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Foreword

This volume is dedicated to the memory of the late Professor WERNER BRAUN, one of the most devoted and active members of the Editorial Board of the Current Topics in Microbiology and Immunology, who passed away, after suffering a heart attack, in November 1972.

Dr. WERNER BRAUN was born in Berlin, Germany, on November 16, 1914. During his highschool days in Berlin he did research work on problems of genetics as a young guest in the Kaiser-Wilhelm-Institut für Biologie, in the department of Prof. R. GOLDSCHMIDT. I remember his colourful description of his discussions during this period, while still a teen-ager, with OTTO WARBURG. He studied biology and medicine at the University of Göttingen and received a Ph.D. degree in biology in 1936.

In the same year he left Nazi Germany and came to the United States first as a Guest Investigator in Genetics at the University of Michigan at Ann Arbor, and then in Berkeley, where he carried out his work in the Departments of Zoology and of Veterinary Science until 1948. He was engaged during this period in the study of problems concerned with physiological genetics, bacterial variation, immunology and biochemistry.

In July 1948 he became Chief of the Microbial Genetics Branch of the Biological Laboratories in Camp Detrick. In 1955 he was appointed Professor of Microbiology in the Institute of Microbiology at the Rutgers University in New Brunswick, New Jersey, a position he held until his death.

Dr. WERNER BRAUN also served for many years (1957–1968) as a Visiting Professor, University of Puerto Rico Medical School, and held a similar position at the Hebrew University-Hadassah Medical School in Jerusalem during 1962–1963. In 1969–1970 he divided a sabbatical year between my laboratory at the Weizmann Institute of Science in Rehovot, Israel, and the Pasteur Institute in Paris and the Karolinska Institute in Stockholm.

He is the author of over 200 publications in scientific journals, editor of several books, a contributor to textbooks and encyclopedias, and the author of "Bacterial Genetics", first published in 1953 and in its second edition in 1965. He received many awards, including honorary lectureship awards at several leading universities. He served as chairman of the American Academy of Microbiology (1968–1969), as Chairman of the Board of Scientific Counselors of the National Institute of Allergy and Infectious Diseases, National Institutes of Health (1969–1970), and a member of the National Advisory Allergy and Infectious Diseases Council (1970–1971). He also served as a member of several editorial boards and was a consultant to many government agencies and pharmaceutical corporations.

Dr. WERNER BRAUN was a renowned microbial geneticist whose research interests centered on problems of cellular immunology, physiological genetics, microbial genetics, and oncology, with special reference to the nature and regulation of immune responses, control of tumor growth, host-parasite interactions and microbial population changes. During the past few years he applied microbiological approaches with great originality to the study of cellular immunology and cancer. His studies on microbial population changes, especially in the case of *Brucella*, are classical, and led him to the investigation of the nature and regulation of immune response with possible application to the control of the growth of tumors.

WERNER BRAUN had abundant enthusiasm, enjoyed an intelligent and lively conversation, and was a born story-teller, be it an anecdote or a vivid and colourful description of some exciting new laboratory experiments or of the latest scientific achievements in some laboratory he had recently visited. He loved art, and possessed an interesting collection of modern painting, including, i.a., work by several modern Czech and Israeli artists. He had a tremendous personal vitality, and everything he did was done with gusto. He enjoyed life, whether teaching or planning research, travelling for fun or attending conferences.

WERNER BRAUN was an outstanding leader, a great scholar, a successful teacher, a beloved colleague, a devoted friend. He is survived by his wife and lifelong companion, Barbara, and by three daughters. His memory will be cherished by all those who had the opportunity to be exposed to his thoughts, his enthusiasm and his leadership, and this certainly includes his colleagues on the Editorial Board of the Current Topics of Microbiology and Immunology as well as the directors of the publisher, Springer Verlag.

MICHAEL SELA

Influenza Viral Proteins: Identification and Synthesis

DAVID O. WHITE

With 2 Figures

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I. Introduction

So much has been learnt about the proteins of influenza virus during the last three years that most of the gene products can now be tentatively assigned a structural or functional role in the virion or in the infected cell. Indeed, it could be said that influenza vies with reovirus and poliovirus for the privilege of becoming the first mammalian virus to be completely characterized chemically and genetically. In the case of influenza, however, this is more than an exercise in molecular biology, for influenza heads the list of pandemic infectious diseases that still pose a major threat to modern man. A precise understanding of the evolution of novel variants of influenza virus, by antigenic drift or genetic reassortment, is crucial to the development of satisfactory combative measures, whether based on immunization, chemoprophylaxis, chemotherapy, or even ecological control.

The epidemiology and immunology of influenza have been well reviewed (PEREIRA, 1969; WEBSTER and LAVER, 1971) and will not be dealt with in this essay. In addition a number of excellent reviews provide a detailed coverage of what was known up till 1969 of the structure of the influenza virion and its multiplication (ROTT and SCHOLTISSEK, 1967; HOYLE, 1968; ROBINSON and DUESBERG, 1968; SCHOLTISSEK et al., 1969; BARRY and MAHY, 1970; COMPANS and CHOPPIN, 1971; SHATKIN, 1971). The aims of this essay are strictly limited: (1) to bring together the mass of new information that has accumulated during 1970–1972, on the proteins of influenza virus and their intracellular synthesis; (2) to try to extract from these data valid generalizations about the structural and functional role of each virus-coded protein; and (3) to point to areas where uncertainty still exists, to speculate on likely explanations of the findings, and to suggest questions that still need to be answered.

No attempt is made to review the literature on the morphology or multiplication of influenza viruses, nor even to deal comprehensively with papers on influenza proteins published prior to 1969. For background information, the reader is referred to the earlier reviews; only a brief paragraph of orientation will be provided here. The myxovirus (or, more strictly, orthomyxovirus) genus as now defined includes only the viruses of influenza types A, B and C (see FENNER et al., 1973). They are filamentous or spherical enveloped viruses about 80 to 120 nm in diameter, containing a ribonucleoprotein (RNP) "nucleocapsid" generally described as displaying helical symmetry (ALMEIDA and WATERSON, 1967, 1970; ARCHETTI et al., 1967; APOSTOLOV and FLEWETT, 1969; BÄCHI et al., 1969; APOSTOLOV et al., 1970; NERMUT and FRANK, 1971; NERMUT, 1972). An RNA-dependent RNA polymerase (transcriptase) is present (CHOW and SIMPSON, 1971; PENHOET et al., 1971; SKEHEL, 1971b) in close association with the RNP (BISHOP et al., 1972; COMPANS and CALIGUIRI, 1973). Glycoprotein peplomers ("spikes") (DRZENIEK et al., 1968; LAVER and VALENTINE, 1969; TIFFANY and BLOUGH, 1970) of two varieties, hemagglutinin (HA) and neuraminidase (NA), arranged in a hexagonal pattern (ALMEIDA and WATERSON, 1967; ARCHETTI et al., 1967; NERMUT and FRANK, 1971), project

from the lipid of the envelope, the inside of which is lined by a layer of "membrane" protein (M) (APOSTOLOV and FLEWETT, 1969; BÄCHI et al., 1969; COMPANS and DIMMOCK, 1969; KENDAL et al., 1969). The RNA occurs as seven or more separate and unique molecules of single "negative" strands of molecular weight (MW) at least 4.8 million daltons per virion (BLAIR and DUESBERG, 1970; KINGSBURY, 1970; PONS, 1970; BISHOP et al., 1971a; LEWANDOWSKI et al., 1971; SKEHEL, 1971c; YOUNG and CONTENT, 1971; PONS, 1972).

II. The Proteins of the Virion

1. Designation

Virtually all recent work on influenza proteins has utilized the technique of polyacrylamide gel electrophoresis in neutral sodium dodecyl sulfate (MAIZEL, 1971). Direct comparison of results from different laboratories is therefore feasible provided careful note is taken of significant technical differences, notably the presence or absence of reducing conditions. At a recent meeting of the N.I.H. Workshop 1 on Influenza Virus Polypeptides and Antigens (KILBOURNE et al., 1972) informal agreement was reached on a common nomenclature for the various polypeptides now recognized. Table 1 gives the

Table 1. The polypeptides of influenza virus[a]

Designation	Function	MW[b]
P	Polymerase?	90,000
HA	Hemagglutinin	80,000 ↗ HA_1 55,000[c] ↘ HA_2 25,000[c]
NA	Neuraminidase	60,000[c]
NP	Nucleoprotein	60,000
M	Membrane (or matrix) protein	25,000
NS	Nonstructural protein	25,000

[a] Adapted from the report on the N.I.H. Influenza Workshop 1 (KILBOURNE et al., 1972).
[b] Molecular weight in daltons (rounded to nearest 5,000).
[c] Molecular weight of these glycoproteins varies strikingly with the viral strain.

recommended symbols. Table 2 represents an attempt to translate the designations allocated by all authors to the peaks on their electrophoretograms into this new nomenclature. The rationale of this allocation will become apparent below. In virtually every instance the identification of all electrophoretic peaks can be deduced in retrospect, even though it was often not apparent in the light of data available at the time. Despite the impeccable logic of the several sets of symbols employed by the different laboratories, the reader confronted with this baffling array will need little convincing that the time has come when they must all give way to the proposed new nomenclature.

Table 2. Nomenclature of influenza proteins

Authors	P	HA	NA	NP	HA_1	HA_2	M	NS
DIMMOCK and WATSON (1969)								N2
JOSS et al. (1969)				1	2	3	4	4
TAYLOR et al. (1969)		VP1		VP2			VP3	VP3
COMPANS et al. (1970b)	1	2	4	3	5	6	7	
CONTENT and DUESBERG (1970)		3, III	II	2	II	I	1	
HASLAM et al. (1970b)		VP1		VP2			VP3	
SCHULZE (1970, 1972)	VPI	VGP1	VGP2	VPII	VGP3	VGP4	VPIII	
ETCHISON et al. (1971)		C		B			A	A
LAVER (1971)		HA	N	RNP	HA_{slow}	HA_{fast}	IP	
LAZAROWITZ et al. (1971)	1	2	4	3	5	6	7	NNP
SKEHEL and SCHILD (1971)	1, 2		3	5	4	6	7	
STANLEY and HASLAM (1971)		HAU		RNP	HAR_L	HAR_S	IP	
KLENK et al. (1972a)	1		4	2	3	5	6	

2. Molecular Weights

Most of the dozen or so laboratories that have now analyzed the proteins of the influenza virion have calculated their molecular weights by the method described by MAIZEL (1971). These estimates can be no more than approximate because the straight-line relationship between electrophoretic mobility and log MW is not strictly valid for proteins of very high or very low MW, and may

Table 3. Molecular weights of influenza viral polypeptides[a]

Authors	Virus	P	HA	NA	NP	HA_1	HA_2	M	NS
HASLAM et al. (1970b)	A/BEL		77	65	50	65	25	21	
LAVER (1971)	A/BEL		79	58		60	21	24	
SKEHEL and SCHILD (1971)	A/BEL	94/81		72	53	58	28	25	
STANLEY and HASLAM (1971)	A/BEL		79			65	23		
JOSS et al. (1969)	A/FPV				56	49	28	24	
KLENK et al. (1972a)	A/FPV	84		45	60	49	32	26	
SKEHEL (1972)	A/FPV	94/81		45	53	46	29	25	23[b]
ETCHISON et al (1971)	A/NWS		85		62			30	
PONS et al. (1969)	A/WSN				50				
COMPANS et al. (1970b)	A/WSN	84	75	55	60	50	30	26	
CONTENT and DUESBERG (1970)	A/WSN				60				
SCHULZE (1970)	A/WSN	92	78	58	65	52	26	26	
LAZAROWITZ et al. (1971)	A/WSN								25
GREGORIADES (1972)	A/WSN			58					
WEBSTER (1970)	A/X-7			58/58					
BUCHER and KILBOURNE (1972)	A/X-7			66/58					
KENDAL and ECKERT (1972)	A/X-7			50					
HASLAM et al. (1970b)	B/LEE		78	56	63	63	27	21	
LAZDINS et al. (1972)	B/LEE			63					

[a] In thousands of daltons.
[b] Plus a second nonstructural protein of MW 11,000.

be in considerable error for glycoproteins. Nevertheless, it is instructive to bring all the results together (Table 3), and certain obvious generalizations emerge from simple inspection of the collated data.

