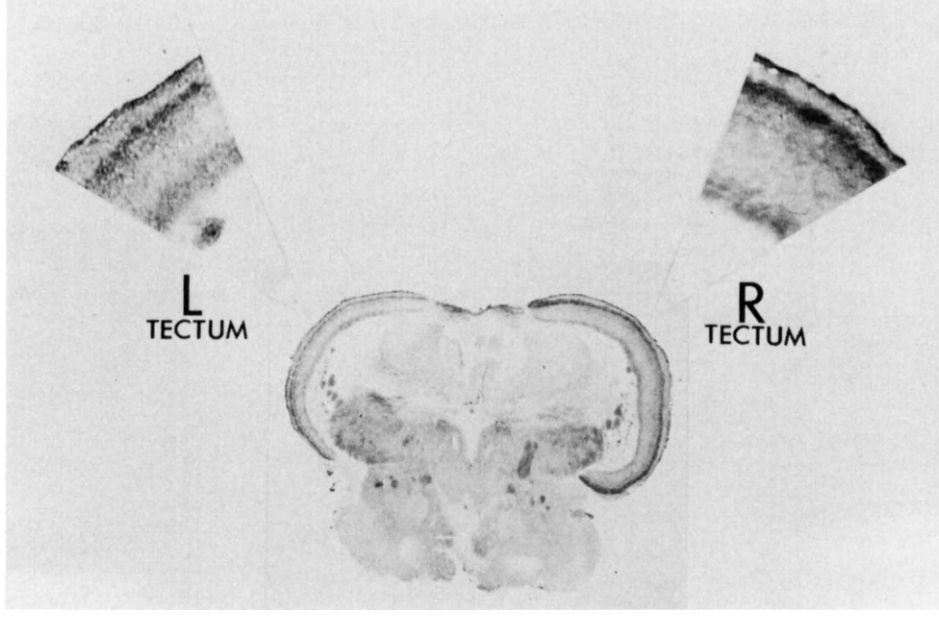

Fig. 4. Effect of eye enucleation on acetylcholine receptor antigenic sites in the tectum. Immunoperoxidase staining for AChR in goldfish brain section 3 months after enucleation of right eye.

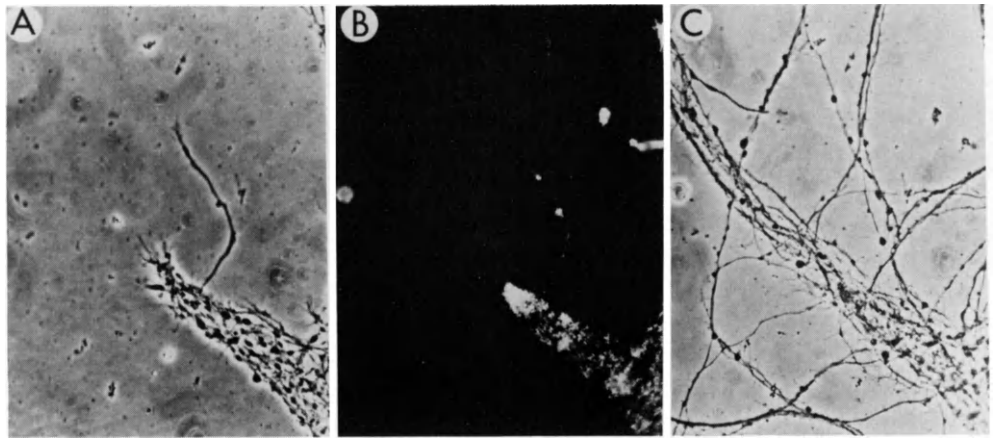

Fig. 5. Lectin binding identifies the site of new membrane addition at the growing tip. Explants were incubated with Con A (25 ug/ml) then anti-Con A (100 ug/ml). Twenty-four hours later the lectin bound portion of the neurite membrane was visualized by fluorescence microscopy following treatment with rhodamine-labeled goat anti-rabbit immunoglobulin (200 ug/ml). Extent of growth was monitored by phase microscopy.
A. Phase photomicrograph of neurites taken at zero time.
B. Fluorescence photomicrograph of same field taken 24 h later.
C. Phase photomicrograph of same field as in B.

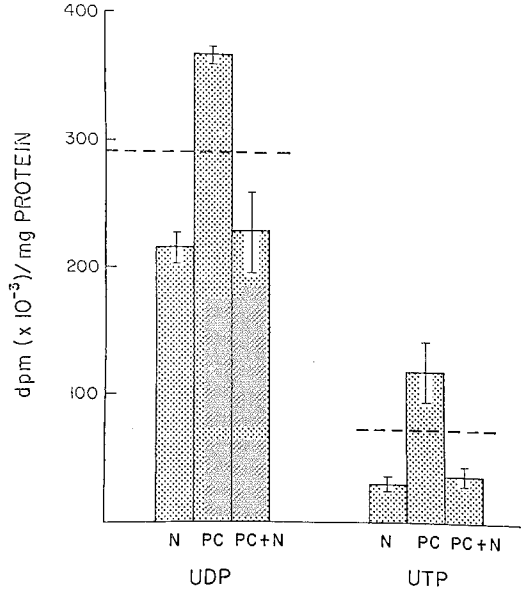

Fig. 6. Characterization of enhanced uridine nucleotide kinase activity in day 3 post-crush post-mitochondrial supernatants, by means of a mixing experiment. Retinal post-mitochondrial supernatants were prepared and aliquots were incubated with 2 uCi of [^{3}H]orotic acid, PPRP and ATP, and an average of 110 ug protein from 3 day PC(post-crush), N (normal) or a mixture of PC and N preparations. The labeling of UDP and UTP was determined by high voltage electrophoresis. Each bar is the average dpm/mg protein ± SEM in each nucleotide from 3 assays of each preparation. PC+N assays containing equal proportions of PC and N enzyme preparations. The dotted line represents the expected dpm/mg protein in UDP or UTP numerical averaging of PC and N preparations. The result suggests that an inhibitory substance is normally present in retina, but is inactive or absent in the retina following optic nerve crush (From Dokas, Burrell and Agranoff, 1979).

Whether the new membrane protein migrates by axonal transport and is inserted at the growth cone or whether post-translational alterations, including carbohydrate addition, occurs at the time of externalization, is not presently known.

BIOCHEMISTRY OF THE RETINA DURING REGENERATION

As stated earlier, higher vertebrates do not regenerate the visual system, and hypotheses to explain the failure include intrinsic differences among species such as inability of the cut axon to initiate the necessary perikaryal repair mechanism in the cell body, and extrinsic mechanisms, such as the formation of an impassable glial scar (Lieberman, 1971; Grafstein, 1975). Some light on biochemical events that accompany axotomy may be shed by experiments in our laboratory in which control retina (from the unoperated eye) has been compared with post-crush retina at various times following the lesion in the goldfish. We found an increase in labeling of tubulin following crush (Heacock and Agranoff, 1976) and more recently, an increase in mRNA for tubulin in post-crush retina (Burrell and coworkers, 1979). The earliest biochemical event that we have thus far detected is an increase in retinal nucleotide phosphorylation that occurs 2-3 days after crush (Dokas, Burrell and Agranoff, 1979). Results in the latter studies are consistent with the hypothesis that an inhibitory substance normally present is reduced following crush (Fig. 6).

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ACETYLCHOLINE IN THE RETINA

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ABSTRACT

Acetylcholine in the retina is synthesized and released by a sparse group of amacrine cells that symmetrically line both margins of the inner plexiform layer. The action of these neurons is at once very selective (they affect ganglion cells of some functional classes, but not others) and rather diffuse (the dependence of the acetylcholine-sensitive ganglion cells on cholinergic input is partial, and cholinergic synapses do not seem to affect the intrinsic structure of the signal transmitted by the ganglion cell). The most interesting aspect of these results is that one cannot yet fit the action of the cholinergic neurons to one of the physiological events thought to occur in the inner plexiform layer. Identification of the cholinergic cells thus seems to isolate a neural interaction that is not included among our concepts of the retina's internal working.

KEYWORDS

Retina; acetylcholine; amacrine cells; ganglion cells.

INTRODUCTION

One of the motives for studying the neurotransmitters of the central nervous system is the possibility that they will serve as markers for function—that neurotransmitters distinguish physiological subclasses among neurons not initially distinguishable by morphological criteria. The classification of neurons on the basis of their neurotransmitters has, as an immediate corollary, a second feature: it provides a means of manipulating the function of the identified cells, by the use of pharmacological agonists and antagonists of the naturally secreted compound. A major goal of such studies thus extends beyond the purely taxonomic level; one seeks both the identification of classes of neurons and an understanding of their role in the neural circuits of which they are part.

The retina contains most of the neurotransmitters found elsewhere in the central nervous system. Here I shall focus on studies, done in our laboratory and others, of the retina's cholinergic system; for it is the one that has been most analyzed by

physiological methods. These studies provide evidence for the existence of cholinergic synapses in the retina, an identification of the acetylcholine-releasing cells and the location of their synapses, and a description of some of the cells' effects on the retina's function.

ACETYLCHOLINE SYNTHESIS AND RELEASE

Most retinas are moderately rich in acetylcholine, choline acetyltransferase, and acetylcholinesterase, and the synthesis of acetylcholine from exogenous choline by intact retinas (Fig. 1) is easily demonstrated (Lam, 1972; Neal and Gilroy, 1975; Masland and Livingstone, 1976; Baughman and Bader, 1977).

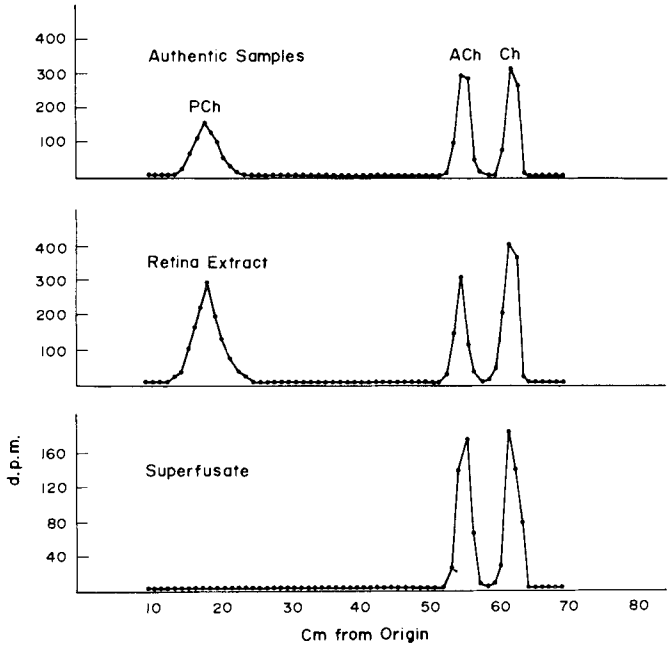

Fig. 1. Choline metabolites synthesized and released by the rabbit retina. High-voltage paper electrophoresis was carried out at pH 1.9 for 2 h. these retinas were exposed to 10 μM ^{14}C-choline. (The distribution of choline metabolites is essentially constant at choline concentrations from 0.1 to 300 μM.) Figures 1-7 presented here are reprinted, by permission, from the Journal of Neurophysiology (Masland and Livingstone, 1976; Masland and Ames, 1976).

When rabbit retinas superfused under physiological conditions were stimulated by light, acetylcholine was released into the superfusate, and the release was prevented if the incubating medium contained an elevated concentration of Mg^{++} and a lowered concentration of Ca^{++} (Fig. 2). An anticholinesterase was added to the superfusing medium in these experiments; if no anticholinesterase was added, a Ca^{++}-dependent choline release was observed, indicating that the retinal synapses contain an effective cholinesterase. The pattern of light- stimulated choline release was the same as the pattern of acetylcholine release in

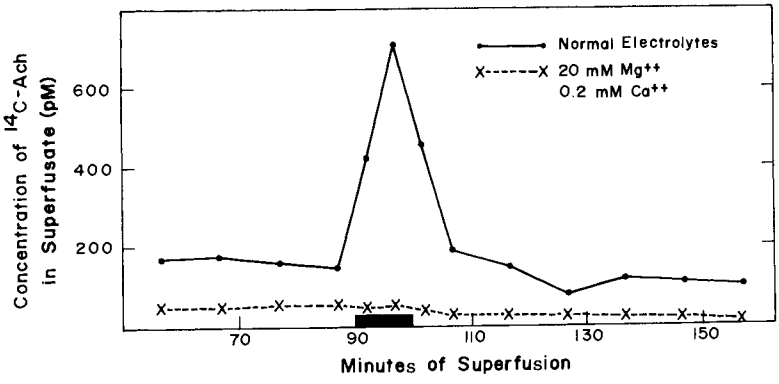

Fig. 2. Release of acetylcholine from the rabbit retina, and its dependence on calcium. At the time indicated by the black bar, the retinas were stimulated by flashing light.

the presence of anticholinesterase, indicating that the anticholinesterase, which might have altered the retina's acetylcholine release by potentiating intra-retinal cholinergic synapses, had not affected the physiological release of acetylcholine. That it is unaffected by isolation of the retina from the eye has been shown by more recent experiments demonstrating light-evoked acetylcholine release from a superfused rabbit eyecup superfused in vivo (Massey and Neal, 1979).

These studies provide evidence that retinal cells can synthesize acetylcholine; that they release it to physiological stimulation; and that the retina contains an effective means of inactivating the released acetylcholine. They also gave a functional clue to the site of acetylcholine's release: Photic stimulation depolarizes many of the cells synapsing in the inner plexiform layer, but hyperpolarizes cells synapsing in the outer plexiform layer. Since the signal for neurotransmitter release is depolarization of the cell, an increased rate of acetylcholine release during stimulation suggests that acetylcholine is released by amacrine or bipolar cells. Such a conclusion was consistent with earlier work showing that acetylcholinesterase and choline acetyltransferase are concentrated in the inner plexiform layer (Raviola and Raviola, 1962; Nichols and Koelle, 1967; Reale, Luciano and Spitznas, 1971; Ross, Cohen, and McDougall, 1975).

EFFECTS OF ACETYLCHOLINE ON RETINAL GANGLION CELL ACTIVITY

In order to evaluate acetylcholine's physiological effects, we used the preparation of Ames and Pollen (1969), in which rabbit retinas are spread over an inflatable dome, which provides support during recording and displayed the retinal surface for photic stimulation. The activity of single ganglion cells was recorded extracellularly. An optical system with two independent channels of stimulation was used to focus stimuli on the retinal surface, and the receptive fields of ganglion cells were mapped and classified. Flowing superfusion preserved the retina's viability and allowed the introduction of test agents to the tissue (Masland and Ames, 1976; the preparation and evidence of its viability are described by Ames elsewhere in this volume).

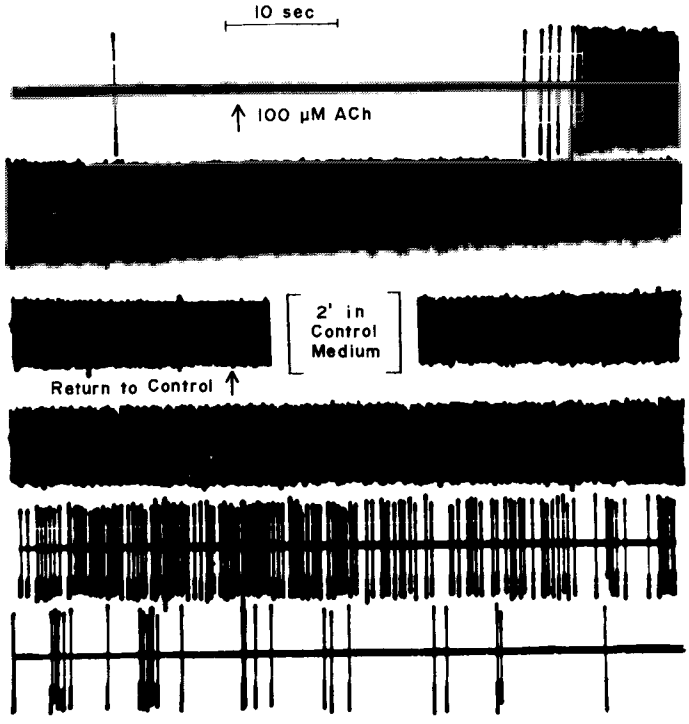

Fig. 3. Response of a single retinal ganglion cell to acetylcholine. This neuron had a directionally selective receptive field. The time at which superfusion medium containing acetylcholine began to enter the incubation chamber is indicated by an arrow. Most of the apparent latency of the response is due to time taken for the test medium to replace the control medium originally present.