The proteins fall into three distinct MW groups, as do the viral RNA fragments (DUESBERG, 1968; PONS and HIRST, 1968). The membrane protein, the nonstructural protein, and the smaller of the two HA cleavage products (HA_2) each have a MW of around 20,000–30,000. The second group contains neuraminidase, nucleoprotein and HA_1 with MWs in the 45,000–65,000 range; the individual MWs and even the order in which these three proteins migrate varies with the particular strain involved. For example, the NP of B/LEE had a substantially higher MW than that of A_0/BEL when the two strains were subjected to co-electrophoresis in the same laboratory. The neuraminidase glycoprotein from fowl plague virus (FPV) is very much smaller than the corresponding glycoprotein from other influenza strains. Furthermore, the HA_1 of A_0/BEL is consistently larger than that of A_0/WSN or FPV, suggesting different HA cleavage sites or substantial differences in carbohydrate content. The absence of the uncleaved hemagglutinin glycoprotein from electrophoretograms of FPV, but not other strains, reflects the fact that the extent of proteolytic cleavage of HA $\rightarrow$ HA_1+HA_2 is a function of the viral strain (as well as the cell type in which it is grown); this phenomenon will be discussed below.

3. Number of Molecules in the Virion

From information extractable from gel electrophoretograms of pure virus it is possible to calculate the percentage of virion protein contributed by each polypeptide, thence the molecular ratio, and finally the number of molecules of each protein in a typical influenza virus particle (WHITE et al., 1970). A number of authors have now attempted this exercise from their own data using essentially the same formula, except that the assumed mean particle weight ranges from $2.5–3.2 \times 10^8$ daltons (REIMER et al., 1966; HOYLE, 1968; SCHOLTISSEK et al., 1969) of which 70–75% is protein (FROMMHAGEN et al., 1959; REIMER et al., 1966). Considering the uncertainty of this basic figure upon which all calculations depend, the degree of agreement between the five laboratories is quite remarkable (Tables 4 and 5).

It can be seen that the membrane protein (M) and the hemagglutinin glycoprotein (HA) (with its cleavage products) each contribute about one third of the total mass of virion protein, and nucleoprotein (NP) most of the remainder. Rather surprisingly to some perhaps, neuraminidase (NA) turns out to be a relatively minor constituent of the virion, contributing only about 3–7% of the protein of the strains examined here.

The calculated number of molecules of each protein in the virion must be accepted with caution, despite the apparent concordance between laboratories. Much depends on what one takes to be the diameter of the "typical" influenza

Table 4. Percentage of virion protein contributed by each polypeptide

Authors	Virus	P	HA[a]	NA	NP	M
WHITE et al. (1970)	A/BEL		38		17	27
SKEHEL and SCHILD (1971)	A/BEL	0.5+1.2	33	7	26	33
KLENK et al. (1972a)	A/FPV	1.8	29	7	22	34
SKEHEL (1972)	A/FPV	1.1–4.5	28–35	2.5	29–31	30–35
COMPANS et al. (1970b)	A/WSN	2.5	24	7	24	42
SCHULZE (1972)	A/WSN	1.5	35	3	17	40

[a] On the assumption that $HA = HA_1 + HA_2$, figures for these three proteins have been pooled to construct this column.

Table 5. Number of molecules of each protein in the influenza virion

Authors	Virus	P	HA	NA	NP	M
WHITE et al. (1970)	A/BEL		1,000		700	2,700
SKEHEL and SCHILD (1971)	A/BEL	12+35	940	220	1,170	3,170
KLENK et al. (1972a)	A/FPV	40	900	300	800	3,000
COMPANS et al. (1970b)	A/WSN	52	557	220	703	2,780
SCHULZE (1972)	A/WSN	25	1,106	115	550	3,300

virion. A particle of diameter 120 nm could accommodate over three times as many protein molecules as one of 80 nm. Furthermore, though the modal and the median diameter may be taken to fall within this range, the mean would be significantly higher if one were to include the larger pleomorphic spheres and filaments of fresh human isolates. Virtually all the data in the literature apply, strictly speaking, only to the typical small round particle of laboratory-adapted strains which NERMUT and FRANK (1971) have recently demonstrated by freeze-drying and freeze-etching to be a "plastic icosahedron" of mean diameter 108 nm. Nevertheless, examination of these crude data enables us to draw some useful conclusions about the contribution each protein makes to the virion.

On a molecular basis, M is by far the most abundant polypeptide in the virion; the general estimate of about 3000 molecules is consistent with the number (3400) calculated by SCHULZE (1970) to occupy a shell 60 Å thick and 800 Å in outer diameter, i.e. the dimensions derived by electron microscopy for the outer protein layer of the viral core.

The NP molecules total rather less than 1000, or perhaps slightly more if one considers that a certain amount of incomplete virus is inevitably present in most preparations. Essentially the same answer can be reached from another direction. The best current estimate of the total RNA content of the average infectious virion is 4.8×10^6 daltons (LEWANDOWSKI et al., 1971; SKEHEL, 1971c; BISHOP et al., 1971a), though for reasons that will be discussed later it is felt that the real figure may turn out to be higher. Since purified RNP

contains 10–12% RNA (PONS et al., 1969; KRUG, 1971), it must contain at least 35–43 $\times 10^6$ daltons of protein. Assuming each NP molecule to have a MW of 50,000–65,000 (Table 3), the virion would carry at least 540–860 NP molecules. Certain inferences that can be drawn about the structure of influenza RNP will be reserved for the detailed discussion of that nucleoprotein.

The virion contains about 1000 HA molecules. As two such molecules are required to construct a single peplomer (LAVER, 1971; SHEKEL and SCHILD, 1971; STANLEY and HASLAM, 1971), there are only about 500 HA spikes. TIFFANY and BLOUGH (1970a) measured the dimensions of the peplomers and the distance between them on the surface of A_0/PR8 particles and calculated that an influenza virion of diameter 100 nm carries a total of 550 spikes; an 80 nm virion could accommodate about 300, and a 120 nm virion about 900. As is apparent below, about 90% of the visible peplomers are hemagglutinin. Earlier studies reviewed by WEBSTER et al. (1968) and WEBSTER and LAVER (1971) had indicated a substantially higher number (2000) of binding sites for monospecific anti-HA antibody. This suggests the possibility that each HA spike carries more than one antibody-binding site (and can accommodate at least two IgG molecules simultaneously), but the errors inherent in all the calculations are too great for one to be dogmatic. The nature of the HA antibody-binding site is discussed in a later section.

The 200 or so NA molecules associate as tetramers (BUCHER and KILBOURNE, 1972; KENDAL and ECKERT, 1972; LAZDINS et al., 1972) to form active neuraminidase, hence the average virion of BEL, FPV or WSN carries only about 50 neuraminidase peplomers. Certain less thoroughly studied strains, e.g. A/X-7 (Fl) and B/LEE, which have about twice the normal neuraminidase content (WEBSTER et al., 1968; WEBSTER and LAVER, 1971; KILBOURNE et al., 1972), probably carry up to 100 neuraminidase molecules, but earlier estimates of 500 (WEBSTER et al., 1968) and even 118 (WEBSTER and LAVER, 1971) are clearly too high. It is now apparent that the great majority of peplomers are hemagglutinin.

The function of protein(s) P is completely unknown, but in view of the fact that less than 50 molecules are present in the virion it is hard to imagine that it has a structural role. Speculation about its function is reserved for discussion of the virion transcriptase.

III. The Hemagglutinin

1. Isolation, Purification and Identification of the Peplomer

Hemagglutinin peplomers released from the viral envelope by sodium deoxycholate (LAVER, 1963) or Tween (WEBSTER and DARLINGTON, 1969; KLENK et al., 1972) cannot be completely separated from neuraminidase by rate zonal centrifugation (DRZENIEK et al., 1968; WEBSTER and DARLINGTON, 1969) but have been purified by adsorption to erythrocytes (HASLAM et al., 1970b). Sodium dodecyl sulfate (SDS) denatures both the hemagglutinin and neuraminidase of many influenza strains, but partially SDS-resistant hemag-

glutinin from certain strains (e.g. A_0/BEL) has been separated from all the denatured proteins by electrophoresis in SDS on cellulose acetate strips (LAVER, 1963, 1964). Hemagglutinin peplomers obtained in this way adsorb to red blood cells ("hemadsorbin") but being "monovalent" do not hemagglutinate until the SDS is dialyzed away and the spikes aggregate by their hydrophobic proximal ends (LAVER, 1964; LAVER and VALENTINE, 1969).

Intact hemagglutinin peplomers isolated from the virion by means of SDS or Tween can be clearly visualized by electron microscopy (DRZENIEK et al., 1968; LAVER and VALENTINE, 1969; WEBSTER and DARLINGTON, 1969). Probably the best pictures were obtained by LAVER and VALENTINE (1969) who demonstrated the peplomer to be an oblong spike 140 Å long and 40 Å wide; in the absence of SDS these spikes aggregate to form rosettes. By direct measurement in the electron microscope (LAVER and VALENTINE, 1969) as well as sedimentation coefficient in sucrose gradients (DRZENIEK et al., 1968; WEBSTER and DARLINGTON, 1969) the spikes were calculated to have a MW of 150,000 or slightly higher.

2. Batch Methods for Vaccine Production

Preparative techniques have recently been developed for the purification of hemagglutinin in large batches. These methods can provide enough material for detailed chemical analyses such as automated amino-acid sequencing and could be scaled up for the commercial production of purified hemagglutinin as a nontoxic vaccine. BRAND and SKEHEL (1972) stripped hemagglutinin from X–31 with bromelain, spun off the cores, banded the HA spikes by rate zonal centrifugation to separate them from solubilized and degraded protein, and crystallized the product by vacuum dialysis against distilled water. The purified spikes are immunogenic and antigenic, though they have lost a small segment of each HA_2 molecule. STANLEY et al. (1973a) extracted HA spikes with the anionic detergent, Sarkosyl NL 30, and separated them from neuraminidase by chromatography on DEAE-cellulose columns; the resulting HA yielded pure preparations of HA_1 and HA_2 for chemical analysis following reduction, carboxymethylation, and molecular sieve chromatography on Biogel A5M columns in the presence of $6M$ guanidine hydrochloride. HAYMAN et al. (1973) have devised what may be the most useful method of all. Deoxycholate-disrupted virus is passed through a Sepharose 4B column to which a lectin has been conjugated. The glycoproteins of the viral envelope adhere to the column and are subsequently eluted with methyl-α-D-mannopyranoside.

3. The Hemagglutinin Protein

Acrylamide gel electrophoresis of hemagglutinin purified by erythrocyte-adsorption of peplomers released by deoxycholate established that this peplomer is composed exclusively of a glycoprotein (HA) of MW 77,000 (HASLAM et al., 1970b) which on reduction could be partially or wholly dissociated into

two smaller glycoproteins (HASLAM et al., 1970a). HA glycoprotein purified from SDS-disrupted, egg-grown A_0/BEL by SDS-acrylamide gel electrophoresis in the absence of reducing agents, could be completely dissociated by 2-mercaptoethanol into equimolar amounts of the two smaller glycoproteins, HA_1 and HA_2 (STANLEY and HASLAM, 1971). Virtually identical findings were reported on egg-grown A_0/BEL hemagglutinin purified by cellulose acetate electrophoresis (LAVER, 1971; SKEHEL and SCHILD, 1971). All three laboratories concluded that the hemagglutinin peplomer, which from EM measurements and sedimentation coefficient had been calculated to have a MW of about 150,000 (LAVER and VALENTINE, 1969), is composed of two molecules of HA held together by non-covalent bonds, and that each HA molecule is in turn made up of HA_1 and HA_2 which are firmly joined by disulfide bonds (Fig. 1).

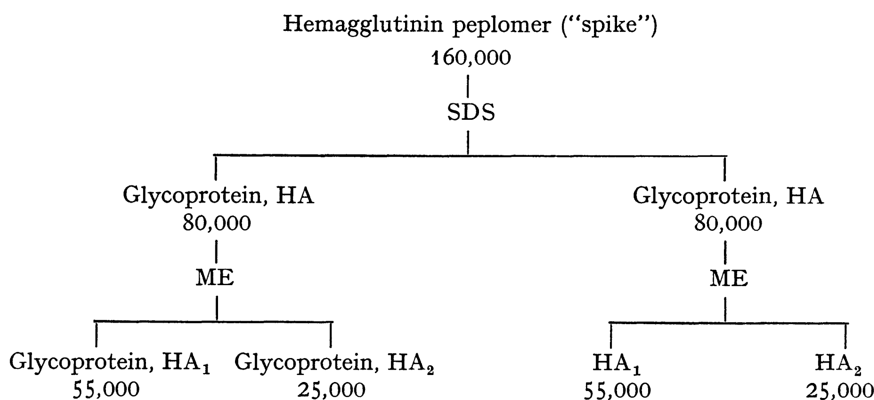

Fig. 1. Structure of influenza viral hemagglutinin

However, it had also been observed that the HA glycoprotein of A_0/BEL grown in calf kidney cells could not be dissociated into HA_1 and HA_2 even by the most stringent reduction conditions (WHITE et al., 1970). The hypothesis was put forward that HA is synthesized as a single molecule and then adopts a tertiary structure involving intramolecular disulfide bonding before being cleaved enzymatically into HA_1 and HA_2 in certain virus-cell systems but not others (STANLEY and HASLAM, 1971). From intracellular pulse-chase experiments in which HA_1 and HA_2 were found to increase in plasma membranes subsequently to HA a similar hypothesis was derived, with the additional important suggestion that the cleavage step may occur in the plasma membrane (LAZAROWITZ et al., 1971). This and subsequent work will be discussed later in the context of viral protein synthesis. Suffice it to say here that cleavage of HA $\rightarrow HA_1+HA_2$ may be biologically irrelevant; virus containing uncleaved HA infects cells and agglutinates erythrocytes normally (STANLEY et al., 1973b).

LAVER (1971) and LAVER and BAKER (1972) have separated HA_1 and HA_2 on a preparative scale by centrifugation on a density gradient containing

guanidine hydrochloride and dithiothreitol and then analyzed their tryptic peptides and amino-acid composition. The peptide maps proved to be absolutely distinct. The only really striking difference in amino-acid content was that the percentage of proline was four to nine times higher in HA_1 than in HA_2. More recent analysis of HA_1 and HA_2 separated by molecular sieve chromatography in the presence of SDS, dithiothreitol and guanidine, indicated a somewhat smaller proline difference (STANLEY et al., 1973a). Nevertheless, the fact that this particular amino-acid is relatively more plentiful in HA_1 than in HA_2 is consistent with the finding from iodination studies using lactoperoxidase that HA_1 has a more tightly folded structure (STANLEY and HASLAM, 1971).

The commonest amino-acids in both HA_1 and HA_2 are aspartic-acid, glutamic-acid, glycine, leucine, serine (and threonine in the case of HA_1) (LAVER and BAKER, 1972; STANLEY et al., 1973a). This is entirely compatible with the fact that both are glycoproteins (LAVER, 1971; STANLEY and HASLAM, 1971); it seems likely that the carbohydrate side chains are attached to the polypeptide via N-glycosidic linkage of N-acetylglucosamine to asparagine, and/or O-glycosidic linkage to serine or threonine.

4. The Carbohydrate

For many years it has been known that influenza virus contains 5–7% by weight of carbohydrate, made up of N-acetylglucosamine 2.6%, mannose 1.3%, galactose 1.3%, and fucose 0.5% (FROMMHAGEN et al., 1959). As might be expected for a virus that carries neuraminidase, influenza carbohydrate has no neuraminic-acid. This has now been demonstrated by electron microscopy following staining with colloidal iron hydroxide at pH 1.75 (KLENK et al., 1970).

LAVER and WEBSTER (1966) showed that most of the viral carbohydrate is indistinguishable from the "host antigen" (HARBOE, 1963) detectable in normal cells of the type in which the virus was grown, and that it is covalently attached hemagglutinin. More recently LAVER (1971) has estimated that the glycoprotein HA_1 contains at least 17% and probably 20% by weight of carbohydrate. Detailed analyses of the sugar content of HA_2 are not yet available, but preliminary estimates of glucosamine alone suggest that this amino sugar is 3–5 times less plentiful in HA_2 than in HA_1 (LAVER, 1971; STANLEY and HASLAM, 1971); the solitary report that carbohydrate is not present in HA_2 (SKEHEL and SCHILD, 1971) was found to be incorrect (BRAND and SKEHEL, 1972).

If one assumes (i) that carbohydrate contributes 6% of the mass of the virion (FROMMHAGEN et al., 1959), (ii) that about two thirds of this is present in glycoprotein and one third in glycolipid (LANDSBERGER et al., 1971), as is the case for certain paramyxoviruses (KLENK and CHOPPIN, 1970), (iii) that about 70–75% of the particle weight is protein (FROMMHAGEN et al., 1959), and (iv) that about 38% of this is glycoprotein (Table 4), it can be calculated

that the glycoproteins of the influenza virion contain approximately 15 % by weight of carbohydrate. About 80 % of this fraction is present in HA_1, which comprises about 60 % of the total glycoprotein of the virus and also contains a substantially higher proportion of carbohydrate than either HA_2 or NA.

Nothing is known of the nature or number of carbohydrate side chains but certain calculations can be made from the analysis by LAVER (1971) of the sugar content of HA_1, which provided the following data: N-acetylglucosamine 9.4 %, mannose 4.8 %, galactose 1.5 %, fucose 1.1 %. The molar ratio of these four sugars is therefore roughly 6:3:1:1. If the terminal sugar is fucose and the proximal sugar N-acetylglucosamine, it is immediately apparent that either there is heterogeneity among the side chains, or glucosamine and probably mannose recur several times. A side chain with this molar ratio would contain about 11 sugars or some multiple of that figure and each HA_1 molecule could carry up to six side chains of MW $\sim$ 2000, or a single huge branched side chain of MW $\sim$ 12,000. In this connection it is interesting that the carbohydrate "host antigen" isolated by LAVER and WEBSTER (1966) from a pronase digest of dehydrated, defatted, boiled, chick-grown A_0/MEL or B/LEE comprised 5 % or less of the dry weight of the virion and had a sedimentation coefficient of 1.9 S, calculated to represent a mass of very roughly 15,000 daltons. This indeed suggests that the carbohydrate of HA_1 may occur as a single giant side chain, but the matter should be reinvestigated with newer techniques because it is very unlikely that this host-antigen preparation was homogeneous; for example, the carbohydrate moiety of HA_2 (total MW 25,000, carbohydrate content about 5 %) could not greatly exceed 1000 daltons. It is entirely possible that the carbohydrate side chains of HA_1, HA_2 and/or NA are heterogeneous and attached to the protein backbone through serine, threonine, aspartic or glutamic acid, which were the four amino-acids present in the "host-antigen" preparation (LAVER and WEBSTER, 1966). Now that methods are available for the large-scale purification of HA and its constituents, HA_1 and HA_2 (LAVER, 1971; STANLEY et al., 1973a), it should be possible to separate and analyse the glycopeptides from each.

5. Attachment of Peplomer to Envelope

The picture has emerged of a hemagglutinin "spike" composed of two non-covalently associated HA molecules, which under certain circumstances may be cleaved into HA_1 and HA_2, but generally remain held together *in situ* by intramolecular disulfide bonds (Fig. 1). There is good evidence that HA_2 is situated at the proximal end of the peplomer in direct association with the lipid of the viral envelope. While *in situ* in the virion, HA_2 is less accessible than HA_1, to radio-iodination by lactoperoxidase (STANLEY and HASLAM, 1971). It is also the most difficult of the glycoproteins to remove from the lipid-coated viral core with proteolytic enzymes (COMPANS et al., 1970b; SCHULZE, 1970, 1972). Bromelain releases HA peplomers which sediment at 9S, behave as monovalent hemadsorbin in antibody-blocking tests, and on dis-

sociation and electrophoretic analysis contain HA_1 and HA_2 except that a small segment (3000 daltons) of HA_2 has been cleaved off (BRAND and SKEHEL, 1972); presumably this is the lipid-associated end of the molecule. Such bromelain-released hemagglutinin spikes will crystallize but do not aggregate via their proximal (hydrophobic) ends to form the usual hemagglutinating rosettes, which once again suggests that the piece of HA_2 that has been lost is involved in the attachment of the spikes to the viral envelope. Intact molecules of HA_2 (but not HA_1) have a marked tendency to aggregate even in the presence of dithiothreitol and guanidine (LAVER, 1971).

The mechanism of attachment of HA_2 to the viral envelope is not known. The most plausible possibility seemed to be that a relatively hydrophobic portion of HA_2 binds to the fatty-acid side chains of envelope lipid (LAVER and VALENTINE, 1969). However, HA_2 is not notably rich in hydrophobic amino-acids (LAVER and BAKER, 1972). An interesting alternative is that the carbohydrate moiety of HA_2 associates with the carbohydrate of the envelope glycolipid (KLENK and CHOPPIN, 1970). KLENK et al. (1972a) have demonstrated the existence of this glycolipid by showing that a phytagglutinin which specifically reacts with N-acetylgalactosamine, the terminal sugar in blood-group-A glycolipid, flocculates the spikeless lipid-coated cores produced by bromelain digestion, whereas concanavalin A flocculates intact virions only. It now appears that the peplomers do not penetrate the lipid to make contact with the membrane protein beneath, as was once proposed (TIFFANY and BLOUGH, 1970b). An esr study of the influenza envelope using three spin labels revealed that the viral lipid is arranged in a 56 Å bilayer of cholesterol and phospholipid in equimolar amounts. The bilayer is not disturbed following removal of the glycoprotein peplomers with protease (LANDSBERGER et al., 1971). Unpublished results obtained by HARRISON and COMPANS with the technique of X-ray diffraction which had shown that the envelope of a togavirus has a deep minimum of electron density at a radius corresponding to the center of the lipid layer, indicating a bilayer organization (HARRISON et al., 1971) also suggested that the lipid of the influenza envelope is not deeply penetrated by the spikes nor by the protein that forms the inner layer of the membrane.