We found many, but not all, retinal ganglion cells to be sensitive to acetylcholine. Sensitivity to acetylcholine was correlated with the functional class of the ganglion cell. Those with on-center or directionally selective receptive fields were excited when acetylcholine was added to the superfusing medium, with thresholds as low as 3 μM (Fig. 3). Perhaps more significantly, the same cells were also excited when physostigmine was applied (Fig. 4). At low concentrations of physostigmine (1 μM) the light-evoked response was greatly prolonged; at higher concentrations of the anticholinesterase (30 μM) the cells often attained a high, sustained level of firing, even in the dark. The response to acetylcholine or physostigmine could be prevented by pre-incubating the tissue in most of a variety of cholinergic antagonists (Fig. 5); with varying effectiveness, these also depressed the cells' response to light.

Such experiments confirmed that the retina contains one or more cholinergic synapses, but did not indicate which neurons are sensitive to acetylcholine: the ganglion cells from which we record directly or more distal cells which then affect

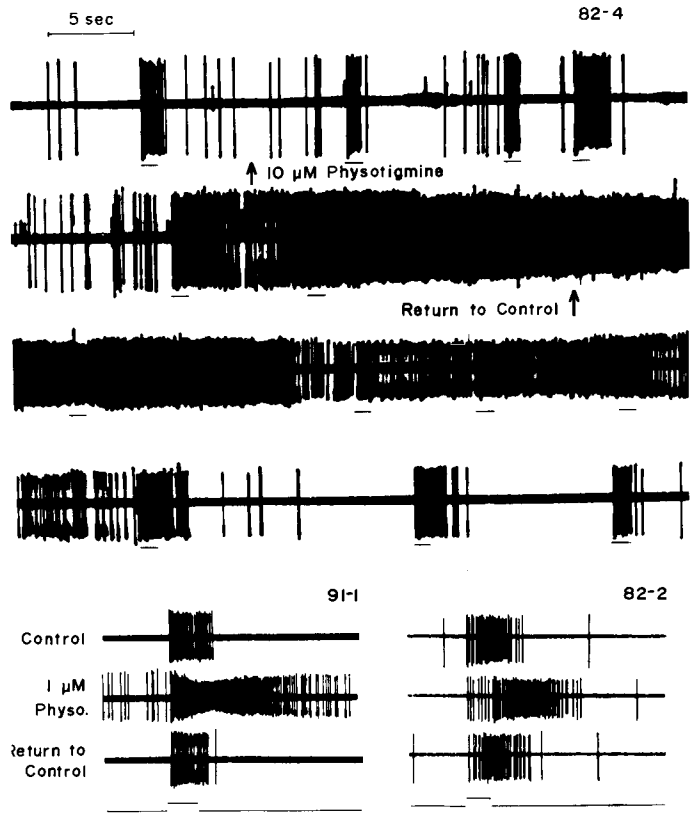

Fig. 4. Responses of two on-center cells and a directionally selective cell (cell 82-2, lower right) to physostigmine. Stimulus in upper records, 1 sec; in lower records, 0.5 sec.

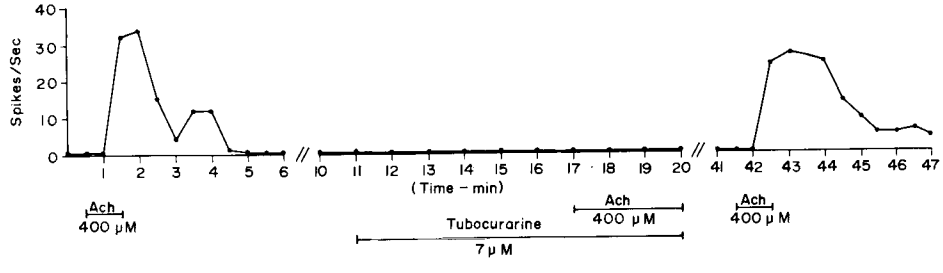

Fig. 5. Prevention of a directionally selective cell's response to acetylcholine by tubocurarine

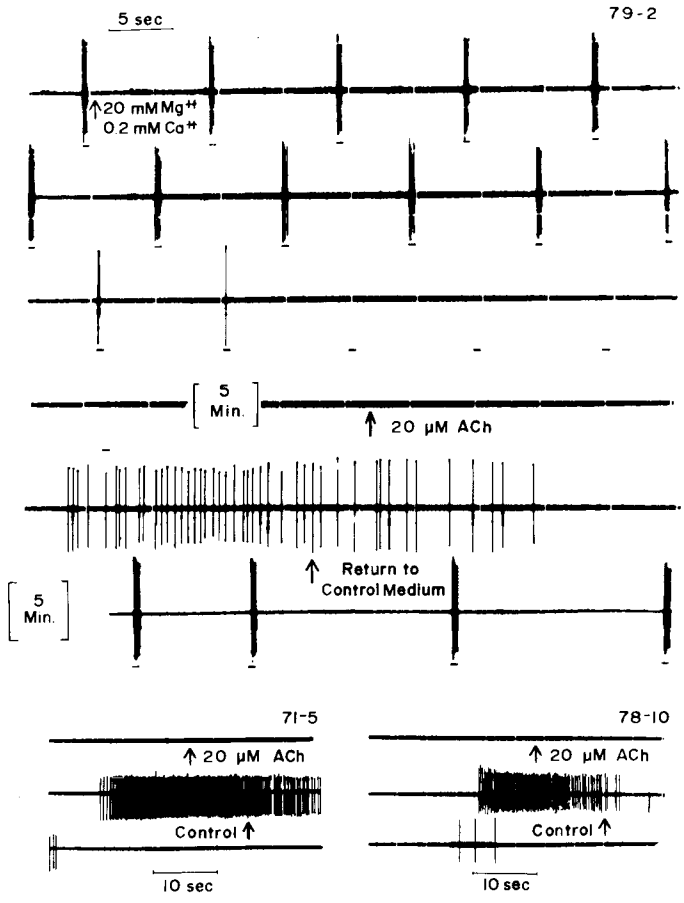

Fig. 6. The response to acetylcholine in medium containing 20 mM Mg^{++} and 0.2 mM Ca^{++}. The upper traces show a complete run. The lower traces (cells 71-5 and 78-10) show only the response to acetylcholine after synaptic transmission had been suppressed.

the ganglion cells transsynaptically. Acetylcholine could have been acting at any synapse between the photoreceptor and ganglion cell. We therefore tested the acetylcholine sensitivity of ganglion cells in retinas whose synaptic transmission was temporarily interrupted by superfusion with a medium containing a high concentration of Mg^{++} and a lowered concentration of Ca^{++}. This allowed an experiment in which (1) ganglion cells were located electrophysiologically in control medium, and their receptive fields classified, (2) their response to a test dose of acetylcholine was evaluated, (3) the control medium was replaced by medium containing high Mg^{++} and low Ca^{++}, and (4) the response to acetylcholine was again evaluated, this time in the absence of synaptic function. The results were unequivocal: almost all of the cells that were sensitive to acetylcholine in control medium remained sensitive to it in the medium that suppresses synaptic function (Fig. 6). Most ganglion cells with on-center or directionally selective receptive fields thus appear to receive input via one or more cholinergic synapses, at least one of which is upon

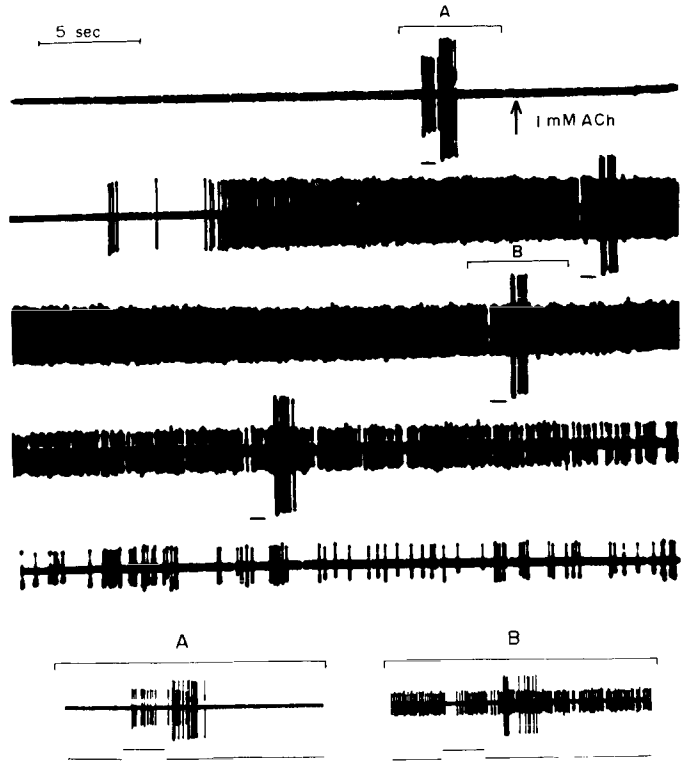

Fig. 7. Effect of a high concentration of acetylcholine on an on-center cell and an off-center cell, simultaneously recorded. The off-cell was unaffected by acetylcholine. Two portions of the continuous record are reproduced below with an expanded time scale and a slightly reduced gain.

the ganglion cell itself. This observation complemented well the studies localizing acetylcholinesterase and the measurements of acetylcholine release mentioned above, which had suggested that acetylcholine is released by amacrine or bipolar cells.

But some ganglion cells, most commonly those with off-center receptive fields, were insensitive to acetylcholine. The majority of off cells responded very faintly to acetylcholine in control medium and not at all in the medium containing high Mg^{++}; and a substantial fraction of them were unaffected by 1 mM acetylcholine, by levels of physostigmine that inactivate virtually all of the retina's acetylcholinesterase, and even by physostigmine and acetylcholine in combination (Fig. 7). These cells' responses to light were unaffected by cholinergic antagonists. Acetylcholine thus appears to be used in the circuits afferent to some retinal ganglion cells, but not all; and the presence or absence of a cholinergic synapse depends to some extent on the functional class of the ganglion cell.

That only a fraction of the ganglion cells are sensitive to acetylcholine has been confirmed in the perfused cat eye by Niemeyer and Albani (1977), in the rabbit retina by Ariel and Daw (1978), and in the superfused carp retina by Glickman and Adolph (1979). (It was in fact implicit in the results of early studies by Straschill, 1968, and by Noell and Lasanksy, 1969, but these could not be interpreted with confidence because the experiments were carried out in vivo.) The implications of this finding, and the detailed character of acetylcholine's affect on the ganglion cells, will be discussed later. For the present, it immediately suggested that acetylcholine plays a quite selective role in retinal function, and made it seem particularly interesting to identify the acetylcholine-releasing neurons morphologically.

IDENTIFICATION OF THE CHOLINERGIC CELLS

The rabbit retina, like all that have been studied (Lam, 1972; Neal and Gilroy,1975; Baughman and Bader, 1977), accumulates choline very effectively from the extra cellular medium (Masland and Livingstone, 1976; Atterwill and Neal, 1978). Baughman and Bader (1977) successfully exploited this fact in studies of the chicken retina, localizing the cells that accumulate choline autoradiographically. For experiments in the rabbit, however, the localization of choline uptake had an important limitation: it depends on most of the choline taken up being destined for acetylcholine. Evan at low extracellular concentrations of choline some tissues—including rabbit retina—incorporate choline more rapidly into phospholipids and their precursors than into acetylcholine (see Fig. 1)

We therefore sought to identify ^{3}H-acetylcholine directly in autoradiographs of retinas exposed to ^{3}H-choline. This required experiments to distinguish silver grains due to ^{3}H-actylcholine from those due to ^{3}H-compounds of the phospholipid pathway. Retinas were pulse-labeled with ^{3}H-choline, after which they were incubated under chase conditions designed either to retain acetylcholine within synapses (high Mg^{++}) or to promote its release (flashing light). This allowed a subtractive identification of acetylcholine, in which retinas that contained radioactivity only in the phospholipid pathway were compared autoradiographically with retinas that contained the same amount of radioactivity in the phospholipid pathway but also contained ^{3}H-acetylcholine (Masland and Mills, 1979).

Figure 8 shows the radiochemical contents of a retina incubated under the acetylcholine-releasing condition, and an autoradiograph of a different piece cut from the same retina and processed histologically. Radioactivity is present in the photoreceptor cells (predominantly the inner segment) and weakly present in the ganglion cells. Figure 9 shows the results obtained in retinas incubated under the acetylcholine-protecting condition. Chemical analysis showed that radioactivity in the phospholipid pathway was essentially the same as that observed in the first group of retinas, but this time a substantial amount of radioactive acetylcholine was also present. In autoradiographs the radioactivity associated with the presence of ^{3}H-acetylcholine appeared in two bands within the inner plexiform layer, and was densely concentrated in a small group of cell bodies at either margin of the inner plexiform layer.

That the pattern of radioactivity just described in fact represented ^{3}H-acetylcholine was independently corroborated by autoradiography of retinas in which the phospholipds were selectively retained during direct fixation with osmium tetroxide; by selective water-extraction of the radioactivity identified as acetylcholine; and by chemical analysis of the radioactive compounds found in photoreceptor cells microdissected from freeze-dried retinas. Hemicholinium-3 eliminated ^{3}H- acetylcholine synthesis and the silver grain localization associated

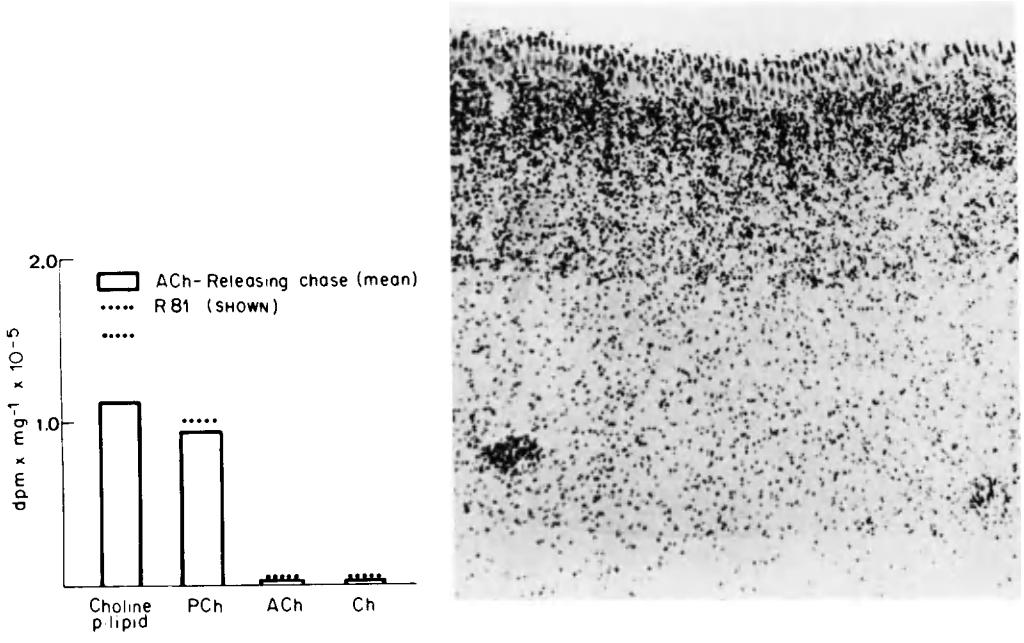

Fig. 8. Radiochemical contents and an autoradiograph of a retina pulse-labeled for 15 minutes with ^{3}H-choline (0.3μM) and then incubated for a subsequent hour under acetylcholine-releasing conditions (1 mM unlabeled choline, flashing light). Bars show means for 6 retinas. Rows of dots show the radiochemical contents of a piece of the retina whose autoradiograph is shown. Standard errors were less than 15% of the means.
Figures 8-10 shown here are reprinted, with permission, from the Journal of Cell Biology (Masland and Mills, 1979).

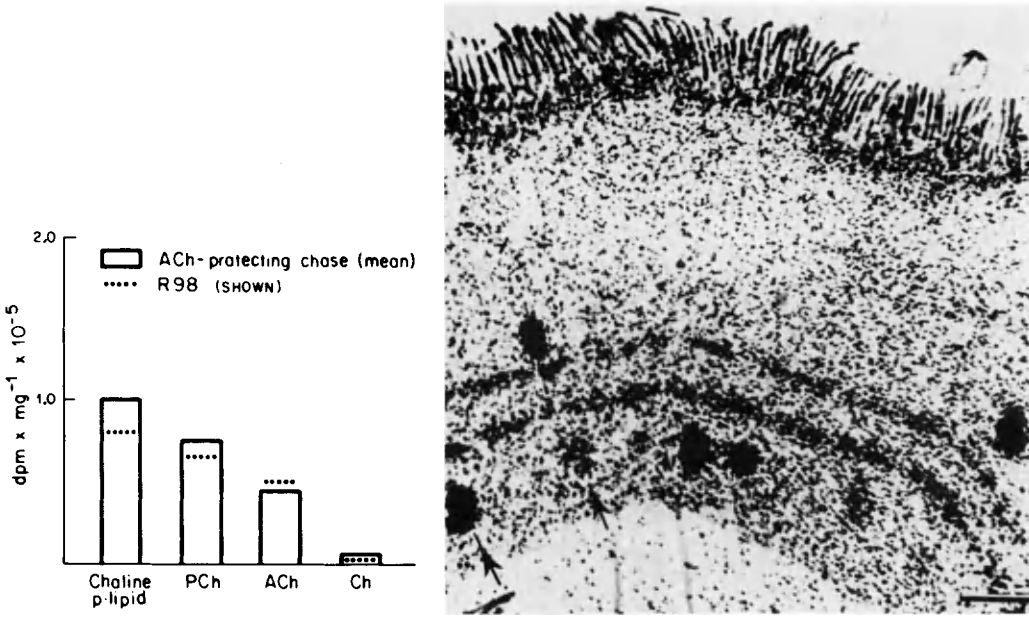

Fig. 9. Radiochemical contents and an autoradiograph of a retina pulse-labeled for 15 minutes with ^{3}H-choline and then incubated for a subsequent hour under acetylcholine-protecting conditions (1 mM unlabeled choline, 30 μM physostigmine, 20 mM Mg^{++}, 0.2 mM Ca^{++}). Calibration 20 μm. Conventions as in Fig. 8.

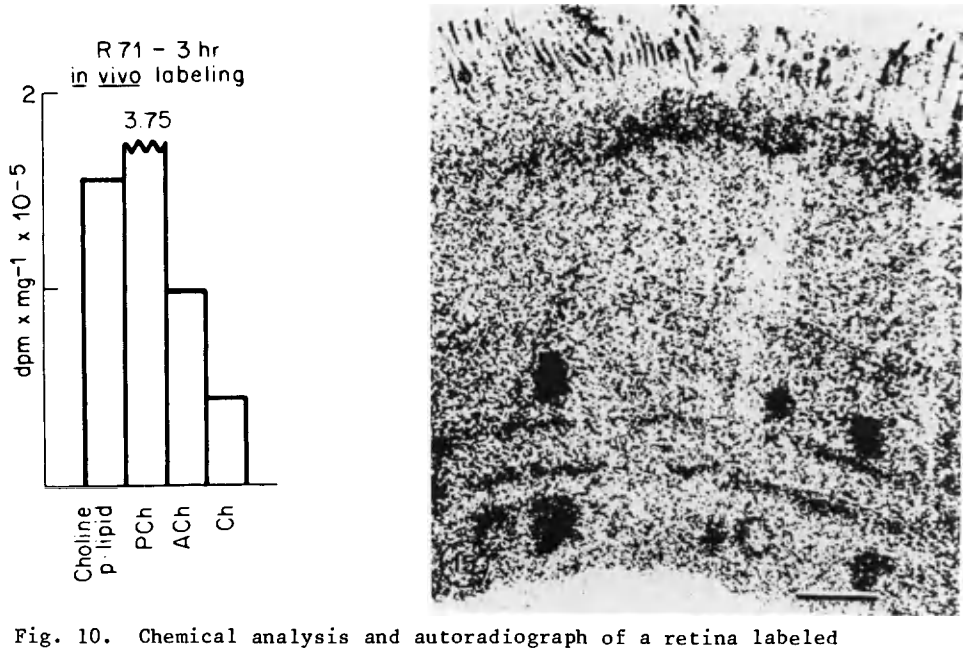

Fig. 10. Chemical analysis and autoradiograph of a retina labeled by injection of choline into the posterior chamber. Calibration 20 μm. Retina was harvested 3 hours after injection.

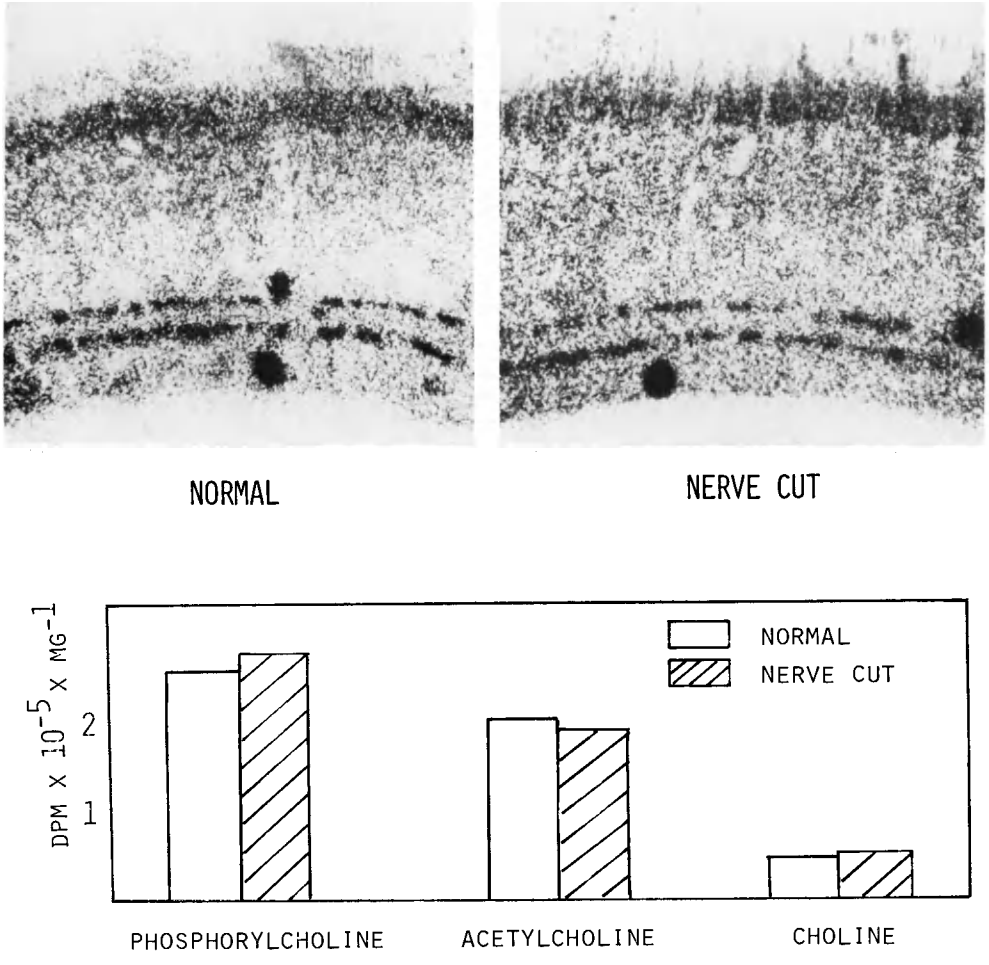

Fig. 12. Acetylcholine synthesis and its autoradiographic localization in normal retinas and retinas whose optic nerve had been sectioned 6-8 months previously. Bottom: Synthesis of radioactive choline metabolites by retinas exposed to 0.8 μM ^{3}H-choline for 15 minutes. Means for 4 normal and 4 operated eyes. Top: Representative results obtained by dry autoradiography of such retinas.

with it. As a control for the possibility that the isolation of the retina (or the pulse-chase procedure) influenced the autoradiographic results, we also studied the pattern of accumulation of radioactivity in retinas exposed to ^{3}H-choline by injection into the posterior chamber of the eye. It was identical to that observed in vitro, except for a higher background radioactivity consequent to the "unchased" situation (Fig. 10). Baughman and Bader localized the sites of ^{3}H-choline accumulation in the chicken (1977) and turtle (personal communication) retinas to two bands within the inner plexiform layer and cells at both of its margins. Although a few neurons deep in the inner nuclear layer of the chicken retina also accumulated some radioactivity, the overall pattern of labeling was very similar to the localization of acetylcholine seen in the rabbit retina. Given the inter-species variability of other retinal neurotransmitters, it is striking that these three vertebrates, differing widely in phylogenetic position, show a similar, and highly distinctive arrangement of cholinergic neurons.

The acetylcholine-synthesizing neurons of the rabbit retina's inner nuclear layer appear from their size and position to be amacrine cells. They make up less than 5% of the total cells in the inner cell row of the layer. The same low frequencies were observed in vivo and in vitro. An internal control for failure of the autoradiographic methods is the fact that the bands of radioactivity in the plexiform

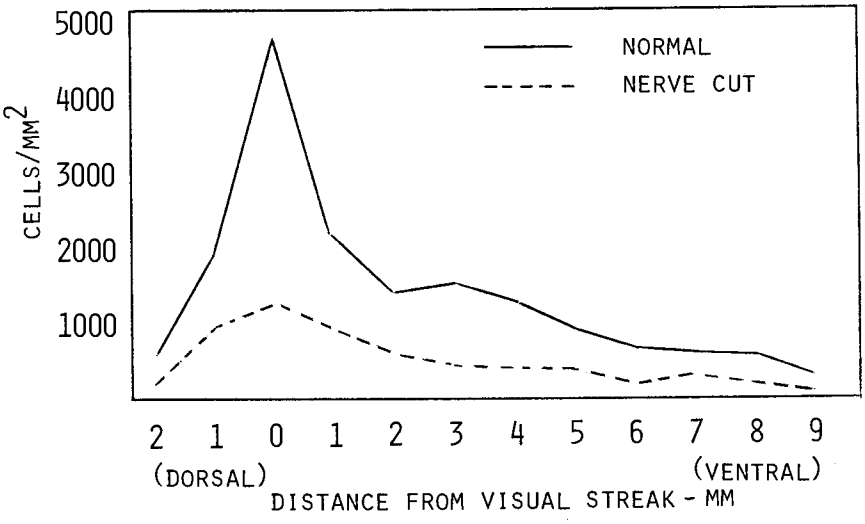

Fig. 11. The density of cells in the ganglion cell layer from a normal eye and an eye whose optic nerve had been sectioned six months previously. Cells were counted in flat mounts of the central 1/3 of the retina, along a vertical line passing near the optic nerve head. These studies wre carried out in collaboration with Shawn A. Hayden and John W. Mills.

layer continued for long stretches where no labeled cells were seen in the adjacent inner nuclear or ganglion cell layers. The acetylcholine-synthesizing cells of the ganglion cell layer are roughly equal in number to those of the inner nuclear layer. From their size and position it was not possible to know whether they were ganglion cells or displaced amacrine cells. In order to answer this question, a different kind of experiment was required.

We compared the rate of acetylcholine synthesis and its localization in normal retinas with those observed in retinas whose optic nerve had been cut many months previously. The nerve was sectioned just behind its exit from the optic foramen. Six to 8 months later the retina was isolated and incubated in ^{3}H-choline. At the end of incubation it was divided, by cuts perpendicular to the visual streak, into three approximately equal pieces. The central one, including the nerve head and fiber bundles as points of reference, was mounted flat and stained with cresyl violet for cell counting. One of the remaining pieces was homogenized and its radioactive choline metabolites evaluated. The third was quick-frozen and processed for dry autoradiography.

Figure 11 shows the degeneration of the retinal ganglion cells consequent to optic nerve section. There was total loss of about 75% of the neurons of the ganglion cell layer. In accord with previous studies of the cat (Stone, 1966), monkey (Stone, Leicester, and Sherman, 1973) and rat (Eayrs, 1952) retinas, the remaining 25% of the cells appeared quite normal in Nissl stained material. Despite severe degeneration of the ganglion cell layer, the synthesis of acetylcholine from exogenous choline was undistinguishable from that observed in normal retina. The density and distribution of acetylcholine-synthesizing neurons in these retinas was entirely identical to that observed in unoperated eyes (Fig. 12). It thus appears that the retina's acetylcholine-synthesizing neurons are all amacrine cells, about half of them displaced to the ganglion cell layer.

Taken together, the identification of the acetylcholine-synthesizing cells of the ganglion cell layer as displaced amacrines, the overall frequency of the cholinergic considerations immediately eliminate many of the amacrine cells seen in Golgi material from candidacy: those with too narrow a dendritic spread, those with bushy dendritic trees, and those that branch in planes near the middle or the extreme edges of the inner plexiform layer.

Descriptions of the displaced amacrine cell from Golgi preparations agree that its dendritic branching is confined to the inner part of the inner plexiform layer (Cajal, 1893; Nelson, Famiglietti, and Kolb, 1978; Perry,1979). Its dendritic spread, as described by Perry in the rat and Famiglietti (personal communication) in the rabbit is enough to meet the requirement given above. The inner band of acetylcholine in the inner plexiform layer thus appears to be due to processes of cholinergic displace amacrine cells. In order for the outer band to exist, it must consist of processes of the conventionally placed acetylcholine-synthesizing amacrine cell. In fact, it seems fairly likely that this cell, too, has dendritic branching confined to a single plane, because the outer band of acetylcholine has approximately the same density as the inner: if the conventionally placed amacrines contributed both to the outer band and the inner, then the inner band (which already contains processes from half the total number of acetylcholine-synthesizing cells) would be denser than the outer. In summary, then, the distribution of the retina's acetylcholine; the known shape of displaced amacrine cells; and the striking regularity with which cholinergic cells bracket the inner plexiform layer all suggest that a single type of cholinergic neuron is symmetrically disposed along both margins of the neuropil.

Such an arrangement is represented in figure 13. It assumes (1) that the displaced neurons, and the location of acetylcholine within the inner plexiform layer, allow certain inferences about the three dimensional shape of the cholinergic neurons. Most of the branching of the acetylcholine-synthesizing amacrines probably occurs in the planes defined by the two bands of acetylcholine observed in the inner plexiform layer--and these planes must be very thin, because the bands of acetylcholine are very narrow. (The process of autoradiography and any diffusion that might occur during processing can only tend to make the bands shown in Figs. 9, 10, and 12 appear wider than they actually are.) Because the bands corresponding to

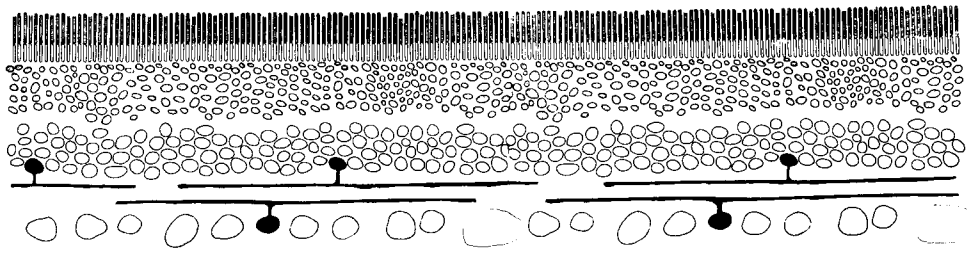

Fig. 13. Distribution and possible shape of the acetylcholine-synthesizing amacrine cells. The drawing is schematic, but preserves the overall dimensions of the retina and correctly shows the frequency of acetylcholine-synthesizing neurons. Spaces between their processes are shown only for clarity. The spaces would be filled by processes of cells out of the plane of the diagram.

acetylcholine-containing processes are continuous throughout the inner plexiform layer, one must conclude that the processes of the acetylcholine-synthesizing cells branch widely enough to cover the entire distance between their cell bodies. These amacrine cells—relatively few of which have been directly studied in Golgi material—form a homogeneous class of neurons, and (2) that the bands of radioactivity seen in autoradiographs define the course of neuronal processes. Final proof of this scheme will thus require more direct evidence than is presented here; but the constraints imposed by the available facts seem to assure that its major features are not far wrong.

FUNCTIONS OF THE CHOLINERGIC CELLS

With knowledge of the identity of the acetylcholine-releasing neurons, it becomes worthwhile to consider in more detail their effects on retinal function. Four statements may be made, the last of which leads to a paradox.

First, as was described earlier, acetylcholine seems to be involved in some circuits of the inner plexiform layer and not in others. Ganglion cells with some functional classes of receptive field are affected by acetylcholine and the related agents, while others are not.