These several findings all point to the fact that the peplomers of the influenza envelope are not deeply embedded in the lipid bilayer but it cannot be concluded that they do not penetrate at all. Comparison of reported electron-microscope measurements of the lengths of isolated peplomers (DRZENIEK et al., 1968; LAVER and VALENTINE, 1969; WEBSTER and DARLINGTON, 1969) with those of peplomers projecting from the virion *in situ* (ALMEIDA and WATERSON, 1967, 1970; APOSTOLOV and FLEWETT, 1969; ARCHETTI et al., 1967; TIFFANY and BLOUGH, 1970a; NERMUT and FRANK, 1971; NERMUT, 1972) indicate an average discrepancy of about 4 nm. The range of estimates (12–22 nm for isolated peplomers, 9–17 nm *in situ*) is so large that the difference may well be an artefact of the preparative procedures, but a careful comparison of spike length in a single strain of virus at various stages of degradation may pay dividends.

6. The Antigenic Site

The central endeavor of influenza research over the last several decades has been and still is the identification of the nature, number and distribution of antigenic sites on influenza hemagglutinin. The history of the debate surrounding the various theories competing to explain the mechanism of antigenic drift has been well reviewed (PEREIRA, 1969; WEBSTER and LAVER, 1971). Recent advances in our knowledge of the chemistry of the hemagglutinin now make it feasible to discuss the nature of the antigenic site in molecular terms.

The genetic information in the virion limits the number of different molecular species of hemagglutinin that any given strain of influenza virus can carry in its envelope. Gel electrophoretograms, insofar as they can resolve molecules of different MW, indicate that the HA glycoproteins of all 500 hemagglutinin peplomers in any given virion are almost certainly identical; there is no "mosaic" of different types of hemagglutinin spikes. Moreover, only a very small number of different antigenic determinants (perhaps only one) could be exposed at the tip of each spike. Consider for a moment the dimensions of an HA peplomer (140 ×40 Å) (LAVER and VALENTINE, 1969) and the amount of space between them (40 Å) (TIFFANY and BLOUGH, 1970a; NERMUT and FRANK, 1971). It is hardly conceivable that an immunoglobulin molecule could make firm contact with any part of the peplomer other than the tip. An HA molecule of MW 80000, 20% of which is carbohydrate, contains about 600 amino-acids of which a few dozen at most could conceivably be exposed on the tip of an oblong spike of dimensions 140 ×40 Å. This would correspond to a very small number of antigenic determinants, perhaps only one. Moreover, the dimensions of the tip of the peplomer would accommodate an IgG molecule almost exactly; indeed there is a striking complementarity in size, shape and substructure between IgG and a hemagglutinin spike.

It has been clearly shown that human strains arising in the field within a few months of one another by antigenic drift rarely differ by more than one or two HA peptides (LAVER and WEBSTER, 1972; WEBSTER and LAVER, 1972). The differences between mutants selected artificially by passage of virus in the presence of sublimiting concentrations of homologous antibody of low avidity are also minimal (LAVER and WEBSTER, 1968). In nature as well as in the laboratory, antibody presumably selects for particular single-step mutants which have the altered amino-acid situated in the key antigenic determinant at the tip of the hemagglutinin peplomer, or sufficiently close to it to influence the folding of that region of the polypeptide. The antigenic change need only be sufficient to confer on the virus a survival advantage in the presence of low titers of IgA (against the wild type) in the respiratory mucus. The antibody may still bind to the altered antigenic site of the new virus but, since the fit will not be good, it will bind with too low an affinity to neutralize it successfully. Neutralization or hemagglutination-inhibition (HI) titrations *in vitro* will reveal a degree of serological overlap between the strains but this need not be interpreted as meaning that the HA peplomers carry a mosaic of additional "older" antigens.

"Original antigenic sin", the phenomenon whereby infection or immunization with a current strain of influenza virus elicits an antibody response directed against an earlier strain of virus that the individual has previously encountered, can be explained in terms of this model. Though the key antigenic determinant on the hemagglutinin of the new strain is demonstrably different from that of older strains, it may be similar enough to bind to sensitized lymphocytes bearing receptors (antibody) for the first antigen and so will trigger an immune response to the original strain. The situation can also be viewed in terms of eliciting a secondary response to a hapten by boosting with the carrier with which the animal was originally primed (MITCHISON, 1971). The "hapten" (i.e. the key antigenic determinant) of the second strain differs from that of the first, but the "carrier" (i.e. the rest of the HA glycoprotein molecule) is essentially unchanged; hence challenge with the recent strain recalls antibodies directed against the earlier one.

Another important question is whether HA_2 makes any contribution to the antibody-binding site(s) at the distal end of the peplomer. The evidence that HA_2 is present at the base of the spike is strong, but it is entirely possible that, despite its relatively small size, HA_2 could also extend to the tip. The amount of ^{125}I incorporated into HA_2 by the high-MW enzyme lactoperoxidase is much less influenced by destruction of the tertiary structure with reducing agents than is the case with HA_1; this suggests that HA_2 may stretch the whole length of the spike whereas HA_1 does not (STANLEY and HASLAM, 1971). LAVER and WEBSTER (1972) have reported "antigenic drift" (amino-acid substitutions) in HA_2 as well as HA_1 from antigenically and temporally closely related strains of A_2. The frequency of change in HA_2 is substantially lower than in HA_1, but then so is the probability of random mutation in a nucleotide sequence less than half as long.

However, the evidence that only HA_1 contributes to the antigenic site is now formidable. Bromelain-derived crystalline hemagglutinin, despite the loss of a small portion of HA_2 exhibits normal immunogenic and antigenic behavior, forming a single precipitin line in gel-diffusion tests with antisera raised against whole virus, purified hemagglutinin, bromelain-released hemagglutinin, or HA_1, but not HA_2 (BRAND and SKEHEL, 1972). Furthermore, ECKERT (1969, 1973) has isolated an immunogenic material which appears to be a dimer of HA_1. Following lipid extraction, virus is dissociated with guanidine (or urea) and 0.01 M dithiothreitol and chromatographed through Sephadex columns. If the eluent contains guanidine, a glycoprotein of MW 40,000 is recovered and dimerizes in the absence of guanidine to form an 80,000 MW molecule. ECKERT calls the dimer HABA (hemagglutinin-binding antigen) because it is capable of adsorbing all the HI antibody from immune sera passed through an immunoaffinity column of HABA protein attached to a cyanogen bromide-treated Sepharose matrix. HABA is also immunogenic, eliciting the formation of HI antibodies when injected into mice. It is not clear what has happened to HA_2 in ECKERT's preparations, but his HABA is almost certainly a dimer of HA_1, even though his estimates of the MW of the monomer (40,000) are considerably lower than those of other workers.

ECKERT's work strongly suggests that HA_2 makes no influential contribution to the antigenic site of the hemagglutinin. However, it does not indicate whether the antigenic site requires the apposition of two HA_1 molecules or whether it is present separately on each HA_1. The finding that free HABA dimers have nearly twice the antibody-binding capacity of the same amount of HABA protein still associated with virions does not necessarily indicate that each free HABA dimer possesses two antibody-binding sites. However, HI antibodies can be raised in rabbits by immunizing them with SDS-treated HA monomers eluted from SDS-acrylamide gels, and the same material forms a precipitin line in immunodiffusion against HI antibodies (SCHILD, 1972; STANLEY et al., 1973a). While one cannot formally exclude the possible reassociation of HA molecules to form peplomers even in the presence of SDS, there is a distinct likelihood that each HA_1 monomer carries a single antigenic determinant and that each peplomer carries two. If so, the two binding sites of an IgG molecule would be confronted with the choice of attaching to the two antigenic sites on a single hemagglutinin spike or of spanning two adjacent spikes.

Let us turn to the carbohydrate. One possibility that has not been widely considered is that the carbohydrate of the HA (and/or NA) influences the conformation of the polypeptide in the region of the antigenic site(s). A single amino-acid change involving cysteine, proline, or any of several others could so alter the folding of the polypeptide that a particular carbohydrate side chain would project in a different direction and play a different role in the antigenic site. Moreover, a mutation involving a serine, threonine or asparagine residue could result in the addition or deletion of a whole carbohydrate side chain. It would be interesting to look specifically for single-step mutations involving these particular amino-acids to see whether they can be correlated with changes in glycopeptide content of HA_1 or HA_2.

Considerable attention has been given to the question of whether the carbohydrate contributes directly to the antigenic site. Certainly antibodies raised against purified chick allantoic "host antigen" (carbohydrate) can inhibit hemagglutination by chick-allantoic-grown virus (LAVER and WEBSTER, 1966). Sera from animals inoculated with carbohydrate from normal duck-embryo allantois, mouse lung, or cultured calf-kidney cells did not inhibit hemagglutination by viruses grown in the homologous (or heterologous) host; possible explanations have been discussed in the literature (WEBSTER and LAVER, 1971). The positive finding does indicate that at least some of the carbohydrate of the HA_1 of some strains of virus grown in some hosts is situated sufficiently close to the tip of the peplomer to be accessible to "anti-host" antibodies. The recent demonstration that influenza virions are precipitable by concanavalin A leads to the same conclusion (BECHT et al., 1972; KLENK et al., 1972a). However, none of this proves that the carbohydrate makes any direct contribution to the antigenic site. The more likely explanation is that the occasional instance of hemagglutination inhibition by antiserum to cellular carbohydrate is attributable to steric hindrance, in much the same way as

anti-NA antibodies (raised against neuraminidase from virus grown in a heterologous host) will sometimes obstruct hemagglutination or neutralize viral infectivity even though both properties are strictly associated with the hemagglutinin (WEBSTER et al., 1968; SCHULMAN and KILBOURNE, 1969; WEBSTER and LAVER, 1971, 1972). Entirely compatible with this view is the finding that the efficiency (titer) of the three types of antibody in blocking hemagglutination decreases in the order anti-HA, anti-"host carbohydrate", anti-neuraminidase. Now that pure carbohydrases are becoming available it should be feasible to digest away the carbohydrate side chains of HA_1 systematically from the fucose termini so as to determine whether they play any role at all in determining the antigenic specificity of the hemagglutinin. Very recently it has been shown that N-acetylglucosaminidase did not affect the hemagglutinating activity of intact egg-grown NDV, nor the neuraminidase of NDV or X-7 (F1), but abolished the hemagglutinating activity of PR8 by releasing sugar from HA_1 (BIKEL and KNIGHT, 1972). Further work will be needed to prove whether the carbohydrate is actually part of the erythrocyte binding site of PR8 hemagglutinin or whether it influences the conformation indirectly, and whether the antibody-binding site (which, of course, is not necessarily identical) is also affected.

It has been clearly established that the active sites responsible for the binding of erythrocytes, infectible cells, hemagglutination-inhibiting (HI) antibodies and neutralizing antibodies are all situated at the distal end of the hemagglutinin peplomer (WEBSTER et al., 1968; LAVER and VALENTINE, 1969; WEBSTER and LAVER, 1971). It has not yet been shown whether all four sites are identical. Competition studies will not answer the question because the relative dimensions of peplomers and even the smallest antibody molecules (IgG) are such that steric hindrance would ensure blockage of access. However, the techniques of iodination already used to study the conformation of the HA molecule and the accessibility of other proteins in the virion (STANLEY and HASLAM, 1971) suggest a means of attacking the problem. Purified virions, isolated peplomers, or purified HA, HA_1 and HA_2 molecules could be labeled with ^{125}I, preferably using the lactoperoxidase technique, before or after attachment of erythrocytes or antibody. The portion of the molecule remaining unlabeled in bound HA (but labeled in unbound HA) would represent the binding site. Mapping of the relevant peptides obtained from HA_1 and HA_2 with e.g. cyanogen bromide, followed by amino-acid sequencing, might well be the most practicable way of determining the key chemical difference between successively emerging strains of influenza.