While cholinergic input contributes to the response of light of many retinal ganglion cells, however, such input is not essential for a response: the cholinergic antagonists depressed, but never entirely prevented, the light-evoked activity of even the cells most sensitive to acetylcholine or physostigmine (Fig. 14). This was true despite the fact that the same antagonists could entirely block the response to a test dose of exogenous acetylcholine (Fig.5), showing that the antagonists were appropriate to the retina's acetylcholine receptor. It thus appears that the acetylcholine-sensitive ganglion cell also receives input via neurotransmitters other than acetylcholine, and that the other neurotransmitters can sustain a substantial part of the cell's response to light. When one remembers that some ganglion cells are not sensitive to acetylcholine at all, the evidence seems clear that the small group of acetylcholine-releasing amacrine cells is not part of an obligatory through-pathway from photoreceptor to ganglion cell.

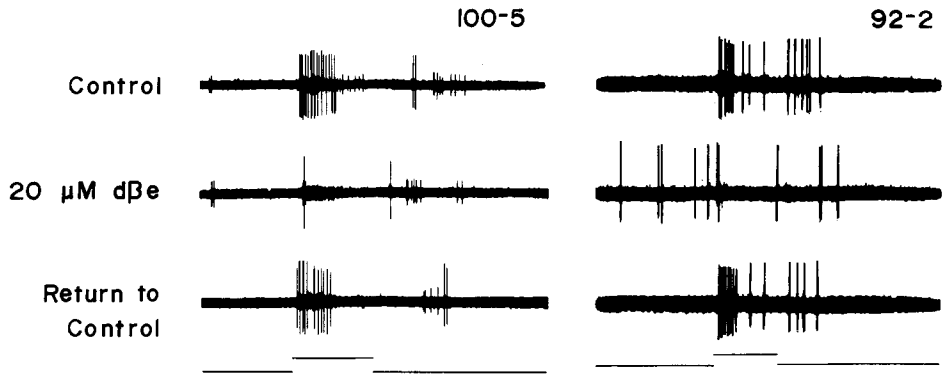

Fig. 14. Effect of dihydro-β-erythroidine on the light evoked activity of ganglion cells. Dihydro-β-erythroidine was the most effective of seven cholinergic antagonists tested. The block of the light-evoked response was never absolutely complete: note that a few spikes remain in the presence of the drug for both of the cells illustrated, and that unresolved cells still responsive to light can be seen as a thickening of the baseline during recording of cell 100-5.

The cholinergic cells thus appear to play some specific internal role in the functions of the inner plexiform layer. Curiously, though, the effect of drugs that modulate cholinergic function upon the acetylcholine-sensitive ganglion cell is a rather general one. Anticholinesterase strengthens and prolongs the cell's reponse to light, and increases the background rate of spontaneous firing; but it does not alter the balance between center and surround. Cholinergic antagonists depress the response to light, but these again leave the nature and balance of the receptive field unchanged. In some way acetylcholine is controlling the absolute level of activity of these cells without changing the intrinsic structure of the signal transmitted by them.

Finally, Ariel and Daw (1978, 1979) have made a detailed study of the response of ganglion cells in the rabbit to physostigmine and mecamylamine injected close-arterially in vivo. They find ON center ganglion cells that receive a major tonic input to be more dependent on cholinergic input than those whose response to light is primarily phasic. Our experience using in vitro recording confirms this conclusion. This raises a paradox: biochemical studies from three laboratories show that only flashing light is effective in causing retinas to release acetylcholine—the release caused by a maintained photic stimulus is so weak as to be nearly undetectable (Masland and Livingstone, 1976; Baughman and Tso, 1979; Massey and Neal, 1979). Thus the more acetylcholine-sensitive of the concentrically organized retinal ganglion cells is one that is tonically affected by illumination; but the cell that releases acetylcholine would appear to do so primarily at the ON or OFF transients.

CONCLUSIONS

At the outset I stated that one seeks, by the identification of neurotransmitters, to segregate classes of neurons from the whole. The cholinergic amacrine cells surely form a distinct grouping, not only because of their common neurotransmitter but because of the precision with which they are positioned in the tissue. The symmetry with which they bracket the inner plexiform layer, and the thin planes in

which their synapses are apparently made, suggest that they play a correspondingly precise physiological role. Yet their apparent actions are at once highly specific and very diffuse: they selectively contact ganglion cells of some functional classes, but serve—within the limits of our experimental imagination—merely to drive up or tone down the activity of those ganglion cells to which they provide input.

The lesson is that one should not expect easily interpretable functions for retinal neurotransmitters. Biochemical and morphological techniques seem to have run ahead of the skills and vision of physiologists. We do not understand, in any serious sense of the word, the role of the cholinergic neurons in the workings of the inner plexiform layer; and the reason is that these cells appear to perform a function for which we do not have a concept, a function beyond a simple picture of the retina as a machine that transduces light, varies its sensitivity to it, and forms receptive fields. There is every indication that present concepts will be equally ineffective in describing the action of the neurons containing other retinal neurotransmitters. The identification of neurotransmitters thus promises to raise a new generation of questions, in which one is forced to imagine not only the traffic that proceeds down the main lines of neural communication but the large and small means by which those signals are regulated.

ACKNOWLEDGMENTS

I am grateful to the colleagues who have collaborated in the experiments described here: Adelbert Ames III, Carol Livingstone, John Mills, and Shawn Hayden. Supported by National Institutes of Health grant EY 01075 and a Research Career Development Award to the author.

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DOPAMINERGIC MECHANISMS IN THE CARP RETINA: EFFECTS OF DOPAMINE, K^+ AND LIGHT ON CYCLIC AMP SYNTHESIS

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ABSTRACT

The ability of dopamine, other proposed retinal transmitters, depolarizing agents, and light to stimulate adenylate cyclase activity in the carp retina has been examined. In both homogenates and pieces of intact tissue, all of the evidence suggests that a dopamine-sensitive adenylate cyclase is the only neurotransmitter activated adenylate cyclase in the carp retina. Experiments involving the use of several dopaminergic agonist and antagonist drugs indicate that this system possesses similar, if not identical pharmacological properties to those reported in various areas of the mammalian central nervous system. Furthermore, binding studies with ^{3}H-domperidone suggest that all dopamine receptors in the retina are linked to adenylate cyclase, implying that the activation of the retinal dopaminergic neurones increases cyclic AMP levels in all postsynaptic neurones. Depolarizing agents, such as K^+, also appear to increase retinal cyclic AMP levels via dopamine. Evidence is provided that K^+ releases endogenous stores of dopamine through a Co^{2+}-sensitive mechanism. Finally, flashing lights slightly increase cyclic AMP levels in the retina, an effect that is abolished by haloperidol.

KEYWORDS

Retina; neurotransmitters; cyclic nucleotides; depolarizing agents; dopamine-sensitive adenylate cyclase; interplexiform cells; dopamine-receptors.

INTRODUCTION

There is substantial evidence that dopamine functions as a neurotransmitter in the vertebrate retina. For example, it has been shown that the retina contains dopamine, synthesizes and degrades it, and releases it upon light stimulation (see Ehinger, 1976; Kramer, 1976). Furthermore dopamine has been found to have physiological effects on a number of retinal neurones (Ames and Pollen, 1969; Hedden and Dowling, 1978).

Two types of retinal neurones contain dopamine. One type is a subclass of amacrine cell, which makes synapses on other, mainly non-dopaminergic, amacrine cells and their processes in the inner plexiform layer of the retina (Dowling and Ehinger,

1978a). The other type is an interplexiform cell, which makes synapses in both plexiform layers of the retina on horizontal, bipolar and amacrine cells and their processes (Dowling and Ehinger, 1978b). Dopamine-containing interplexiform cells are found only in teleost fish and New World Monkeys. Interplexiform cells in other species appear to use another, as yet unidentified, neurotransmitter (see Boycott and others, 1975; Dowling and Ehinger, 1978b).

In teleost fish, there are abundant synapses made by the dopamine-containing interplexiform cells, and the majority of these contacts are found in the outer plexiform layer on the very large external horizontal cells (Dowling and Ehinger, 1978b). These large post-synaptic neurones are easily penetrated by micropipettes so that the fish retina is a particularly favourable tissue in which to study dopaminergic mechanisms. For example, it has been shown that dopamine applied to the retina transiently depolarizes carp horizontal cells and reduces the amplitude of their light-evoked responses (Hedden and Dowling, 1978).

How dopamine mediates its synaptic effects is unknown, However, it is well established that dopamine potently stimulates adenylate cyclase activity in several areas of the nervous system, including the retina (Brown and Makman, 1972; Kebabian, Petzold and Greengard, 1972; Clement-Cormier and others, 1974; Schorderet, 1977), and it has been proposed that membrane permeability may be altered by raised cyclic AMP levels in neurones (Greengard, 1976). Evidence in support of this idea has been contradictory, however, (Libet, 1979) and what role cyclic AMP may play in neurones remains an enigma (see Greengard, 1978).

As a first step in attempting to understand the mode of action of dopamine in the retina and the possible role of cyclic AMP in retinal function, we have characterized the dopamine-sensitive adenylate cyclase system in the carp retina. Studies on both cell-free homogenates and intact retinae have been carried out, and the effects of dopamine, dopaminergic agonists and antagonists, other neurotransmitters, depolarizing agents, and light-dark regimes have been examined. This report summarises the results of these experiments; a more detailed description of them will be published subsequently.

METHODS AND MATERIALS

Mirror or common carp (Cyprinis carpio), 5-7 inches long, were used for most studies. Following partial (10-30 minute) or complete (12 hours) dark adaptation, animals were decapitated, the eyes enucleated and hemisected and the retinae dissected free from the remaining eyecups. All dissection procedures were performed under dim red light.

Adenylate cyclase activity in homogenates of carp retinae was assayed using the method of Kebabian, Petzold and Greengard (1972). Twenty microlitre aliquots of the homogenate (1 retina/500 µl homogenising buffer) were incubated at 30°for 5 min in the presence of 1mM ATP.

For studies on intact tissue, retinae were mounted on small pieces of filter paper receptor side up and quartered. Each retinal piece was placed in an oxygenated vial and incubated at 27-30°for 5-10 min in 1 ml of Ringers (Wu and Dowling, 1978).

In experiments employing both homogenates of carp retina and intact tissue, incubations were terminated by boiling for 3 min. Following microcentrifugation for 5 min, supernatants were assayed for cyclic AMP content using the method of Brown, Ekins and Albano (1972). Results were calculated as pmol cyclic AMP/mg protein, with protein contents being determined according to the method of Lowry and others (1951).

For radioligand binding studies, membrane preparations from either carp or guinea pig retina or guinea pig striatum were prepared as previously described (Leysen, Gommeren and Laduron, 1978; Creese, Schneider and Snyder, 1977; Fields, Reisine and Yamamura, 1977). Incubation tubes containing 50 µl ^{3}H-domperidone (Janssen Pharmaceutica, specific activity 10 Ci/mmol^{-1}) to give a final concentration of 1nM, 50µl dopamine or distilled water and 900 µl homogenate (equivalent to approximately 5 mg wet weight of original tissue for striatum and 7-10 mg wet weight of original tissue for retina) were incubated for 20 min at 30°C. Membranes were collected by filtration, washed and the bound radioactivity determined using the above methods. Binding of ^{3}H-domperidone (corrected for filter blanks) to retinal or striatal membranes, in the absence or presence of varying concentrations of dopamine, were expressed as fmol bound/mg wet weight tissue.

RESULTS

Adenylate Cyclase Activity in Homogenates

The dopamine-sensitive adenylate cyclase in retinal homogenates appears very similar in its response to adrenergic agonists to that found in homogenates of other brain regions (Kebabian, Petzold and Greengard, 1972; Iversen, 1975). Figure1 shows the effect on adenylate cyclase activity of dopamine, adrenaline, noradrenaline and the specific α-andβ -adrenoreceptor agents, phenylephrine and isoprenaline. The adenylate cyclase is stimulated most potently by dopamine, although both adrenaline and noradrenaline are capable of maximally activating the cyclase if high enough concentrations of these agents are used. The adenylate cyclase activity is half-maximally stimulated with a concentration of 1 µM dopamine, whereas a concentration of 10 µM of adrenaline or noradrenaline is required to achieve the same degree of stimulation.

Three lines of evidence suggest that the stimulations of adenylate cyclase activity in carp homogenates produced by either noradrenaline or adrenaline result from an interaction of these agents with dopamine receptors, rather than with α- or β-adrenoreceptors. First the specific β-and α-adrenoreceptor agents, isoprenaline and phenylephrine respectively, have no detectable effect on cyclase activity in retinal homogenates (Fig. 1). Second, the addition of dopamine and noradrenaline or adrenaline together at concentrations sufficient for each to maximally activate the cyclase do not increase activity in the homogenate. And third, the response to noradrenaline is blocked by low doses of dopamine antagonists.

Other substances known to stimulate adenylate cyclase activity in brain homogenates have also been tested (Iversen, 1975). Those believed to stimulate through the dopamine receptor, such as apomorphine, certain ergot derivatives and ADTN (2-amino-6,7-dihydroxy-tetralin) are highly active. Agents which in other systems are thought to activate cyclase activity through their own specific receptors, such as 5-hydroxytrypt amine (serotonin) and histamine were found to be completely inactive.

An interesting feature of carp retinal homogenates is that they exhibit very low levels of basal adenylate cyclase activity. An average amount of 6.1±0.5 pmol cyclic AMP/mg protein/min was found in unstimulated aliquots of 36 homogenates. When maximally activated with 100 µM dopamine on the other hand, the cyclic AMP levels increased to an average of 41.3± 3.3 pmol/mg protein/min in aliquots of the same homogenates. Thus, in carp retinal homogenates, dopamine causes about a 7-fold increase in adenylate cyclase activity. This is a considerably larger percentage response than is observed in homogenates from other regions of the brain, and indicates that this system is a particularly good one for the study of the action of drugs on dopamine receptors.

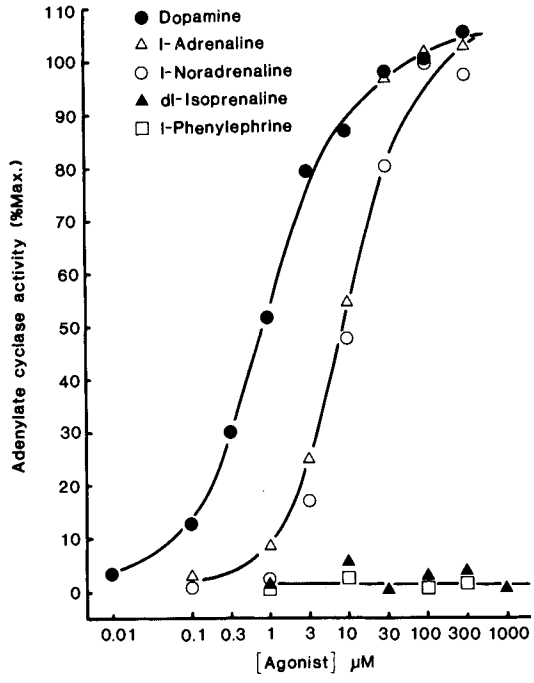

Fig. 1. Effect of adrenergic agonists on adenylate cyclase activity in homogenates of carp retina. Each point is the mean of 2-6 experiments involving quadruplicate determinations. SEM values were less than 10% of the mean. Results are expressed as per cent maximum response elicited by 100 µM dopamine.

The dopamine-sensitive adenylate cyclase in the carp homogenate can be blocked by a variety of agents known to antagonize dopaminergic activity. Of these the phenothiazine derivative, fluphenazine, and the thioxanthine derivatives, α-piflutixol and α-flupenthixol, are the most effective. All other antagonists are less effective in antagonizing the response and Table 1 lists the calculated inhibition constants (Ki) for the agents tested in descending order of potency. These data are very similar to those previously obtained employing homogenates of rat striatum (see Table 1).

Figure 2 shows inhibition curves for a few of the antagonists examined. As already noted α-flupenthixol and fluphenazine are particularly effective. They produce 50% inhibition of the maximum dopamine response at a concentration of 1 µM and almost complete inhibition at 10 µM. Haloperidol and chlorpromazine, on the other hand, are about 10 times less effective at inhibiting the response, while propranolol and phentolamine, β- and α-adrenoreceptor blocking agents, significantly

inhibit the response only at concentrations of 100 μM or more.