IV. The Neuraminidase

As neuraminidase is present in normal mammalian cells there was originally some question about whether influenza neuraminidase is virus-coded. All doubts were resolved by the demonstration that the viral enzyme differs from its cellular counterpart in a wide variety of properties including substrate specificity, optimum pH, Michaelis constant, divalent cation requirements,

susceptibility to inhibitors, and most importantly, antigenicity. Then it became apparent that the neuraminidases of different strains of influenza virus differed from one another in a number of other characteristics, including antigenic specificity (for reviews see HOYLE, 1968; PEREIRA, 1969; SCHOLTISSEK et al., 1969; LAVER and WEBSTER, 1971; KILBOURNE et al., 1972). Another notion that enjoyed wide currency until the mid-1960's was that hemagglutinin and neuraminidase activity are associated with the same molecule or the same peplomer (see HOYLE, 1968). Proof of their total separateness came with the demonstration that the two peplomers can be separated and purified by cellulose-acetate electrophoresis (LAVER, 1964), independently exchanged by genetic recombination (LAVER and KILBOURNE, 1966), and differentiated both serologically (DRZENIEK et al., 1966; WEBSTER et al., 1968) and morphologically (DRZENIEK et al., 1968; LAVER and VALENTINE, 1969).

1. Isolation, Purification and Identification

Neuraminidase in enzymatically active form is quite readily released from the viral envelope by detergents, lipid solvents or even proteolytic enzymes, but has proved very difficult to separate from hemagglutinin. There are three reasons for this. Firstly, the two are physically adjacent in the envelope and hence remain associated if the membrane is not totally disrupted (HOYLE, 1968). Secondly, even when properly dispersed as free peplomers, they tend to cosediment in rate zonal centrifugation (DRZENIEK et al., 1968; LAVER and VALENTINE, 1969; WEBSTER and DARLINGTON, 1969) and elute together in molecular sieve chromatography (WEBSTER and DARLINGTON, 1969) because they are of similar size. Thirdly, isolated peplomers of both types tend to aggregate together via their hydrophobic "feet" to form mixed "rosettes" (HOYLE, 1952, 1968; DRZENIEK et al., 1968; LAVER and VALENTINE, 1969; WEBSTER, 1970a) of hemagglutinin and neuraminidase. Such aggregation occurs when ether or other lipid solvents are used to dissolve the envelope, but can be prevented by using trypsin (NOLL et al., 1962), SDS (LAVER, 1963), Tween (WEBSTER and DARLINGTON, 1969), NP40 (GREGORIADES, 1972) or sarkosyl (STANLEY et al., 1973a).

SDS denatures most of the viral proteins, but certain strains (e.g. B/LEE and A_2/Singapore or X-7) have a neuraminidase that is partially resistant to the detergent (LAVER, 1963; LAVER and KILBOURNE, 1966). Hence it is possible to isolate single undenatured neuraminidase peplomers which do not aggregate unless the SDS is dialysed away (DRZENIEK et al., 1968; LAVER and VALENTINE, 1969; LAZDINS et al., 1972). Sarkosyl releases active hemagglutinin and neuraminidase from several influenza strains (STANLEY et al., 1973a) and has the added virtue of not precipitating at 4° C, hence it may turn out in the long run to be a more generally useful detergent than SDS.

Proteolytic enzymes such as trypsin (NOLL et al., 1962; HASLAM et al., 1970b; LAZDINS et al., 1972), nagarse (KENDAL and ECKERT, 1972) and pronase (SETO et al., 1966; ROTT et al., 1972) have also been employed to

liberate active neuraminidase from virions. Trypsin cleaves the stalk of the mushroom-shaped peplomer, leaving most of the structure including the active site intact, but removing the envelope-associated base (LAZDINS et al., 1972) with the consequence that the molecule is no longer capable of aggregation (DRZENIEK et al., 1968; LAVER and VALENTINE, 1969). The fate of the hemagglutinin seems to vary with the experimental conditions. SCHLOER found that under controlled conditions, selective removal of neuraminidase by trypsin does not detectably affect the infectivity, hemagglutinating capacity, buoyant density, sedimentation coefficient, or even the electron-microscopic appearance of the virion (KILBOURNE et al., 1972). SKEHEL and SCHILD (1971) reported that brief treatment with bromelain released (and presumably destroyed) the hemagglutinin of A_0/BEL, leaving half the neuraminidase intact and still attached to the viral core, whereas SCHULZE (1970) found conditions under which the neuraminidase of A_0/WSN was removed or destroyed, and the HA glycoprotein cleaved *in situ* into HA_1 plus HA_2 without affecting the hemagglutinating capacity or the infectivity of the virion. Pronase released active neuraminidase from several strains but destroyed the hemagglutinin of A_0/NWS only. The susceptibility of A_0/NWS hemagglutinin to trypsin has been reported to be inherited in genetic crosses independently of its strain-specific antigenicity (KILBOURNE et al., 1972).

Several methods have been used to purify neuraminidase isolated from the virion by detergents or proteolytic enzymes: rate zonal centrifugation (NOLL et al., 1962), cellulose-acetate electrophoresis (LAVER, 1964), acrylamide-gel electrophoresis (LAZDINS et al., 1972), molecular-sieve chromatography (GREGORIADES, 1972), DEAE-cellulose chromatography (STANLEY et al., 1973a), electrofocusing (GREGORIADES, 1972), immunosorption (STANLEY et al., 1973a), or various combinations of these techniques.

The enzyme has a sedimentation coefficient of 8.5–11 S in sucrose gradients (NOLL et al., 1962; LAVER, 1963; DRZENIEK et al., 1966; LAVER and VALENTINE, 1969; WEBSTER and DARLINGTON, 1969; ROTT et al., 1970; BUCHER and KILBOURNE, 1972) whether the proximal end of the peplomer has been removed by proteolytic enzymes or not. SDS or NP40 may be included in the gradient to prevent aggregation of peplomers derived by SDS or NP40 treatment, respectively (GREGORIADES, 1972; LAZDINS et al., 1972), but can be omitted for trypsin-derived neuraminidase, which cannot aggregate anyway (LAZDINS et al., 1972). All the other viral proteins, including the hemagglutinin from most strains, are solubilized by SDS so that they remain at the top of the gradient (DRZENIEK et al., 1968; LAVER and VALENTINE, 1969; HASLAM et al., 1970b). In the case of A_2 strains with partially SDS-resistant hemagglutinin and neuraminidase, however, the two cosediment (ROTT et al., 1970). Neuraminidase released from the virion with proteolytic enzymes can also be satisfactorily separated by rate zonal centrifugation, but in this case only a small amount of low-MW degraded material remains at the top of the gradient, while what remains of the virus particle sediments rapidly to the bottom (HASLAM et al., 1970b; KENDAL and ECKERT, 1972; LAZDINS et al., 1972).

Cellulose-acetate electrophoresis of SDS-dissociated virus (LAVER, 1963, 1964) clearly separates active neuraminidase from all the other viral proteins of strains such as B/LEE in which the enzyme is partially SDS-resistant; resolution is not possible in the case of those strains in which the hemagglutinin as well as the neuraminidase is partially SDS-resistant e.g. A/X–31 (SKEHEL and SCHILD, 1971). Acrylamide gel electrophoresis has been used as the final step in the purification protocol to obtain highly pure neuraminidase (BUCHER and KILBOURNE, 1972; LAZDINS et al., 1972). The recovery of active enzyme from these SDS gels was far too small for it to be a useful preparative procedure but enough to enable the protein to be analyzed following radio-iodination with ^{125}I (LAZDINS et al., 1972). Chromatography on DEAE-cellulose columns is a far more practicable way of recovering active neuraminidase in large amounts from virus disrupted with SDS (GREGORIADES, 1972) or Sarkosyl NL30 (STANLEY et al., 1973a). This can be followed by electrofocusing and chromatography on Sepharose 2B if highly pure preparations are desired (GREGORIADES, 1972).

The first electron micrographs of isolated peplomers were presented by DRZENIEK et al. (1968) who described the SDS-resistant neuraminidase of A_2/Singapore and B/LEE as a "ring-like" structure 8–9 nm in diameter and demonstrated the polymeric nature of the enzyme. LAVER and VALENTINE (1969) using more highly purified preparations of SDS- resistant A_2 neuraminidase from the recombinant X–7(F1) later obtained pictures which clearly showed a mushroom-shaped molecule consisting of an oblong "head" measuring 8.5 ×5 nm, connected by a thin "stalk" 10 nm long to a smaller "foot" of diameter 4 nm. WEBSTER and DARLINGTON (1969) published virtually identical pictures of the neuraminidase extracted from several A strains using Tween 20 at pH 10, while APOSTOLOV et al. (1970) described the A_2/Singapore enzyme as consisting of a 12 nm fiber with a 5 nm head.

2. One Protein or Two?

Purified preparations of active neuraminidase from several strains of influenza have recently been analyzed by acrylamide gel electrophoresis or exclusion chromatography following dissociation with SDS, reducing agents, and urea or guanidine, and shown to be composed of a fundamental subunit of MW about 60,000 (HASLAM et al., 1970b; WEBSTER, 1970a; BUCHER and KILBOURNE, 1972; GREGORIADES, 1972; KENDAL and ECKERT, 1972; LAZDINS et al., 1972). This monomer is a glycoprotein (KENDAL and ECKERT, 1972; LAZDINS et al., 1972).

There is considerable uncertainty, however, about whether the monomer is a single glycoprotein or a mixture of two species of closely similar MW. Four independent laboratories have reported on the SDS-resistant A_2 neuraminidase from the recombinant A/X–7 (HON2). WEBSTER (1970a) recovered X–7 neuraminidase from SDS-disrupted virus run on cellulose acetate strips, dissociated it with guanidine and dithiothreitol, and passed it through a Biogel

2 % beaded agarose column; the protein of MW 58,000 which he eluted from the column resolved into two bands on analysis by SDS-acrylamide gel electrophoresis. Using the same virus and similar techniques BUCHER and KILBOURNE (1972) found two proteins of MW 58,000 and 66,000, while SKEHEL and SCHILD (1971) and LAVER and BAKER (1972) reported two proteins of MW 70,000 and 80,000 from X-7 neuraminidase isolated from cellulose acetate strips. By contrast, KENDAL and ECKERT (1972) reported only a single protein of MW 50,000 from X-7 (F1) neuraminidase released from the virion with nagarse protease (subtilisin); if nagarse, like trypsin, cleaves about 7000 daltons of glycoprotein from the proximal end of the molecule (LAZDINS et al., 1972) their estimate of 50,000–54,000 for the MW is probably too low and the possible existence of two species of glycoprotein may have been obscured.

By contrast with X-7, only one NA protein has been reported for all other strains of influenza examined. HASLAM et al. (1970b) electrophoretically resolved only a single glycoprotein from the B/LEE neuraminidase released either by SDS or trypsin, as also did LAVER and BAKER (1972) from SDS-disrupted LEE and BEL enzymes. SKEHEL and SCHILD (1971) found only one in NA from A_0/BEL and, unexpectedly, from the recombinant A/X-31, which contains neuraminidase of the same subtype (N2) as the A/X-7 strain in which they reported two. Recently GREGORIADES (1972) has shown that highly purified neuraminidase from WSN also contains only a single species of glycoprotein. The spikes projecting from the envelope of WSN had previously been shown to contain four glycoproteins (COMPANS et al., 1970; SCHULZE, 1970) and, although none of these had been positively identified, it was quite clear from comparison with other work (HASLAM et al., 1970b; LAVER, 1971; SKEHEL and SCHILD, 1971; STANLEY and HASLAM, 1971) that three of them represented HA, HA_1 and HA_2, so that only the one remaining glycoprotein (MW 55,000–58,000) could be assumed to represent NA. The same argument can be applied to fowl plague virus where only three envelope glycoproteins have been described (KLENK et al., 1972a) and two of them represent HA_1 and HA_2 (HA, being completely cleaved in this strain, is not detected at all in virions under reducing conditions).

Taken at face value the data from several laboratories would therefore suggest that the neuraminidase from strain X-7 contains two species of glycoprotein whereas that from other strains (LEE, BEL, WSN) contains only one. It must not be overlooked, however, that an extra NA protein would imply both a radical difference in the structure of the enzyme and an extra cistron in the genome. It may be worth looking at X-7 RNA for an extra molecule in the middle size range, but in the absence of any evidence that the genomes of influenza strains differ in the number of RNA pieces they contain, one should perhaps consider alternative explanations of the data.

Two possibilities suggest themselves. Firstly it is quite conceivable that the neuraminidase of all influenza strains is composed of two distinct species of glycoprotein and that for some strains their MWs are so similar that they cannot readily be resolved by conventional procedures. The second, equally plau-

sible interpretation of the data is that the neuraminidase of all influenza strains contains only a single type of glycoprotein and that the second band reported for some strains is a non-glycosylated (or less glycosylated) form or is derived by proteolytic cleavage from the first. Some recent work points in this direction (LAZDINS et al., 1972). Neuraminidase extracted from B/LEE with SDS and separated from the hemagglutinin and the other viral proteins by rate zonal centrifugation contained two glycoproteins of MW 63,000 and 56,000, but after further purification by acrylamide gel electrophoresis the active enzyme was found to contain only the 63,000 glycoprotein. Neuraminidase extracted from the virion by trypsin, or by SDS followed by trypsin, was found to contain only a single glycoprotein species of MW 56,000. It is pertinent to note that most laboratories describing two NA components find that the amount of the lower-MW molecule (NA_2) varies from experiment to experiment and is usually a relatively minor constituent (WEBSTER, 1970a; SKEHEL and SCHILD, 1971; LAZDINS et al., 1972). BUCHER and KILBOURNE (1972) found NA_1 and NA_2 in approximately equimolar amounts but reported that the NA_2 band was diffuse.

Resolution of this question demands new experimental approaches. Firstly, peptide mapping or amino-acid sequencing would indicate whether NA_2 is derived by proteolytic cleavage of NA_1. Secondly, reconstitution experiments could be designed to demonstrate unequivocally whether the active enzyme requires both NA_1 and NA_2. Such an experiment should be feasible because BUCHER and KILBOURNE (1972) have recovered active enzyme from protein with an estimated MW of 53,000 eluted from a Bio-Gel A-5 column. If their 66,000 and 58,000 monomers could be clearly separated from one another by gel electrophoresis or column chromatography in an equally nondenatured form, it would be important to determine whether functional neuraminidase can be reconstituted from NA_1 alone.

3. The Carbohydrate

Neuraminidase contains carbohydrate (KENDAL and ECKERT, 1972; LAZDINS et al., 1972) but nothing is known about the number or nature of the carbohydrate side chains. It is worth noting, however, that aspartic acid and serine (together with glutamic-acid) are the commonest amino-acid residues in the NA from X-7 (F1), BEL and LEE (KENDAL and ECKERT, 1972; LAVER and BAKER, 1972).

It has been estimated that there are 5.7 glycosamine residues per X-7 (F1) NA molecule of MW 54,000 (KENDAL and ECKERT, 1972). Non-amino sugars were not measured, but if one were to assume, for want of contradictory evidence, that glucosamine comprises about half the total carbohydrate of NA, as it does for HA (LAVER, 1971), then only about 2000 daltons of the NA molecule would be attributable to carbohydrate. This would mean a carbohydrate content of 5 % or so, which is similar to HA_2, but about four times less than HA_1.

However, there is no real reason to assume that the carbohydrate moiety of NA resembles that of HA_1 or HA_2. Though the particular combination of

transferases present in the cell of origin will determine which sugars may be added and in what order, the nature of the "host antigen" may also be influenced by the amino-acid sequence and tertiary structure of the protein to which it is attached. ROTT et al. (1972) have recently presented evidence that concanavalin A selectively precipitates neuraminidase rather than hemagglutinin from a mixture of the two.

A high proportion of the carbohydrate in neuraminidase is associated with the base of the stalk which is removed from the molecule by trypsin (LAZDINS et al., 1972) or pronase (ROTT et al., 1972). This suggests that the peplomer may not adhere to the lipid of the viral envelope via interaction between hydrophobic amino-acids and fatty-acid side chains, as is generally assumed, but rather by association between the carbohydrate of glycoprotein and glycolipid, respectively.

4. Structure of the Enzyme

Estimates of the MW of influenza viral neuraminidase vary from 150,000 to 300,000, with most of the recent figures in the 180,000–270,000 range (SETO et al., 1966; KENDAL et al., 1969; LAVER and VALENTINE, 1969; WEBSTER and DARLINGTON, 1969; APOSTOLOV et al., 1970; ROTT et al., 1970; BUCHER and KILBOURNE, 1972; GREGORIADES, 1972; KENDAL and ECKERT, 1972). Most of these determinations are based on sedimentation coefficients in sucrose gradients. This method tends to underestimate the true MW of a nonspherical molecule such as neuraminidase which will be somewhat retarded by virtue of its "mushroom" shape. Some support for this view comes from the consistent finding that neuraminidase liberated from the virion by trypsin sediments as rapidly as that liberated by SDS, despite the fact that the former has lost the proximal end of its stalk (LAZDINS et al., 1972). The lowest MW estimate (150,000) is based on measurements from electron micrographs (LAVER and VALENTINE, 1969). A value of 180,000 was obtained by Sepharose chromatography (GREGORIADES, 1972). Identification of active enzyme on SDS-acrylamide gels by color reaction with the chromogenic substrate 2-(3′-methoxyphenyl)-N-acetyl-α-neuraminic-acid gave a figure of 220,000–250,000 (BUCHER and KILBOURNE, 1972).

Since the active neuraminidase molecule of influenza A/X-7 or B/LEE has a MW averaging just over 200,000, and the individual glycoprotein molecules of which the enzyme from those strains is composed have a MW of around 60,000, it has been calculated that the peplomer is probably a tetramer (BUCHER and KILBOURNE, 1972; KENDAL and ECKERT, 1972; LAZDINS et al., 1972). Disulfide bonds link the monomers firmly into pairs which in turn associate with another pair, mainly by non-covalent bonds (LAZDINS et al., 1972). Strong reducing conditions are needed to dissociate the enzyme into its 60,000 MW subunits (BUCHER and KILBOURNE, 1972; LAZDINS et al., 1972). The native enzyme has about 85 cysteine residues, none of which is available as a free sulfhydryl group for carboxamidomethylation by ^{14}C-iodoacetamide (KENDAL and ECKERT, 1972).

As will be seen from Fig. 2, the substructure of the molecule bears a striking resemblance to that of hemagglutinin. The analogy would be even closer if the two disulfide-linked NA molecules are in fact distinct species, NA_1 and NA_2.

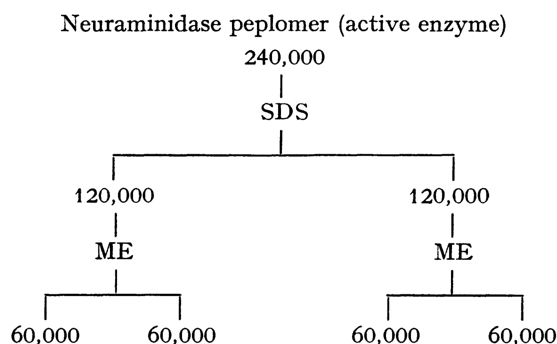

Fig. 2. Structure of influenza viral neuraminidase

V. The Membrane Protein

Perhaps the most unexpected finding to emerge from the first gel electrophoretograms of purified influenza virus was the existence of a major new protein of MW 20,000–25,000 comprising about one third of the total mass of the virion protein and by far the most plentiful molecule in the virion, but quite distinct from all three of the known antigens, hemagglutinin, neuraminidase and ribonucleoprotein (HASLAM et al., 1970a, b). From the fact that this protein was readily released from the viral RNP by deoxycholate it was postulated to form a layer situated between the RNP and the spikes of the envelope. Such a layer had been visualized in electron micrographs as an electron-opaque zone 4–6 nm in diameter lining the inside of the lipid of the viral envelope: the "nanogranular layer" of APOSTOLOV and colleagues (APOSTOLOV and FLEWETT, 1969; KENDAL et al., 1969; APOSTOLOV et al., 1970), the "inner apposition" of BÄCHI et al. (1969) and the "inner leaflet" of COMPANS and DIMMOCK (1969, 1970a).

Unequivocal proof that protein M forms a layer surrounding the RNP followed the preparation of viral "cores" by removal of the peplomers of the envelope with proteolytic enzymes. Smooth round cores are obtained by stripping the peplomers from influenza virions with caseinase C (REGINSTER, 1965), nagarse (subtilisin) (BIDDLE, 1968; KENDAL et al., 1969; APOSTOLOV et al., 1970), bromelain (COMPANS et al., 1970b) or chymotrypsin (SCHULZE, 1970). Such cores were found to be still surrounded by an intact layer of lipid (COMPANS et al., 1970b; SCHULZE, 1970; LANDSBERGER et al., 1971; KLENK et al., 1972a) but to have lost no NP and no M protein. "Cores" obtained with deoxycholate (SKEHEL et al., 1971a) were much less stable, having lost their lipid and swollen to a diameter of 150 nm to look rather like the dimethyl sulfoxide-derived cores of NERMUT (1970), but once again they retained both M and RNP as well as the minor P protein(s). Following uranyl acetate staining of chymo-

trypsin-derived cores, a 60 Å layer of protein was clearly visible beneath a 60 Å layer of lipid (SCHULZE, 1970, 1972). If the core so derived was fixed with glutaraldehyde and then treated with the detergent NP40 to remove the lipid, a smaller, more dense core still surrounded by M protein was obtained. Similarly, phospholipase C rendered chymotrypsin cores susceptible to protease. These findings led SCHULZE to propose the term "membrane protein", which is now widely employed. LAZAROWITZ et al. (1971) and COMPANS (1973) have provided evidence that the M protein associates with infected plasma membranes destined to become the envelope of budding virions.

The M protein has been purified by chromatography and peptide maps obtained (LAZAROWITZ et al., 1971). More recently amino-acid analyses have been conducted on material isolated by cellulose-acetate electrophoresis (LAVER and BAKER, 1972). Following purification by elution from SDS-acrylamide gels of pure virus, M cannot be completely freed of SDS, even by prolonged dialysis, but is still moderately immunogenic and antigenic. Immunodiffusion tests conducted with such reagents have demonstrated that M is serologically unrelated to NP or any other viral protein and that it is a type-specific, not a strain-specific antigen (SCHILD, 1972). One would not expect an internal antigen such as M to undergo antigenic drift like HA and NA because it is inaccessible to antibody, therefore changes due to spontaneous mutation would not be subject to natural selection. Presumably, antibodies to M have no influence on the establishment or progress of natural infection.

VI. The Nucleoprotein

1. Isolation, Purification and Identification

The ribonucleoprotein (RNP) can be extracted from the virion by using lipid solvents or detergents to solubilize the envelope. The first solvent to be employed for this purpose was ether or a mixture of ether and Tween 80 (HOYLE, 1952, 1968), but more commonly used in recent years have been deoxycholate (LAVER, 1963; KINGSBURY and WEBSTER, 1969; PONS et al., 1969), NP40 (PONS et al., 1969), or Triton X-100 (KRUG, 1971). All these detergents release intact RNP from the interior of the particle, whereas SDS usually solubilizes the RNP as well as the rest of the virion to liberate free RNA and NP monomers (LAVER, 1963; SCHILD, 1972).