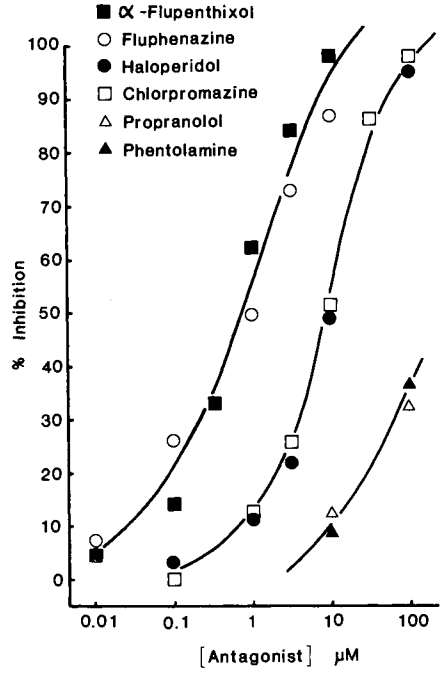

Fig. 2. Inhibition of dopamine-stimulated adenylate cyclase activity in carp retinal homogenates by various antagonists. Each point is the mean of 3 separate experiments involving quadruplicate determinations. SEM values were less than 10% of the mean. Results are expressed as the percentage inhibition of the normal maximum response produced by 100 μM dopamine.

Are all Dopamine Receptors in the Retina Linked to Adenylate Cyclase?

The experiments employing cell-free homogenates indicate that there is an abundant amount of adenylate cyclase in the carp retina that is specifically stimulated by dopamine. This suggests that one effect of dopamine on postsynaptic neurones is to increase cyclic AMP levels. However, there is increasing evidence that at least two types of dopamine receptors exist in many parts of the central nervous system (Cools and van Rossum, 1976; Iversen, 1975). A recent classification suggests that receptor type D1 is linked to adenylate cyclase whereas the other, D2, is not (Kebabian and Calne, 1979). It is possible, therefore, that some, if not all of the neurones postsynaptic to the dopamine-containing neurones may not possess a dopamine-sensitive adenylate cyclase, i.e. they possess D2 receptors.

To approach this question, we have taken advantage of the fact that the D2 receptors are known to have a high affinity for certain butyrophenone derivatives and are labelled by radioligands such as ^{3}H-haloperidol, ^{3}H-spiperone and ^{3}H-domperi-

Drug	Ki(M) Carp retina	Rat striatum
Fluphenazine	3.0×10^{-9}	4.3×10^{-9}
α-Piflutixol	4.5×10^{-9}	
α-Flupenthixol	5.1×10^{-9}	1.0×10^{-9}
(+)-Butaclamol	2.5×10^{-8}	8.8×10^{-9}
Chlorpromazine	7.3×10^{-8}	4.8×10^{-8}
Thioridazine	8.4×10^{-8}	1.3×10^{-7}
Haloperidol	9.4×10^{-8}	9.1×10^{-8}
Pimozide	1.1×10^{-7}	1.4×10^{-7}
Clozapine	1.7×10^{-7}	1.7×10^{-7}
Spiperone	6.1×10^{-7}	9.5×10^{-8}
Phentolamine	$> 1 \times 10^{-6}$	$> 1 \times 10^{-6}$
Propranolol	$> 1 \times 10^{-6}$	$> 1 \times 10^{-6}$
(-)-Butaclamol	$> 1 \times 10^{-6}$	$> 1 \times 10^{-6}$
Domperidone	$> 1 \times 10^{-6}$	$> 1 \times 10^{-6}$

The effect of each drug on dopamine-stimulated adenylate cyclase activity (100 μM) was examined at 6 concentrations in the range 0.01-100 μM. Each point is the mean of 2-4 separate experiments involving quadruplicate determinations. SEM values were less than 10% of the mean. Ki values were calculated from the relationship;

$$Ki = \frac{IC50}{1 + S/Km}$$

when IC50 is the concentration of drug required to produce 50% inhibition of the maximum dopamine-induced response, S is the concentration of dopamine used (100 μM) and Km is the concentration of dopamine producing half-maximal stimulation of adenylate cyclase (1 μM).
$>1 \times 10^{-6}$M = less than 50% inhibition 100 μM. Results for carp retina are compared with previously published data for dopamine-sensitive adenylate cyclase in homogenates of rat striatum (Iversen, 1975 ; Laduron and Leysen, 1979).

For these experiments, retinal homogenates were incubated with ^{3}H-domperidone along with varying concentrations of unlabelled dopamine. The left side of Fig.3 shows the result for retinal homogenates prepared from both carp and guinea pig retinae. Both homogenates bind a small amount of ^{3}H-domperidone but in neither homogenate is

any of the bound drug displaceable with dopamine at concentrations up to 10^{-5}M. This indicates that the binding observed is non-specific and probably represents binding of the ligand to non-receptor sites. A small amount of displacement of the bound drug appears to occur with dopamine concentrations of 10^{-4} and 10^{-3}M, but this displaceable binding is not statistically significant.

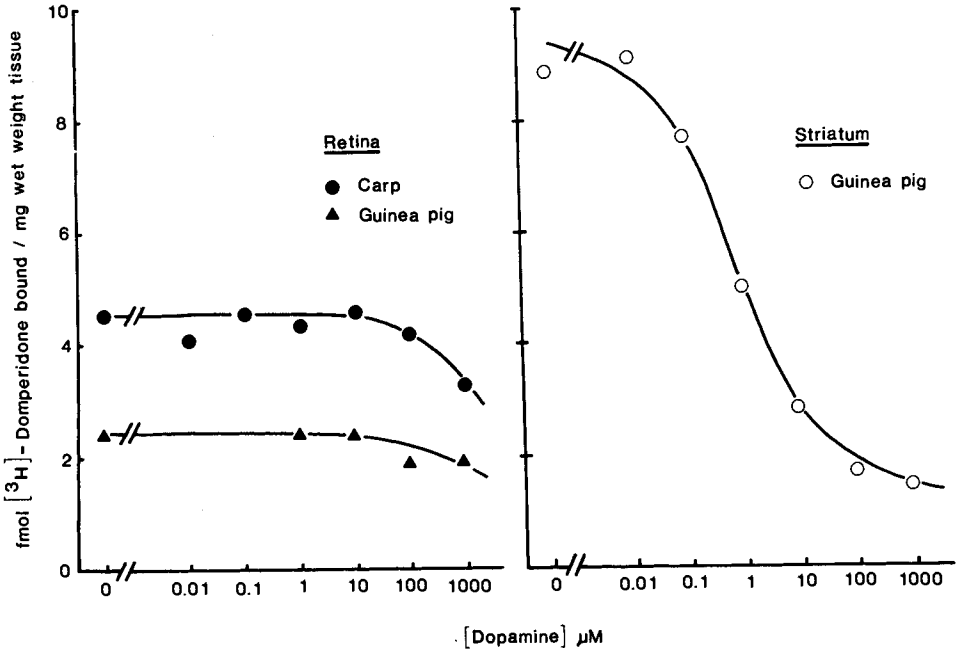

Fig. 3. Effect of dopamine on the binding of ^{3}H-domperidone to guinea pig and carp retinal homogenates and guinea pig striatal homogenates. Binding of ^{3}H-domperidone to striatal or retinal membranes, in the absence or presence of dopamine, is expressed as fmol bound/mg wet weight tissue. Each point represents the mean of 4 experiments involving triplicate determinations. SEM values were less than 15% and 30% of the mean in the striatum and retina respectively.

In contrast, a significant amount of dopamine displaceable binding of ^{3}H-domperidone from membranes of the corpus striatum can be demonstrated readily in similarly prepared homogenates from guinea pig (right side of Fig. 3). In the striatal homogenates there is 2-4 times as much total binding per mg of tissue as compared to the retinal homogenates, and 85-90% of this binding is displaced by dopamine at a concentration of 10^{-3}M. Indeed, a concentration of 10^{-6}M dopamine displaces about 50% of the ^{3}H-domperidone.

These experiments suggest that the retina, in contrast to the striatum and other parts of the central nervous system, does not contain D2 receptors. Thus all dopamine receptors in the retina may be of the D1 type, i.e. linked to adenylate cyclase, which implies that all neurones postsynaptic to the retinal dopaminergic neurones possess dopamine-sensitive adenylate cyclase.

Factors Affecting Cyclic AMP Levels in Intact Retinae

Dopamine Agonists and Antagonists

To study cyclic AMP formation and regulation in the retina under more physiological conditions, a series of experiments were undertaken to examine the effects of various agents on cyclic AMP levels in pieces of retina prepared as though for intracellular recording and maintained in an oxygen-rich environment (see Methods and Wu and Dowling, 1978). The retinae were incubated in oxygenated Ringers to which drugs were added. Recordings from pieces of tissue prepared in this way and incubated for up to 15 minutes in oxygenated Ringers showed that all components of the electroretinogram (ERG) survived and remained in good condition.

When pieces of retina are incubated for 5-10 min in Ringers containing 100 μM dopamine, no changes in cyclic AMP levels are detected. However, if phosphodiesterase inhibitors such as isobutylmethylxanthine (IBMX) or theophylline are added to the incubating Ringers, significant increases in cyclic AMP levels are observed, whose extent depends on the inhibitor used and its concentration. Preliminary experiments indicated that 2mM IBMX appears to inhibit much of the retinal phosphodiesterase and, therefore, to test the effects of various agents on cyclic AMP levels in the intact retina, this agent was routinely added to the Ringers.

Figure 4 shows that under conditions in which retinal phosphodiesterases are inhibited by 2mM IBMX, very large increases in cyclic AMP levels are induced in the retina by dopamine. After 5 min incubation, basal levels are approximately 100 pmol/mg protein but these amounts are increased by about 10-fold to nearly 1000 pmol/mg protein when dopamine at a concentration of 100 μM is added to the Ringers. Half maximal stimulation occurs with a dopamine concentration of about 5 μM, which is somewhat more than that observed in retinal homogenates (1 μM). Noradrenaline also effectively stimulates cyclic AMP levels in pieces of intact retina, but as in homogenates, it is significantly less potent; i.e. larger concentrations of noradrenaline are required to increase cyclic AMP levels to the same extent as dopamine. ADTN and apomorphine are also effective in raising cyclic AMP levels in pieces of carp retina. ADTN is as potent as dopamine and stimulates to the same extent, while apomorphine is somewhat less potent, and maximally stimulates cyclic AMP levels only to about 60 percent that stimulated by 100 μM dopamine. These results are also similar to those obtained with homogenates.

Unlike the situation in homogenates, isoprenaline causes a significant increase in cyclic AMP levels in the intact carp retina (Fig. 4). However, this agent is considerably less potent than either dopamine or noradrenaline, and concentrations of 1 mM isoprenaline increase cyclic AMP levels only to about 35% of that amount induced by 100 μM dopamine. The increase in cyclic AMP levels induced by isoprenaline is antagonized by haloperidol (Fig. 4) but not by propranolol (data not shown) suggesting that this agent is working through the dopamine receptor (see discussion). Phenylephrine on the other hand causes no change in cyclic AMP levels in the intact retina (Fig. 4), a result similar to that observed in homogenates (Fig. 1).

A surprising aspect of the experiments employing the intact carp retinae was the finding that under our standard incubating conditions the increases in cyclic AMP induced by dopamine were poorly antagonized by the agents which potently inhibit dopamine-sensitive adenylate cyclase in homogenates. Fluphenazine, for example, which

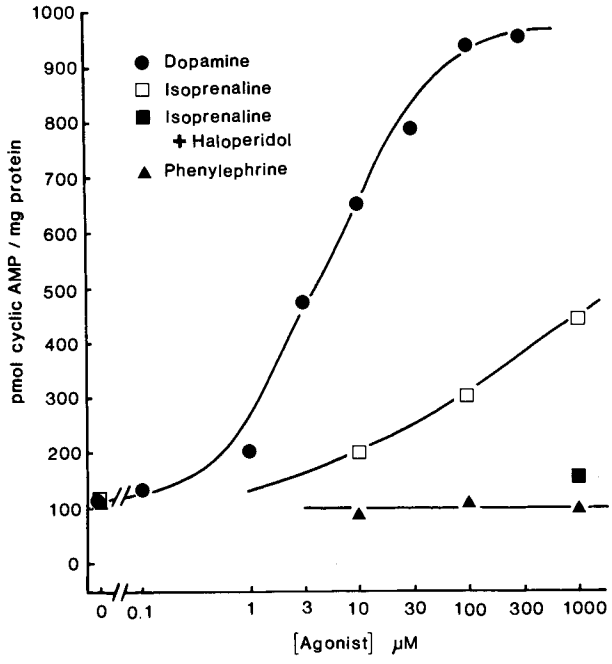

Fig. 4. Effect of dopamine, isoprenaline, isoprenaline plus 100 µM haloperidol, and phenylephrine in pieces of intact carp retinae. Retinae were incubated for 2 min in Ringers containing 2mM IBMX and for an additional 5 min in Ringers containing 2mM IBMX plus drug. These data are taken from representative single experiments involving triplicate determinations. Results are expressed as pmol cyclic AMP/mg protein.

inhibits 50% of cyclic AMP formation in an homogenate at a concentration of 1 µM (Fig. 2), half antagonizes the accumulation of cyclic AMP in the intact retina induced by 100 µM dopamine only at a concentration of 50-100µM (Fig. 5). Chlorpromazine is even less effective, half inhibiting in the intact retina at a concentration of about 300µM (Fig. 5).

For these experiments, the antagonists were routinely applied to the retina 5 min before the dopamine was added. Thus, the retina was exposed to Ringers plus antagonist for 5 min and then to Ringers plus antagonist and dopamine for an additional 5 min. Under such conditions, all inhibitors tested were considerably less effective than in homogenates, but of the agents tried, haloperidol was found to be the most effective antagonist. It caused 50% inhibition of the standard cyclic AMP response induced by 100µM dopamine at a concentration of about 30µM. In the homogenate, on the other hand, about 8µM haloperidol is required to half inhibit the adenylate cyclase. Why haloperidol should be the most effective antagonist in the intact retina is not clear; however, this finding indicates that haloperidol is the antagonist of choice in such experiments. It is of interest to note in this regard that haloperidol is generally found to be the most effective agent in antagonizing dopamine effects in vivo (Janssen and Van Bever, 1978).

If a 2 min preincubation period with haloperidol is used, somewhat larger doses of antagonist are required to inhibit the cyclic AMP accumulation than if a 10 min preincubation is employed (Fig. 5). These are not large differences (~ 20 μM vs 40 μM to achieve half inhibition), but they do suggest that the various antagonists have difficulty in gaining access to synaptic sites in the intact tissue during incubation periods lasting just a few minutes. To test this hypothesis, experiments were carried out employing excessively long preincubation periods. Figure 6 shows results obtained with fluphenazine after preincubation periods of 55 min. In one experiment, the retina was incubated with Ringers and antagonist alone (i.e.

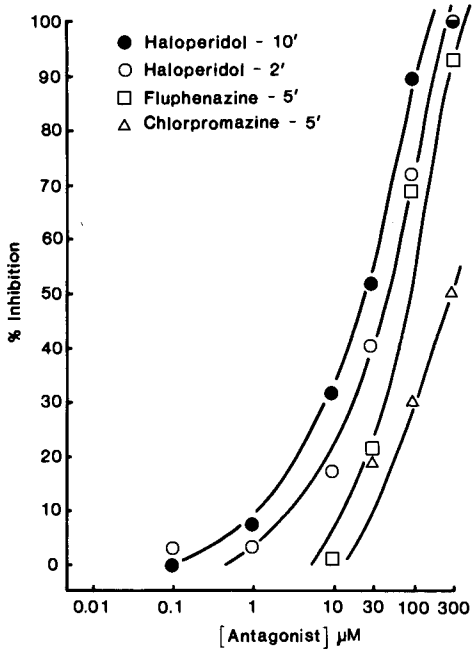

Fig. 5. Inhibition of dopamine-stimulated cyclic AMP production in pieces of intact carp retina. These data are taken from representative single experiments involving triplicate determinations. The retinal pieces were preincubated for 2, 5 or 10 min in Ringers containing antagonist and 2 mM IBMX, and for an additional 5 min in Ringers containing antagonist, 100 μM dopamine, and 2 mM IBMX. Data are expressed as percentage inhibition of those levels of cyclic AMP produced in pieces of retina incubated in 100 μM dopamine and 2 mM IBMX.

no IBMX) for 55 min; in the other, with Ringers alone for 55 min. Following this initial preincubation period, the retinal pieces were incubated in Ringers containing antagonist and IBMX for 5 min and then in Ringers with antagonist, IBMX and dopamine for a final 5 min. It can be seen from Fig. 6 that the long preincubation periods increase substantially the effectiveness of fluphenazine in inhibi

ting the cyclic AMP response. Indeed, following a 55 min preincubation period with fluphenazine, this agent is as effective in the intact retina as in the homogenate (i.e. half inhibition occurs with a concentration of about 1 μM). On the other hand, the cyclic AMP response in retinae incubated for 55 min is virtually identical to that in retinae not preincubated. Preincubation in Ringers alone also enhances the effectiveness of fluphenazine by at least 5-fold. These results suggest that in the intact retina there are barriers to antagonists, which may be broken down by long periods of preincubation. Unfortunately, the carp retina does not tolerate well such long preincubation periods, and, therefore the retina cannot be considered physiologically intact after such treatment.

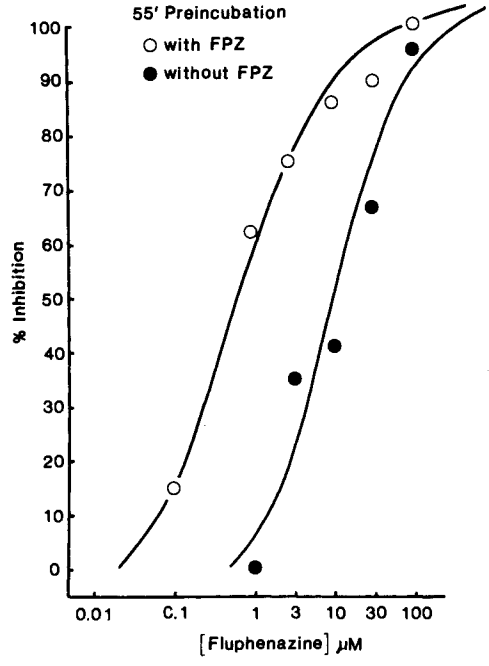

Fig. 6. Inhibition by fluphenazine of dopamine-stimulated cyclic AMP production in pieces of intact carp retina following long term preincubation. The retinal pieces were preincubated in Ringers and fluphenazine for 55 min (open circles) or Ringers alone for 55 min (closed circles). This was followed by a 5 min incubation in Ringers containing fluphenazine and 2 mM IBMX and a final 5 min incubation in Ringers containing fluphenazine, 2 mM IBMX and 100 μM dopamine. Data are expressed as in Fig. 5.

Depolarizing Agents and Other Neurotransmitters

In slice preparations from other parts of the brain (see Daly, 1977) and from various retinae (Brown and Makman, 1972; Wassenaar and Korf, 1976), it has been shown that K^+ ions substantially increase cyclic AMP levels. The same holds true

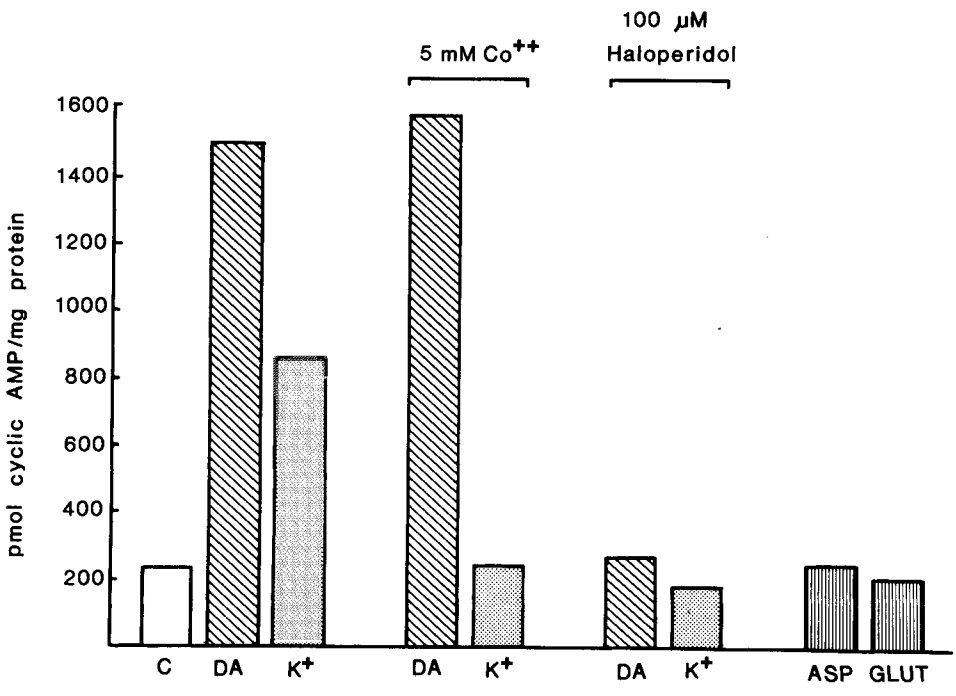

Fig. 7. The effects of 100 μM dopamine, 45 mM K^+ ions, 10 mM aspartate and 10mM glutamate on the production of cyclic AMP in pieces of intact carp retinae, and the effects of 5 mM Co^{2+} and 100 μM haloperidol in antagonizing the responses produced by dopamine and K^+ ions. The retinal pieces were preincubated for 2 min in Ringers containing 2 mM IBMX, or Ringers containing 2 mM IBMX plus 5 mM Co^{2+} or 100 μM haloperidol; and for an additional 10 min in the same Ringers plus stimulating agent. The results are expressed as pmol cyclic AMP/mg protein. The control (C), dopamine (DA) and K^+ response without antagonists are averages from 18, 12 and 17 experiments respectively. SEM values for these experiments were less than 10% of the mean (see text). Four experiments employing Co^{2+} were averaged to give the data shown in this figure, and two experiments employing haloperidol. The responses to aspartate and glutamate represent the averages of two experiments. In all cases triplicate determinations of cyclic AMP were carried out.

for the intact carp retina. Figure 7 shows that following a 10 min incubation in 45 mM K^+, cyclic AMP levels in the retina are increased by about 4-fold. For this increase in cyclic AMP to occur, however, a phosphodiesterase inhibitor must be present in the Ringers. In the experiments shown in Fig. 7, 2 mM IBMX was included in the incubating Ringers. Under these conditions of 10 min incubations in

Ringers with 2mM IBMX added, basal levels of cyclic AMP were on average 237 ± 19 pmol cyclic AMP/mg protein (n = 18 samples). Following stimulation with 100 μM dopamine (n = 12), cyclic AMP levels rose to an average of 1499 ± 93 pmol cyclic AMP/mg protein, and with 45mM K^+, average cyclic AMP levels were 861 ± 72 pmol cyclic AMP/ mg protein (n = 17). Thus, 45mM K^+ increased cyclic AMP levels to about 60 percent of the level to which 100μM dopamine raised them. With lower concentrations of K^+ ion, less stimulation was found; however higher concentrations of K^+ did not consistently increase cyclic AMP levels above those produced by 45mM K^+.

The mechanism by which K^+ increases cyclic AMP content in neural tissues has not been resolved (Daly, 1977; Wassenaar and Korf, 1976). However, it has been proposed that K^+ may be releasing a factor from cells that in turn stimulates adenylate cyclase (Rech and Kalix, 1975; Daly, 1977). To test the idea that in the retina this agent might be dopamine released from the dopamine-containing cells and their terminals by the depolarizing action of K^+, two kinds of experiments were undertaken. First, the release of transmitter agents from presynaptic terminals requires Ca^{2+}, and this Ca^{2+}-dependent release is antagonized by certain divalent cations such as Mg^{2+} or Co^{2+} (Del Castillo and Katz, 1954; Katz and Miledi, 1967). Thus pieces of retina were incubated with 5mM Co^{2+} along with dopamine or K^+. Figure 7 shows that Co^{2+} had no effect on the dopamine-stimulated increase of cyclic AMP levels but completely inhibited the increase of cyclic AMP induced by K^+.

In the second type of experiment, retinae were incubated with 100μM haloperidol along with 100μM dopamine and 45mM K^+. Figure 7 shows that, as expected, this concentration of haloperidol almost completely inhibited the dopamine-stimulated increase of cyclic AMP, and that it also completely antagonized the K^+ mediated increase of cyclic AMP. Both of these experiments therefore support the idea that K^+ exerts its effect by releasing endogenous stores of dopamine within the retina.

It might be supposed that depolarization of neurones can increase their cyclic AMP levels, and that this is one possible way depolarizing agents and transmitter substances may influence cyclic nucleotide concentrations in postsynaptic cells. This is unlikely to be the case in the retina because aspartate and glutamate, at concentrations up to 10mM, have no significant effect on retinal cyclic AMP levels (Fig. 7). Both of these agents are known to substantially depolarize horizontal cells in the retina, and, as noted above, the horizontal cells in the carp retina are richly innervated by the dopamine-containing interplexiform cells.

We have in addition tested a number of other transmitter agents or agonists on pieces of intact carp retinae, including serotonin (5-hydroxytryptamine), carbachol, substance P, GABA (γ-aminobutyric acid), glycine, and a metabolically stable enkephalin analogue (2-D-ala, 5-D-leu-enkephalin). None of these substances significantly increased cyclic AMP levels. The only substance tried which was effective in raising cyclic AMP levels was veratridine, which depolarizes neurones by opening Na^+ channels in cell membranes (Ulbricht, 1969). The retinal response to veratridine (but not to K^+) was entirely abolished by TTX (tetrodotoxin), providing evidence that veratridine acts in the retina as it does in other systems and most likely exerts its effect by depolarizing the dopamine-containing neurones, causing the release of dopamine.

Light and dark. The dopamine-containing neurones of the retina receive synaptic

input in the inner plexiform layer from amacrine cells (Dowling and Ehinger, 1978a, b). The amacrine cells are driven by bipolar cells which in turn connect with the photoreceptors. It is likely, therefore, that light and/or dark modulates the activity of these neurones, and that dopamine synaptically released from these cells influences the level of cyclic AMP in postsynaptic neurones.

Experiments to test the effects of light and dark on dopamine-mediated changes in retinal cyclic AMP levels are complicated by two factors, however. First we have shown above that to demonstrate increases in cyclic AMP by dopamine or any other agent, a phosphodiesterase inhibitor must be introduced into the retina. However, we have also found that 2 mM IBMX appears to disrupt synaptic transmission in the carp retina (i.e. the b-wave of the ERG is lost after 5 min of incubation in Ringers containing this agent), and therefore retinas incubated in 2 mM IBMX do not show dopamine-mediated light-dark differences. To surmount this difficulty we have tried other phosphodiesterase inhibitors and have found that the b-wave of the ERG will survive up to 10 min incubation in Ringers containing 10 mM theophylline. This phosphodiesterase inhibitor is much less effective than is IBMX, but significant increases in cyclic AMP levels do occur upon dopamine-stimulation in the presence of this agent. Thus retinae tested for light-dark differences were in all cases incubated in Ringers containing 10 mM theophylline.

The second complicating factor is that there are substantial levels of cyclic AMP in the vertebrate photoreceptors, although the major cyclic nucleotide within receptors is cyclic GMP (Orr and others, 1976; DeVries and others, 1978). Light activates phosphodiesterases in the photoreceptors which results in a precipitous fall of cyclic nucleotide levels (Miki and others, 1973). Thus, changes in cyclic AMP levels occur in the photoreceptors during light and dark adaptation and these changes must be distinguished from those attributable to the effects of light-and dark on the dopamine-releasing neurones. Figure 8 shows the results of experiments designed to do this.

Earlier work suggested that the interplexiform cells in carp are best stimulated by flashing lights and that the interplexiform cell system relates more to cone receptors than to rods (Hedden and Dowling, 1978). Therefore, relatively bright (~ 2 log units above b-wave threshold) flashing (1 Hz) and red light (> 620 nM) was used which, it was hoped, would stimulate selectively interplexiform cell activity. This was compared to the effects of continuous red light of the same intensity on retinal pieces which is more likely to activate the receptors preferentially.

Small, but statistically significant changes in cyclic AMP levels were observed after exposure of incubated retinas to 7 min of these lighting regimes. Variability in cyclic AMP levels in individual retinae were found to be higher than the changes induced by light, and to combat this factor, all experiments were carried out with paired pieces from the same retina. One piece was left in darkness, the other exposed to light.

Cyclic AMP levels increased by 24.2 $\pm$ 6.1% in those pieces of retina exposed to flashing light (n = 25) as compared to their equivalent pieces left in darkness. This change is highly significant ($P = <.001$, paired sample t-test). On the other hand, pieces of retina (n = 10) exposed to continuous red light showed a decline in cyclic AMP content of 11.5 $\pm$ 5.4% as compared to equivalent pieces left in darkness. This change too is statistically significant ($P= <0.25$).

It might be supposed that the increase in cyclic AMP content induced by flickering light reflects changes mediated by the dopamine-containing cells, whereas the decrease in cyclic AMP content observed after continuous light exposure reflects a

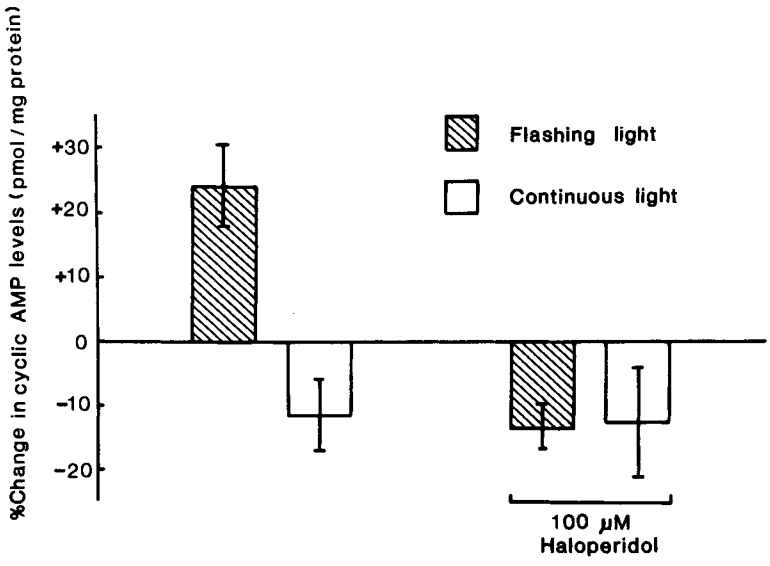

Fig.8. Effects of flashing and continuous light on cyclic AMP levels in intact pieces of carp retina in the absence or presence of 100 µM haloperidol. Equivalent pieces of retina from the same eye were incubated in Ringers containing 10 mM theophylline and left in darkness or exposed to flashing red light or continuous red light for 7 min. These data are expressed as percent change in cyclic AMP production with respect to darkness levels. See text for further details and statistical analysis.

decline of cyclic AMP in the receptors. To test this, in 8 experiments retinal pieces were exposed to flashing and continuous illumination in Ringers containing 100 µM haloperidol. Under these conditions a decline of cyclic AMP levels was observed in retinas exposed to either light regime. Those exposed to flashing light showed a decline of 13.3 $\pm$ 3.6% whereas those exposed to continuous light showed a decline of 12.4 $\pm$ 8.5%. These results were significant at the $P = <.002$ and $P = <.01$ level respectively, and provide evidence that the increase in cyclic AMP level in the retina following stimulation with flashing light results from activation of the dopamine-containing cells, whereas the decline of cyclic AMP levels following stimulation with steady light reflects the decrease of cyclic nucleotide levels in the photoreceptors upon illumination.

DISCUSSION

The experiments described here indicate that there is abundant amount of dopamine-sensitive adenylate cyclase present in the carp retina. Furthermore, the lack of ^{3}H-domperidone binding to retinal membranes suggests that all dopamine receptors in

the retina are linked to adenylate cyclase, i.e. they are of the D1 type. This implies that all neurones postsynaptic to the dopamine-containing cells are endowed with adenylate-cyclase sensitive to dopamine, and in carp this includes the horizontal, bipolar, and amacrine cells. Thus, activation of the dopamine-containing cells presumably leads to increases in cyclic AMP levels in these postsynaptic neurones, and our experiments on the effects of light on retinal cyclic AMP levels suggest that flickering light is one activating stimulus.