Once released from the virion, intact RNP can be concentrated by precipitation at its isoelectric point (pH 4.5) (HASLAM et al., 1970b; PONS, 1971), then separated from the other viral constituents by equilibrium gradient centrifugation in cesium chloride or potassium tartrate (DUESBERG, 1969; JOSS et al., 1969; PONS et al., 1969; CONTENT and DUESBERG, 1970; HASLAM et al., 1970b), sometimes preceded by glutaraldehyde fixation (KRUG, 1971). Identification rests on electron microscopy, which reveals a characteristic "twisted double-stranded structure with single-stranded loops" (PONS et al., 1969; SCHULZE et al., 1970; PONS, 1970, 1971; COMPANS et al., 1972). Purity can be

assessed by chemical analysis which indicates an RNA content of 10–12% (PONS et al., 1969; KRUG, 1971) and a single electrophoretic peak of protein.

2. Properties of the Nucleoprotein

NP can be readily released from the RNA-NP complex in monomeric form by SDS alone; reducing agents do not influence its release or its subsequent behavior on acrylamide gel electrophoresis. Several laboratories have identified NP as a homogeneous protein of MW 50,000–65,000 (DUESBERG, 1969; JOSS et al., 1969; PONS et al., 1969; CONTENT and DUESBERG, 1970; HASLAM et al., 1970b). NP displays a marked affinity *in vitro* for influenza RNA (both + and –, but not double-stranded material) as well as for a small AMP-rich RNA from normal cells (SCHOLTISSEK and BECHT, 1971); its affinity for Sindbis and NDV RNA is lower but appreciable.

Amino-acid analyses conducted on BEL and LEE NP recovered from cellulose acetate strips reveal them to be totally distinct (LAVER and BAKER 1972). Immunodiffusion using NP recovered from SDS-acrylamide gels indicates that the antigen is type-specific and distinct from protein M (SCHILD, 1972).

3. Structure of the RNP

From the days when helical symmetry was first described in animal viruses it has been accepted that influenza RNP, like that of the paramyxoviruses and rhabdoviruses, occurs as a typical nucleocapsid in which the NP capsomers are spirally arranged around the RNA. However, recent work suggests that the RNP is neither continuous nor a single helix.

Following release from the virion with detergents, influenza RNP is found to occur, not as a continuous strand as in the case of the paramyxoviruses, but as a heterogeneous collection ranging in length from about 30–110 nm (SCHULZE et al., 1970; COMPANS et al., 1972) and falling into size classes corresponding to those of the isolated RNA molecules (DUESBERG, 1969; KINGSBURY and WEBSTER, 1969; PONS et al., 1969; PONS, 1971; COMPANS et al., 1972). Negative staining (PONS et al., 1969; SCHULZE et al., 1970; PONS, 1970, 1971; SCHULZE, 1972) or positive staining (COMPANS et al., 1972) reveals that these RNP fragments have a "twisted double-stranded structure with single-stranded loops where it is turned back on itself". The double helix is 10–15 nm in diameter for most of its length and 5–7.5 nm in the single-stranded loop. A periodicity of about 4.5 nm in the loop and 7.5 nm down the length of the double helix suggests that the NP molecules each measure about 4.5 ×7.5 nm with their short axes aligned along the RNA strand. Morphologically indistinguishable double-helical structures were observed when the RNA was displaced from isolated RNP by polyvinyl sulfate (PONS et al., 1969; GOLDSTEIN and PONS, 1970; PONS, 1970; SCHULZE et al., 1970).

SCHULZE (1972) has carefully examined the RNP of virions in various states of disaggregation and finds it to have the same appearance as isolated

RNP (PONS et al., 1969; PONS, 1970, 1971; SCHULZE et al., 1970). Virions fixed with glutaraldehyde *in situ* and then dissociated briefly with a low concentration of NP40 and negatively stained with PTA, or chymotrypsinderived cores similarly dissociated with NP40 and negatively stained with uranyl acetate, were both found to contain RNP that met the above description. The diameter of the double-stranded structure was 12–14 nm compared with 10–13 nm in thin sections.

The available evidence therefore suggests that the RNP occurs naturally in the virion in such double-helical fragments. Another alternative may be worth consideration however. In the virion, RNP may occur as a continuous single helix which in turn is wound to form a supercoil; extraction from the particle, preparation for electron microscopy, or even environmental changes following budding might place this supercoil under strain and cause it to break between the separate RNA molecules yielding RNP fragments which then spontaneously twist back on themselves to form double-helical structures. Occasional virions penetrated by negative stain do show a large apparently continuous RNP supercoil about 50 nm in diameter (ALMEIDA and WATERSON, 1967, 1970; NERMUT and FRANK, 1971; NERMUT, 1972). It is now generally considered that these symmetrical coils must represent an aberrant state atypical of the population as a whole (SCHULZE et al., 1970; SCHULZE, 1972) because they are seen in less than 0.1 % of virions, even when NP40 is applied to the EM grid to cause partial disruption of the particles and allow the phosphotungstate to penetrate. Yet inspection of the excellent published pictures from several laboratories (see ALMEIDA and WATERSON, 1970, Figs 2–8; SCHULZE et al., 1970, Fig. 12; NERMUT and FRANK, 1971, Fig. 3, d) displaying "nucleocapsids" so closely resembling those of the other major groups of RNA viruses of helical symmetry, the paramyxoviruses and rhabdoviruses (see FENNER et al., 1973), makes it hard to accept the view that these beautifully symmetrical and apparently continuous structures arise by accident in the odd virion that has "gone wrong". One cannot readily envisage the mechanism of an aberrant process leading to the linking together of short fragments of RNP into a continuous coil, but one may readily imagine a continuous coil breaking to yield double-helical fragments following treatment with detergents.

The RNP of the paramyxoviruses has been shown by optical diffraction to consist of 2400–2800 NP molecules (capsomers) of MW 60,000 arranged as a single helix with a diameter of 20 nm or slightly less, a pitch of 5–6 nm and 11–13 hourglass-shaped capsomers per full turn of the helix, to form a nucleocapsid 1100 nm long (FINCH and GIBBS, 1970). It is worth noting that if 800 influenza NP molecules (Table 5), which are almost the same size and shape as those of the paramyxoviruses were spiralled around RNA with a periodicity of 15 nm and with 12 NP molecules per full turn of the single helix, as can be discerned from the micrographs of COMPANS et al. (1972), the length of the single helix would also be 1000 nm, which corresponds well with the median length of the coils described in virions by ALMEIDA and WATERSON (1970) and by SCHULZE et al. (1970). Since this is a much more open type of helix than

that of the paramyxoviruses, it might provide the degree of flexibility needed to allow isolated influenza RNP fragments to twist back on themselves to form a double helix and might also explain why the RNA of influenza RNP, unlike that of paramyxovirus nucleocapsids, is accessible to ribonuclease (DUESBERG, 1969; KINGSBURY and WEBSTER, 1969; PONS et al., 1969) and is readily displaced by polyvinyl sulfate (PONS et al., 1969; PONS, 1970; PONS and GOLDSTEIN, 1970; SCHULZE et al., 1970).

VII. The Transcriptase

1. Enzyme Activity *in vitro*

In 1971 it was discovered that influenza virions contain an RNA-dependent RNA polymerase (CHOW and SIMPSON, 1971; PENHOET et al., 1971; SKEHEL, 1971b), as do all the major genera of RNA viruses in which the virion RNA itself is not infectious (see FENNER et al., 1973). The enzyme is present in cores obtained by removing the peplomers of the envelope with chymotrypsin (CHOW and SIMPSON, 1971) and remains associated with the RNP following extraction and purification (BISHOP et al., 1972; COMPANS and CALIGUIRI, 1973). NP40-treated cores are active in producing RNA *in vitro*. Under these conditions the enzyme was found to operate optimally at a temperature of 28–33° and pH 8.0–8.2, and to require all four ribonucleoside triphosphates plus monovalent cations and the divalent cations Mg^{++} and Mn^{++} (BISHOP et al., 1971a; CHOW and SIMPSON, 1971). It is now considered that Mg^{++} ions are essential (BISHOP et al., 1971a; CHOW and SIMPSON, 1971; PENHOET et al., 1971; SKEHEL, 1971b), as is the case with the RNA-dependent RNA polymerases of other viruses, but that Mn^{++} ions, which were at first considered vital (BISHOP et al., 1971a, b; CHOW and SIMPSON, 1971), merely stimulate activity somewhat (SKEHEL, 1971b). The enzyme activity *in vitro* is totally insensitive to actinomycin D (BISHOP et al., 1971b; SKEHEL, 1971b).

The product of the *in vitro* reaction consists of abbreviated single strands of complementary RNA (cRNA) of MW 50,000–300,000 (BISHOP et al., 1971b; SKEHEL, 1971b) capable of hybridizing to all the resolvable species of viral RNA (BISHOP et al., 1971b; 1972). This cRNA has not yet been demonstrated to have messenger activity in a cell-free protein-synthesizing system. Nevertheless, it almost certainly represents incomplete molecules of mRNA, since the mRNA present in the polyribosomes of influenza-infected cells is also complementary in base sequence to viral RNA (PONS, 1972). The virion polymerase can therefore be designated a "transcriptase".

2. Protein P

The chemical composition and structure of the virion transcriptase is still unknown. The logical contender for the role is protein P (COMPANS et al., 1970b; SCHULZE, 1970; SKEHEL and SCHILD, 1971), the only known virion polypeptide not so far assigned a function. BISHOP et al. (1972) found protein(s) P in trans-

criptase-containing RNP extracted from virions. By contrast, COMPANS and CALIGUIRI (1973) do not detect P in RNP purified from infected cells, and therefore postulate that the NP itself may be the transcriptase.

Clearly it is important that this matter be resolved. If protein P is not associated with RNP the possibility that it is a contaminant accidentally enveloped by the budding virion would have to be considered. Viral transcriptase is present in large amounts in the cytoplasm at the time virions are budding (SCHOLTISSEK and ROTT, 1969, 1970) and could be fortuitously incorporated into the particle, but if P is not associated with the transcriptase either, it could conceivably be a cellular contaminant.

On the other hand, if P is in fact associated with the RNP it might well be concerned with the transcriptase activity. Perhaps P is bound to a specific polymerase-attachment site near the 3′-terminus of each of the several RNA molecules. The protein could also serve as a linker holding the RNA fragments together.

The small number of P molecules in the virion points to the likelihood that this protein has a specialized function rather than a purely structural role. Table 5 indicates that the average virion contains less than 50 molecules of P (though estimates vary widely, as is to be expected, since it is notoriously difficult to assess trace amounts of proteins on gels, and the figure may yet turn out to be somewhat higher than previously thought [SKEHEL, 1972, Table 1]). It would be instructive to look for differences in P content between strains in the light of the finding that *in vitro* transcriptase activity can vary over a 20-fold range (CHOW and SIMPSON, 1971). If the viral transcriptase is a polymer, like known mammalian and bacterial RNA polymerases, there might be only enough P molecules to give less than a dozen transcriptase molecules per virion.

SKEHEL (1971 a, 1972) has reported two separate P proteins whereas other workers found only one (COMPANS et al., 1970b; SCHULZE, 1970; KLENK et al., 1972a). It is important to resolve this question because an RNA molecule equivalent to nearly 20% of the genome would be required to code for each protein of such high MW, regardless of how few molecules of that protein the virion contains. Of course, we cannot discount the possibility that the single P bands found by other laboratories contain two (or more than two) distinct polypeptides of identical MW. Several other transcriptases are composed of more than one type of sub-unit.