We have found no evidence for another neurotransmitter-sensitive adenylate cyclase in the retina. Most neurotransmitter substances tested such as serotonin, aspartate, glutamate, carbachol, substance P, GABA, glycine, an enkephalin analogue and histamine are totally ineffective in stimulating adenylate cyclase activity in homogenates or raising cyclic AMP levels in intact pieces of retina. On the other hand those substances that do activate adenylate cyclase in homogenates and/or increase cyclic AMP levels in the retina, including adrenaline, noradrenaline, ADTN, apomorphine and isoprenaline appear to do so by interacting with the dopamine receptors. Evidence in support of this notion comes from the finding that dopamine-antagonists selectively block the stimulating effect of these agents, while their action is only weakly antagonized by other antagonists such as those that block α- and β- adrenoreceptors. The activity of isoprenaline in this regard is particularly intriguing, in that it significantly activates adenylate cyclase in intact retinal pieces, but not in homogenates. It is effectively blocked in the intact retina by haloperidol (Fig.4), but not by propranolol, indicating that it acts on the dopamine receptor in the intact retina. Why it apparently does not interact with the dopamine receptor in homogenates is not clear, but suggests, perhaps, that the dopamine receptors are somewhat modified in the homogenate.

The depolarizing agents K^+ and veratridine also appear to increase cyclic AMP levels in the retina via dopamine. They do this, presumably, by depolarizing the dopamine-containing cells which results in a release of dopamine from presynaptic terminals. Our evidence for this notion is particularly strong in the case of K^+, where we have shown that Co^{2+}, which inhibits the release of substances from presynaptic terminals, and haloperidol, which inhibits dopamine action, both effectively block the K^+ mediated increase of cyclic AMP. The maximum stimulatory effect of K^+ is considerably less than that obtained with dopamine, and by comparing the relative effectiveness of the two agents it is possible to estimate the concentration of dopamine presumably released in the retina by K^+. Since 45 mM K^+ increases cyclic AMP levels only to about 60% of those levels induced by 100 μM dopamine, this suggests that K^+ maximally releases the equivalent of 10 μM dopamine from endogenous stores.

The low levels of basal activity in carp retinal homogenates may also suggest that the retina possesses relatively few types of adenylate cyclase and that the dopamine-sensitive cyclase represents the only neurotransmitter activated one. That dopamine alone, of all the neurotransmitter agents proposed to play a role in the retina is capable of activating adenylate cyclase in retinal cells suggests a unique physiological function for the dopaminergic neurones.

The changes in cyclic AMP levels observed in retinas exposed to light were found to be very small, and this might suggest that illumination has little effect on dopamine-mediated cyclic AMP changes. However, there are a number of reasons why the measured changes are so small. First, even though synaptic activity in the retina does appear to survive in retinae incubated in Ringers containing 10 mM theophylline, the ERG is not normal. The a-wave (representing mainly receptor activity) is greatly increased in amplitude, while the b-wave (representing activity postsynaptic to receptors) is smaller in amplitude and of somewhat slower dura-

tion. Thus the retina under these conditions is clearly compromised, and the neurones postsynaptic to receptors may not be fully activated upon light stimulation. A second reason the changes are small is that theophylline is not the most effective phosphodiesterase inhibitor. Third, the lighting regimes used may not be ideal, and other rates of flicker, flash colour and flash intensity may be more effective in stimulating selectively the dopamine-containing cells. Fourth, about 20% of the retinal pieces showed no light-evoked changes compared to their equivalent dark-kept pieces due, perhaps, to damage incurred during the dissection or to the use of a retina from an unhealthy animal. These negative results were included in the sample and reduced the average percent increase observed in the retinae showing differences upon light exposure. And finally, there appear to be antagonistic effects of light on cyclic AMP levels in receptors and in the neurones influenced by the dopamine-containing neurones. Thus, the appropriate comparison to make is perhaps not between light-and dark-exposed retinae, but between the retinae exposed to flickering and steady light. If this is done, the increase in cyclic AMP content in retinas exposed to flashing lights as compared to those maintained in steady light is close to 50%. It seems likely, therefore, that in the in vivo, healthy retina the changes in cyclic AMP levels that are induced by light in neurones postsynaptic to the dopamine cells are considerably larger than our results suggest and these changes may have important effects on the postsynaptic cells.

Finally, our observation that a phosphodiesterase inhibitor must be present for dopamine or any other agent to increase cyclic AMP levels in the retina suggests that there is probably an abundant amount of phosphodiesterase present in the system. On the other hand, with 2 mM of the phosphodiesterase inhibitor IBMX present, extraordinarily large amounts of cyclic AMP accumulate upon dopamine stimulation. For example, over a 5 minute incubation period cyclic AMP levels rise in the retina from just over 100 pmol/mg protein to nearly 1000 pmol/mg protein (Fig. 4). Thus, under these conditions cyclic AMP accumulates at the rate of about 180 pmol/mg protein/min. This also is probably not the maximal rate because it is unlikely that 2 mM IBMX inhibits all of the phosphodiesterase in the retina.

The observations suggest, therefore, that dopamine released from the terminals of the dopamine-containing neurones is likely to cause substantial but very transient increases in cyclic AMP levels in postsynaptic cells. Such alterations in cyclic AMP levels would thus appear to parallel the transient physiological changes that occur in carp retinal neurones when they are exposed to dopamine (Hedden and Dowling, 1978). What the link might be between the physiological and biochemical changes that occur in retinal neurones upon dopamine stimulation is unknown. The carp retina, however, appears to be an ideal system in which to investigate this question.

ACKNOWLEDGEMENT

The authors wish to thank Dr.P. Laduron, Janssen Research Laboratoreis, Béerse Belgium, for the generous gift of ^{3}H-domperidone and Dr. B. Ehinger for helpful comments on the manuscript. K.J. Watling was a Fellow of the Parkinson's Disease Society, London. J.E.Dowling was on leave from Harvard University and a Simon A. Guggenheim Fellow, 1978-79.

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Neurochemistry Vol. 1, pp. 539-550.
Pergamon Press Ltd. 1980. Printed in Great Britain.

THE DISK MEMBRANE AS MODEL FOR AN EXCITABLE MEMBRANE

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ABSTRACT

Excitable membranes appear to be biochemically characterized by the presence of a highly specific receptor protein, which undergoes conformational changes upon stimulation and returns to its original conformation for renewed excitability. Our present knowledge of the disk membrane and its receptor protein rhodopsin is reviewed in the light of this model.

The influence of the membrane environment on rhodopsin has been determined in bovine rod outer segment membranes, in rhodopsin preparations which are depleted of the membrane phospholipids, and in reconstituted phospholipid-rhodopsin vesicles. Except for the absorbance spectrum in the visual region, all properties studied change upon lipid depletion. This process starts when 30% of the phospholipids have been removed. Upon reincorporation of lipid-free rhodopsin into lipid bilayer membranes, all properties are restored to the values observed in the native membranes. Hence, removal of the membrane causes deficiencies in the rhodopsin molecule, which are reversible under appropriate conditions.

With respect to the function of rhodopsin in visual excitation, the photolytic sequence seems to be the most relevant parameter presently available. Experimental evidence indicates a partial unfolding of the protein upon illumination, culminating at the metarhodopsin I to II transition, followed by a refolding, which is largely completed in the free opsin stage. While the unfolding phase of the photolytic process apparently is independent of the microenvironment of the rhodopsin molecule, the refolding phase seems to require embedding in a partially hydrophobic environment which allows sufficient thermal motion, e.g. a semifluid biomembrane.

KEYWORDS

Conformational changes, disk membranes, opsin, photolysis, rhodopsin, thermal motion.

INTRODUCTION

In parallel to the historical development and the methodological possibilities of general biochemistry, neurochemistry has successively paid attention to composition

and metabolic pathways of many nervous tissues, both in health and disease. The more recent trends in biochemistry, focussing on molecular mechanisms, can also be traced in neurochemistry, notably in investigations of the mechanisms involved in cholinergic transmission. Although relatively little is known about the molecular mechanisms typical of sensory cells, it appears possible to formulate a generalized hypothesis (Stryer, 1975).

Sensory cells are, like other nerve cells, characterized by the presence of excitable membranes. From the biochemical point of view, the excitable assemblies in these membranes seem to have a number of properties in common: i) they contain a protein receptor molecule, which is highly specific for a particular stimulus, ii) this protein is an integral component of the excitable membrane; it is an intrinsic membrane protein, iii) the specific stimulus elicits a conformational change in the receptor protein, which generates a secondary signal, iv) this conformational change and the corresponding functional modulation are reversible in order to restore the excitability. This general scheme will be used as a guideline in discussing rhodopsin and the disk membrane.

Probably due to their colour and light sensitivity, the visual pigments, notably the rhodopsins, are so far the only reasonably characterized sensory receptor proteins. The rod outer segment, the rhodopsin containing part of the vertebrate photoreceptor cell for dim vision, contains two types of membranes. It consists of some 500-2000 stacked flat membrane disks, surrounded by an outer (plasma) membrane. The disk membranes are initially formed through invagination of the outer membrane, but the large majority of the disks are separate organelles without direct (membrane) connections to outer membrane or other disks. Although the differences and similarities in structure and composition between these two types of membranes are largely unknown, they probably have different functions. Since the primary event of vision, absorbance of light, takes place on the disk membrane, our discussion will be confined to these membranes. Special attention will be given to the work from our own laboratory and, partly owing to this fact, most data refer to bovine disk membranes and rhodopsin.

GENERAL CHARACTERIZATION OF RHODOPSIN

Rhodopsin is a monomeric chromoprotein, composed of an apoprotein (opsin) and the chromophore 11-cis retinal (Hubbard and Wald, 1952). The single protein chain of the apoprotein consists of about 330 amino acids, with a molecular weight of approximately 37,000 Dalton. Due to the strongly hydrophobic nature of large parts of the molecule, its amino acid sequence is only known in part: a C-terminal sequence of 40 amino acids and a N-terminal sequence of 39 amino acids (Hargrave and others, 1979). Near the N-terminus two small oligosaccharides are attached to asparagine residues (Heller and Lawrence, 1970; Hargrave, 1977).

The chromophore 11-cis retinal is a sterically hindered and very condensed isomer of vitamin A aldehyde. In intact rhodopsin it is covalently bound through its aldehyde group to a specific ε-amino group of a lysine residue in a Schiff base linkage (Bownds, 1967; Fager and others, 1972; de Grip and others, 1973a).

Our current knowledge of the three-dimensional structure of rhodopsin in its natural environment, the disk membrane, rests on indirect evidence. Most investigators assume that a large part of the polypeptide chain is in the α-helix conformation, probably alternated with more randomized domains. A considerable part of native rhodopsin seems inaccessible to (small) hydrophilic reagents. Thus both the Schiff base linkage and the polyene system of 11-cis retinal in situ cannot be modified by hydrophilic reagents, unless the protein structure is first denatured. Similarly, only two of the six sulfhydryl groups of rhodopsin can be modified by water-

soluble reagents (Wald and Brown, 1952; de Grip and others, 1973b), although all six can be reached by the more hydrophobic methylmercuric iodide without gross denaturation, i.e. loss of the typical absorbance spectrum (Daemen and others, 1976).

Historically, the primary identity criterion of rhodopsin has always been its absorbance spectrum. Known since a century (Kühne, 1879), it shows an absorbance maximum in the visual region around 500 nm, which shifts to lower wavelengths upon illumination (Fig. 1). Extensive and elegant studies, notably by Wald, Hubbard and their coworkers (see Wald, 1968), have shown that upon illumination of rhodopsin

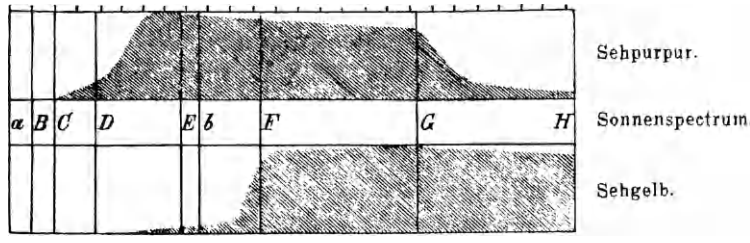

Fig. 1. First absorbance spectrum of (frog) rhodopsin published (Kühne, 1879). "Sehpurpur" is rhodopsin; "Sehgelb" is probably a mixture of metarhodopsins. "Sonnenspectrum" refers to the Fraunhofer lines: C lies at 656 nm, D at 589 nm, E at 530 nm, b at 517 nm, F at 486 nm, G at 430 nm and H at 397 nm.

the 11-cis retinal chromophore is isomerized to the all-trans form. This sets in motion a spontaneous photolysis, resulting in the release of all-trans retinal after a number of spectrally recognizable steps with changing interaction between the chromophore and the apoprotein. This process is referred to as the photolytic sequence of rhodopsin. Under appropriate conditions the apoprotein is able to regenerate rhodopsin when exposed to 11-cis retinal (Wald and Brown, 1956).

As a receptor molecule rhodopsin is highly sensitive as well as specific for light as stimulus. It is fairly stable against denaturing conditions (high temperature, extreme pH). During denaturation nothing can be recognized of the normal photolytic sequence and the chromophore retains (in first instance) its 11-cis configuration, which emphasizes the specificity of the photolytic process.

RHODOPSIN AS A MEMBRANE PROTEIN

Our biochemical knowledge of rhodopsin, the major protein of disk membranes, rests on the fundamental studies of Wald and coworkers, which have been mainly carried out by spectrophotometric methods. As a result rhodopsin and its photolytic intermediates have predominantly been characterized as spectrally defined entities. In fact, the level of our "spectral knowledge" of rhodopsin still exceeds that of our "chemical knowledge", i.e. the spectral data are only very partially understood from a structural point of view. Considering that the other major components of disk membranes, the phospholipids, do not absorb in the visible and near-ultra-violet region, it is understandable that the relation between rhodopsin and the membrane did not receive much attention until the more sophisticated methods of membrane analysis, especially chromatography, became available in the early sixties.

Rhodopsin, either in the membrane or lipid-free, is completely insoluble in aqueous solutions. This serious limitation, e.g. for accurate spectrophotometry, was overcome when Tansley (1931) found that the plant saponin digitonin solubilized rhodopsin with retention of the original absorbance spectrum. The fortunate fact that this very mild detergent did not seriously interfere with the (presently known) properties of rhodopsin in the membrane, did however delay the recognition of the essential role of the membrane. Only after newly developed synthetic detergents like Triton X-100 and cetyltrimethylammonium bromide (CTAB) had become available in the late fifties, the ambiguous nature of detergents for rhodopsin research was realized, since they do not only readily solubilize the pigment, but also may profoundly alter its properties.

Nevertheless, indications for a role of the membrane had been obtained before. Schmidt (1938) observed by polarization microscopy that frog rod outer segments, measured side-on, absorb light polarized perpendicularly to the axis of the rod more extensively than light polarized parallel to this axis. These observations were extended and quantified by other investigators, establishing that rhodopsin in rod outer segments shows linear dichroism when observed side-on, but not when observed end-on. All experiments were consistent with the idea that the chromophore, 11-cis retinal, is predominantly oriented in the plane of the rode disk. This means that the rhodopsin molecule must also be oriented in the photoreceptor membrane. When it was demonstrated more recently that rhodopsin is prone to both rotational (Brown, 1972; Cone, 1972) and lateral movements (Poo and Cone, 1974; Liebman and Entine, 1974) in the disk membrane, the concept of the rhodopsin molecule oriented perpendicularly to the plane of the membrane, was firmly established.

The molecular structure of the disk membrane has been the subject of much controversy. Whereas a classical phospholipid bilayer model, as most recently confirmed by ^{31}P-NMR measurements, has never seriously been doubted, all possible locations of the rhodopsin molecule have been proposed. Present evidence strongly suggests that rhodopsin is a transmembrane protein (Fung and Hubbell, 1978) with the C-terminal end exposed to the cytoplasmic space and the N-terminal end, including the oligosaccharide residues, to the intradiscal space (Röhlich, 1976; Adams and others, 1978; Hargrave and others, 1979). Thus the disk membrane can be viewed as a phospholipid bilayer film, in which the individual rhodopsin molecules are inserted asymmetrically, oriented along an axis perpendicular to the membrane, free to rotate around this axis and to diffuse laterally in the bilayer. The symmetry aspects of the membrane phospholipids, which are highly unsaturated, have been discussed earlier in this volume (Bonting and others, 1979).

In order to study the influence of the membrane environment on the properties of rhodopsin, the phospholipids must be removed in such a manner that the pigment is not drastically denatured. Complete removal of the phospholipids only appears possible in detergents, e.g. by chromatography over hydroxyapatite or affinity chromatography over a concanavalin A column (Hong and Hubbell, 1973; van Breugel and others, 1977). Dialyzable detergents must be used in order to permit their subsequent removal. Alternatively, gradual depletion of up to 85-90% of the phospholipids, without the use of detergents, can be obtained by treatment with phospholipase C (van Breugel and others, 1978). Reconstitution of such delipidated rhodopsin preparations into an artificial membrane system of the desired phospholipid composition is possible by solubilization in detergent, followed by removal of the latter by dialysis (Hong and Hubbell, 1972). Electronmicroscopy shows that the rhodopsin preparations in the delipidated state form two-dimensional aggregates (Olive and others, 1978) and confirms the presence of rhodopsin containing bilayer membranes after reconstitution (Chen and Hubbell, 1973).

Delipidated and reconstituted rhodopsin preparations have been compared with native rhodopsin in disk membranes with respect to some of the properties of the pigment.

The original absorbance spectrum and the sensitivity to light is retained in all these preparations, indicating that the chromophoric centre is not influenced by the microenvironment of the pigment molecule. However, changes in other structural parameters, like the thermal stability and the number of water-exposed sulfhydryl groups per molecule, suggest that removal of the phospholipids causes conformational damage to the molecule (Table 1). At the same time the photolytic sequence and the regeneration capacity of delipidated rhodopsin are affected. Photolysis does not proceed beyond an intermediate, which very much resembles metharhodopsin I (Applebury and others, 1974; van Breugel and others, 1978), and the capacity for regeneration with 11-cis retinal is reduced by 50% (Hong and Hubbell, 1973; van Breugel and others, 1978). When these properties are followed during gradual removal of the phospholipids by phospholipase C, it is noticed that removal of the first 20 of the slightly more than 60 phospholipids present in the disk membrane per molecule of rhodopsin has little effect (Fig. 2). Anyhow, these experiments show that complete or even partial removal of the phospholipids from the disk membrane results in a deficient rhodopsin molecule.

Remarkably, these changes in the rhodopsin molecule are completely reversible

TABLE 1 The Dependence of Rhodopsin Parameters on the Membrane Environment

Rhodopsin parameter \ Rhodopsin preparation	Rod outer segment membranes (62 P/Rh)	Phospholipase C treated ROS membranes (4-6 P/Rh)	Lipid-free rhodopsin (>0.5 P/Rh)	Reconstituted lipid-rhodopsin vesicles (>50 P/Rh)
Absorbance spectrum, light sensitivity	+	+	+	+
Thermal stability ($T_{\frac{1}{2}}$, 10 min)	70°	59°	53°	70°
Water-exposed SH/ rhodopsin	2	4	6	2
Photolytic sequence (Rh→MRI→MRII→MRIII)	+ + +	+ - -	+ - -	+ + +
Regeneration capacity (%)	≡100	52	51	93

Thermal stability expressed as the temperature at which 50% of the rhodopsin is spectrally denatured in 10 min. P/Rh: molar ratio phospholipids/rhodopsin.

upon its reconstitution in a phospholipid bilayer (Hong and Hubbell, 1973; Applebury and others, 1974; van Breugel and others, 1978). The phospholipid composition does not seem to be very critical. Mere addition of phospholipids to delipidated rhodopsin, with or without sonication, which does not lead to its incorporation in a lipid bilayer, is no effective in restoring the properties of the pigment. These experiments show that the changes, induced in rhodopsin by lipid removal, are reversible upon its reincorporation in a lipid bilayer. Most likely the rhodopsin must be (monomerically?) dispersed in the artificial membrane and solvated by the hydrocarbon chains of the membrane lipids, in order to function properly.

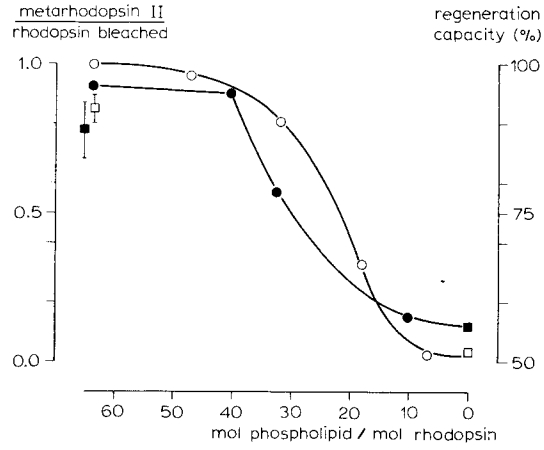

Fig. 2 Dependence of the normal photolytic sequence and the regeneration capacity on the molar ratio: membrane phospholipid/rhodopsin. Gradual lipid depletion (circles) is performed by phospholipase C treatment. Complete lipid removal and reconstitution in a lipid bilayer (squares) are carried out by standard methods. Closed figures refer to the amount of metarhodopsin II formed, as measured at 380 nm one minute after illumination. The amount of bleached rhodopsin is measured at 500 nm after addition of hydroxylamine. Open figures refer to the regeneration capacity. For methodological details, see van Breugel and others (1978).

CONFORMATIONAL CHANGES UPON PHOTOLYSIS OF RHODOPSIN

There is little doubt that conformational changes occur in the rhodopsin molecule after illumination. The cis-trans isomerization of the chromophore represents molecular motion and the spectral changes during the photolytic sequence strongly suggest that this light-induced molecular motion in turn causes conformational changes in the protein part of rhodopsin. The fact that the Schiff base linkage between retinal and the apoprotein becomes accessible to hydrophilic reagents like hydroxylamine and borohydride and that ultimately the chromophore is released leads to the idea that the photolysis of rhodopsin is accompanied by an unfolding of the protein part.

Indications for light-induced protein conformational changes are particularly strong for the transition of metarhodopsin I (λ_{max} = 480 nm) to metarhodopsin II (λ_{max} = 380 nm). First, in addition to the large spectral shift, thermodynamic data suggest a large positive change of entropy of activation in this step (Matthews and others, 1963). Secondly, photoreversal studies (see Williams, 1975) show that the photointermediates through metarhodopsin I instantaneously revert to photopigment with the photoisomerized chromophore, whereas in metarhodopsin II photoreversal is

less efficient and delayed. This suggests that in the latter case substantial protein rearrangement precedes photopigment regeneration. Thirdly, only during the metarhodopsin I to II transition does the Schiff base linkage with the chromophore become accessible. Finally, photolysis of rhodopsin at room temperature is easily blocked at the metarhodopsin I and II transition by a variety of experimental conditions: absence of water (Wald and others, 1950), exhaustive sulfhydryl group modification (Daemen and others, 1976), removal of the membrane phospholipids (Applebury and others, 1974; van Breugel and others, 1978) and glutaraldehyde fixation. The latter observations are most easily explained by assuming that changes in microenvironment or interior of the molecule inhibit the rhodopsin molecule to complete its light-induced conformational changes. The predominant vulnerability of the molecule during the metarhodopsin I to II transition demonstrates that at this stage the most extensive or most specific light-induced conformational changes take place. Since the decay of metarhodopsin I is the last step of rhodopsin photolysis preceding the electrical signal of hyperpolarization of the outer membrane (Hagins, 1972), this suggests that the protein rearrangements during this step represent the secondary signal from rhodopsin stimulation or are closely connected to it.

With respect to the molecular nature of the light-induced conformational changes, little is known with certainty. Older experiments, carried out in detergent solution, can no longer be considered as relevant, in view of the denaturing side-effects in these solutions. Recent evidence, obtained with rhodopsin in its native environment, the membrane, indicates that the light-induced conformational changes are restricted to a comparatively small domain of the molecule in the vicinity of the chromophore binding space (Rafferty and others, 1977; Chabre, 1978; Rafferty, 1979).

REVERSIBILITY OF CONFORMATIONAL CHANGES IN RHODOPSIN

It may seem trivial to state that the conformational changes in rhodopsin following illumination are reversible. The concept of the visual cycle (Dowling, 1960; Wald, 1968) is generally accepted. The classic autoradiographic studies of Young (1976) have shown that rhodopsin has a lifetime of about 10 days in mammals. The fact that rhodopsin after pulse-labeling *in vivo* has a constant specific activity during its lifetime (Hall and others, 1969), implies that the apoprotein can be re-used many times. Studies with tritiated vitamin A (Hall and Bok, 1974) tend to confirm that the chromophore of rhodopsin can be recycled as well, although the mechanism (and even the location) of the reisomerization from the alltrans to the 11-cis configuration remain puzzling (Lion and others, 1975; Bridges, 1977).

The uncertainty with regard to the mechanism of chromophore recycling, of course, brings up the question how the apoprotein of rhodopsin returns to its original conformation, after being exposed to illumination. Two hypotheses can be formulated: i) the conformationally changed (unfolded) apoprotein returns to the original conformation only when 11-cis retinal is offered: the induced-fit model, or ii) the apoprotein returns to its original conformation, when the isomerized chromophore is released, and then simply has to condense with 11-cis retinal: the lock and key model. Strictly speaking, the term opsin, as coined by Wald has a dual meaning: it refers to the apoprotein of intact rhodopsin as well as to the ultimate photolysis product of rhodopsin. The two alternative models can then be rephrased as a single question: to which extent is opsin as an apoprotein conformationally different from or similar to opsin as a photolytic product.

The following evidence is available. First, few differences have so far been observed between membrane suspensions of rhodopsin and opsin by means of thechniques that might be expected to reveal differences in protein structure: circular dichroism (Shichi, 1971; Rafferty and others, 1977), birefringence (Liebman and oth-

ers, 1974), X-ray (Chabre, 1975) and Fourier transform infrared measurements. The sensitivity of rhodopsin and opsin towards proteases and pH changes is very similar. Their conspicuously different stability towards detergents (de Grip and others, 1973b) and temperature (Hubbard, 1958) is easily explained by the mere absence of the chromophore, i.e. the presence of a hydrophobic cleft, in opsin.

Secondly, the ε-amino group of the lysine that bears the chromophore only becomes available to hydrophilic reagents during photolysis, but in opsin this amino group is only slowly attacked by small hydrophilic reagents (de Grip and coworkers, 1973c). This suggests a shielded location of this essential amino group in opsin, as in rhodopsin.

Thirdly, the number of sulfhydryl groups, exposed to the aqueous medium, and their reactivity are very similar in rhodopsin and opsin, whereas clear indications exist that during photolysis more sulfhydryl groups are exposed, which are, in addition, more reactive (Regan and others, 1978; Chen and Hubbell, 1978).

Fourthly, the spontaneous reaction between opsin and 11-cis retinal seems a fairly simple condensation, characterized by an isosbestic point (Wald and Brown, 1956). The likely absence of spectral intermediates in this reaction suggests that 11-cis retinal fits the apoprotein so well, so that no appreciable conformational changes are needed to form rhodopsin. Independent evidence from the effects of structural analogues of 11-cis retinal on the rate of the reaction between opsin with the latter substances supports the idea that post-phospholitic opsin possesses a prefiguration of the chromophore binding space in rhodopsin. It turns out that the competitive effects, seen with short side-chain analogues, decrease and ultimately disappear, when the length of the side-chain surpasses the longest dimension of 11-cis retinal (Daemen, 1978). Therefore the chromophore binding space of opsin seems to be characterized by a hydrophobic binding site for the six-membered ring structure (Matsumoto and Yoshizawa, 1975) and by specific dimensions which exclude the longer analogues.

Thus there is considerable evidence favouring the lock and key model: similarity between pre- and post-photolytic opsin. Summarizing, we propose that rhodopsin upon photolysis undergoes protein-conformational changes, probably peaking at the metarhodopsin I to II transition. Thereupon the molecule spontaneously returns to its original conformation, so that the condensation with 11-cis retinal, which restores the excitability of the visual pigment, can occur according to a lock and key model: 11-cis retinal puts a hydrophobic, excitable seal in the molecule.

FUNCTIONS OF THE DISK MEMBRANE

After having discussed rhodopsin as a receptor protein, the role of the disk membrane deserves attention once again. The classic role of biological membranes is to separate functionally different spaces, respectively to connect them by selective transport facilities. In the disks, the compartmentalization has a very unusual character, inasfar as a structure is formed with a minimal internal volume (at least 100 times smaller than with the disk membrane surrounding a sphere). In an almost complete juxtaposition, the two opposite sites of the membrane approach each other to within 2 nm: a real bis-bilayer arrangement. The narrow internal space must be hydrophilic in view of the inwardly oriented oligosaccharide residues of rhodopsin and the polar head groups of the phospholipids, lining the inward face of the membranes. A simple, though formal calculation, based on the following assumptions - internal width including phospholipid head groups, 3 nm; rhodopsin concentration in outer segments, 3 mM; 62 phospholipids per rhodopsin - shows that the hydrophilic interior of the disk has overall concentrations of about 100 mM oligosaccharide (equivalent to 600 mM "hexose"), 1 M (molar!) phospholipid phos-

phate ions and 1 M phospholipid choline and amino groups. Phosphatidylserine, when evenly distributed over the membrane, contributes 150 mM carboxyl groups, the net negative charge of which may be mainly compensated by calcium ions (Schnetkamp, 1979). Whether a compartment with such high local oligosaccharide and charge densities might still be considered as a normal aqueous space seems doubtful. This may explain why there is little evidence for the presence of selective transmembrane transport systems in the disk membrane.

The bis-bilayer arrangement in each disk and the parallel stacking of all disks (with a repeat distance of 30 nm) yield a structural frame work which serves at least two functions: rhodopsin can be present in high concentration (3 mM; 10% of the wet weight of the outer segment!) and, in addition, the resulting parallel orientation of the rhodopsin molecules increases their effective light absorbance by a factor of 1.5 over that for the case of random orientation.

As we have noticed before, the disk membrane also seems to ensure the structural and functional integrity of rhodopsin. Accepting the normal photolytic sequence as the best criterion for functional integrity (photochemical functionality; O'Brien and others, 1977), we can now attempt to define more specifically the role of the membrane in this process. The photolytic process is blocked at the metarhodopsin I to II transition in the absence of the membrane, more precisely the decay of metarhodopsin I is delayed some orders of magnitude and metarhodopsin II does not occur as an intermediate (Applebury and others, 1974; van Breugel and others, 1978). A simple explanation for this phenomenon could be that the membrane, as in the case of some membrane-bound enzymes, serves as a transport pathway for the in vitro "product" of photolysis, all-trans retinal. It is conceivable that, in the absence of a hydrophobic sink, all-trans retinal has literally no way to go and has to stay on the rhodopsin molecule. A closely related alternative can be imagined if the isomerized chromophore, with or without a hydrophobic part of the protein chain would be extruded from the molecule during the transition to metarhodopsin II. If such an event were prevented by the absence of an appropriate hydrophobic environment, e.g. the membrane, this would explain the blockage at the metarhodopsin I stage.

A second line of reasoning implicates the observation that the decay of metarhodopsin I is also delayed in a bilayer of saturated phospholipids (O'Brien and others, 1977). This suggests that the mobility of the rhodopsin molecules is essential for the last phase of photolysis. In that case, the highly unsaturated, semi-fluid biomembrane might serve the function of lubricant and antiadhesive, assuring the freedom of motion of the rhodopsin molecules and preventing their aggregation. It could mean that thermal motion, both of rhodopsin and the membrane phospholipids, is necessary for the normal photolytic sequence of rhodopsin, namely for the second phase earlier interpreted as the refolding phase of the light-induced conformational changes.

Combining both interpretations on the role of the membrane in rhodopsin photolysis leads to the following hypothesis. While the unfolding phase of the photolytic process is independent of the microenvironment of the rhodopsin molecule, the refolding phase requires embedding in a partly hydrophobic environment which allows sufficient thermal motion, e.g. a semi-fluid biomembrane.

CONCLUSION

We have discussed rhodopsin and the disk membrane from the point of view of neurochemistry. Both the three-dimensional structure of rhodopsin and its photoproducts and the interplay between receptor protein and disk membrane are still poorly characterized. Our present knowledge is fragmentary and a crucial question like the coupling between rhodopsin photolysis and visual excitation is essentially unan-

swered. The efforts of many investigators and a wide variety of experimental approaches will be needed to achieve a more coherent picture.

ACKNOWLEDGEMENTS

The authors are indebted to Prof. Dr. S. L. Bonting for critical comments on the manuscript. The investigations carried out in our laboratory have been supported by the Netherlands Organization for Basic Research (ZWO) through the Netherlands Foundation for Chemical Research (SON).

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Neurochemistry Vol. 1, pp. 557-558.
Pergamon Press Ltd. 1980, Printed in Great Britain.

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Neurochemistry Vol. 1, pp. 559-572.
Pergamon Press Ltd. 1980. Printed in Great Britain.

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