Incomplete virus derived by high-multiplicity passage of influenza has a greatly reduced content of the transcriptase (CHOW and SIMPSON, 1971). Incomplete virus is also known to lack the largest of the several recognized classes of RNA molecules (DUESBERG, 1968; PONS and HIRST, 1969; LI and SETO, 1971). This coincidence tempts one to postulate that the largest of the viral RNA fragments may be the cistron(s) coding for the transcriptase. The MW of this particular class of RNA molecules (800,000–1,000,000) is in the right range to code for protein P (MW 81,000–94,000).

VIII. Synthesis of Viral Proteins

Early studies on the intracellular synthesis of influenza viral proteins using various combinations of microscopy and serology (BREITENFELD and SCHÄFER, 1957; DUC-NGUYEN et al., 1966; DIMMOCK, 1969) have recently been supplemented by the application of acrylamide-gel electrophoresis together with radioactive pulse-chase procedures (TAYLOR et al., 1969; HOLLAND and KIEHN, 1970; LAZAROWITZ et al., 1971) and cell-fractionation techniques (TAYLOR et al., 1969, 1970; HOLLAND and KIEHN, 1970; LAZAROWITZ et al., 1971; COMPANS, 1973; STANLEY et al., 1973b). The new work has thrown light on the site and mechanism of synthesis, maturation and migration of each viral protein, including a major nonstructural protein.

1. Kinetics and Control of Viral Protein Synthesis

Pulse-labeling experiments coupled with gel electrophoresis have shown that the major structural proteins of the virion first become detectable above cell background during the second hour post-infection and the rate of viral protein synthesis increases steadily to reach during the fifth hour a maximum which is maintained for about three hours before gradually declining (WHITE et al., 1970; SKEHEL, 1972). The various viral proteins are not made in anything like equal molecular numbers. This is best illustrated by saying that protein P, since it is the virion protein of highest MW, should be the most conspicuous on acrylamide gels of infected cells, yet is in fact barely detectable, whereas lower MW proteins such as NP and NS are very prominent. In the virion itself the molecular ratio P:NA:NP:HA:M can be calculated to be approximately 1:5:20:25:75 (Table 5). The molecular ratio of the same proteins in the infected cell is only slightly different, NP being synthesized in relative excess (WHITE et al., 1970) while M is in relatively short supply (LAZAROWITZ et al., 1971) as are HA and its cleavage products in some virus-cell systems (STANLEY et al., 1973b). Clearly, transcriptional or translational controls must restrict the expression of the viral cistrons for P and NA in particular.

There is also some evidence that the rates of synthesis of the viral proteins change relative to one another during the course of the multiplication cycle. The ribonucleoprotein antigen was found by immunofluorescence to appear an hour earlier than the hemagglutinin and neuraminidase (BREITENFELD and SCHÄFER, 1957; MAENO and KILBOURNE, 1970), and by gel electrophoresis NP was reported to be synthesized in relatively greater excess late in the cycle (ETCHISON et al., 1971). A recent paper also indicates that M is in short supply only early in the cycle when NS is being made in large amounts, and that the ratio of the two reverses as multiplication progresses (SKEHEL, 1972). Now that techniques of RNA-RNA hybridization have been perfected to the point where mRNA molecules complementary to individual vRNA molecules can be identified (CONTENT and DUESBERG, 1971; PONS, 1971; SCHOLTISSEK and BECHT, 1971), an attempt should be made to measure the amount of each species of

mRNA at various times after infection in order to determine whether the regulation operates at the level of transcription or of translation.

Additional controls must act to suppress cellular protein synthesis. Though the shutdown is not dramatic by the standards of the picornaviruses, it is sufficiently pronounced in some cell lines (e.g. HeLa, BHK21-F, MDCK) to ensure that most of the protein being made after the fourth hour is viral (TAYLOR et al., 1969; WHITE et al., 1970; ETCHISON et al., 1971; LAZAROWITZ et al., 1971; SKEHEL, 1972). The overall rate of protein synthesis is not diminished until quite late in the cycle, i.e. the rate of synthesis of viral proteins increases steadily at the expense of the host. This could conceivably result from simple competition for ribosomes between host and viral mRNA. But there are some cell systems, e.g. MDBK and chick-embryo fibroblasts (CEF), in which the degree of shutdown is reported to be minimal for the first 8 hours (LONG and BURKE, 1970; LAZAROWITZ et al., 1971). The likelihood is therefore, as suggested by LONG and BURKE (1970), that a newly synthesized viral protein is responsible for the shutdown. Presumably this protein acts at the level of translation, not transcription, because cellular RNA synthesis does not decrease (SCHOLTISSEK et al., 1969) and indeed may actually increase (MAHY et al., 1972) during the first 3 hours post infection. There are no firm data incriminating any particular viral protein, but NS would certainly be a candidate (TAYLOR et al., 1970).

2. Site of Viral Protein Synthesis

Over the years studies based on immunofluorescence (BREITENFELD and SCHÄFER, 1957; MAENO and KILBOURNE, 1970), autoradiography (BECHT, 1969) and electrophoresis (JOSS et al., 1969) have consistently indicated that influenza NP is found mainly in the nucleus of the infected cell. This has sometimes been interpreted to mean that at least the NP protein, and perhaps all the viral proteins are made there. Pulse-chase experiments using very short labels were necessary to demonstrate that all the viral proteins are in fact synthesized in the cytoplasm in the usual way but that NP and NS very rapidly migrate into the nucleus and accumulate there (TAYLOR et al., 1969, 1970; BECHT, 1971).

3. Migration of NS and NP to the Nucleus

Accumulation of NS in the nucleus occurs extremely rapidly (half-time <5 minutes after synthesis) whereas that of NP is considerably slower (TAYLOR et al., 1969, 1970). This difference is consistent with the view that the viral proteins move around the cell by a process involving simple diffusion, smaller molecules migrating more rapidly than larger ones. Nuclear accumulation does not require continuing protein synthesis or RNA transcription and is also independent of multiplicity of infection, time after infection or permissiveness of the cell. It is not necessary to assume active transport of these proteins into the nucleus but simply that they are preferentially bound when they arrive there. In the case of NS this binding may be quite particular since

virtually all of it accumulates in the nucleolus (TAYLOR et al., 1970). There is considerable interest in identifying the material with which the two viral proteins become associated. It may not be irrelevant that both NP and NS are richer in arginine than the other major proteins in influenza virus-infected cells (WHITE et al., 1970; BECHT, 1971; LAVER and BAKER, 1972); NP has a marked affinity for nucleic-acid (SCHOLTISSEK and BECHT, 1971) and it is possible that NS may share this property.

The fact that NS and NP accumulate in the nucleus does not necessarily imply that they are needed there. It is quite conceivable that the phenomenon is fortuitous and that any viral protein which drifts into the nucleus is doomed to play no further role in the multiplication cycle. Against this, however, is evidence from enucleation experiments. Influenza virus would not grow in fragments of cytoplasm from which the nucleus had been removed, whereas the paramyxovirus, parainfluenza did (CHEYNE and WHITE, 1969). The consistent finding that multiplication of influenza but not parainfluenza virus is inhibited by actinomycin D, mithramycin, α-amanitin, or pre-irradiation of the cell with ultraviolet light has led to the still unproven hypothesis that the nucleus is essential to influenza because some cellular RNA must be transcribed (see BARRY and MAHY, 1970; MAHY et al., 1972).

4. Insertion and Cleavage of HA in Plasma Membrane

While NS accumulates in the nucleolus and NP in the nucleoplasm, HA finds its way quite rapidly into smooth cytoplasmic membranes (HOLLAND and KIEHN, 1970; WHITE et al., 1970). This occurs with a half-time of about 10 minutes after synthesis (LAZAROWITZ et al., 1971; STANLEY et al., 1973b). Indeed the HA molecule may spend its whole life in membranes for it is not found in any but trace quantities in the soluble fraction of cytoplasmic extracts but is always membrane-associated (COMPANS, 1973; STANLEY et al., 1973b).

Pulse-chase experiments have unequivocally demonstrated the reality of post-translational cleavage of HA → HA_1+HA_2 (LAZAROWITZ et al., 1971; KLENK et al., 1972b; SKEHEL, 1972) and LAZAROWITZ et al. (1971) have presented evidence to show that it takes place in the plasma membrane. Their data suggest that this occurs only after the HA has been in the membrane for a considerable time, usually over an hour, hence cleavage is not a necessary step in the insertion of the HA molecule into the lipid bilayer. Indeed, the HA found in the plasma membranes or virions secreted from BEL-infected BHK21 or calf-kidney cells is not cleaved although the cells hemadsorb and many of the virions are infectious and hemagglutinate (STANLEY et al., 1973b). Hence it seems that cleavage is not necessary for the biological activity of the hemagglutinin nor for infectivity of the virion.

The extent of HA cleavage is influenced by both the virus strain and the type of host cell involved. For example, the HA of A_0/BEL is completely cleaved in chick allantois, only partially cleaved in calf-kidney cells, and not cleaved at all in BHK21 or HeLa cells (HASLAM et al., 1970a; STANLEY et al.,

1973b), whereas that of A_0/WSN is substantially cleaved in most systems examined (except HeLa cells) in the order eggs > MDBK, BHK21 > monkey kidney > HeLa (LAZAROWITZ et al., 1971; STANLEY et al., 1973b). Similarly, the HA of fowl plague virus is cleaved completely and that of WSN hardly at all in chick embryo fibroblasts (KLENK et al., 1972a) while NWS HA is more extensively cleaved in MDCK than is that of A_2 (ETCHISON et al., 1971). Cleavage differences between virus strains presumably reflect differences in HA amino-acid sequence or folding, or carbohydrate side chains. Differences between cell types could result from qualitative, quantitative or topographical differences in the distribution of proteolytic enzymes, or from obstruction of access of such enzymes to potentially vulnerable peptide bonds by the addition of particular carbohydrate side chains to viral HA in some cell types but not others. Indeed, cleavage could be an artefact due to the presence of proteolytic enzymes in cell extracts or elsewhere during the experimental protocol (HOLLAND et al., 1972). It is rather a coincidence that trypsin cleaves HA at the same spot as do cells (SCHULZE, 1970); perhaps trypsin is the enzyme responsible for intracellular cleavage of HA, or more likely the glycoprotein is folded in such a way that only a single region is accessible to any proteolytic enzyme.

With the exception of HA in certain virus-cell systems, all the viral proteins are stable. Short pulses followed by prolonged chases provided no evidence for the existence of any high-MW precursor that is subsequently cleaved to produce HA, NA, NP, M or NS (TAYLOR et al., 1969). The data of ETCHISON et al. (1971) which seemed to indicate such a precursor–product relationship has been interpreted (SKEHEL, 1972) in terms of specific cleavage of HA $\rightarrow$ HA_1 and HA_2 accompanied perhaps by some nonspecific proteolysis (HOLLAND et al., 1972).

5. Glycosylation of HA

The first glucosamine residues are attached to the HA polypeptide almost immediately after it is synthesized on cytoplasmic polyribosomes (STANLEY et al., 1973b). Fucose is added somewhat later. Most of the glycosylation occurs very rapidly while HA is membrane-associated but before it is inserted into the plasma membrane. This suggests that sugars may be added in the membranes of the endoplasmic reticulum and/or the Golgi apparatus.

Several years ago KILBOURNE (1959) reported that the sugar analogue deoxyglucose inhibited the production of influenza virus in embryonated eggs. It has now been found that deoxyglucose and glucosamine selectively block the completion of the viral glycoproteins HA and NA without affecting the synthesis of the non-glycosylated proteins NP, M and S (GANDHI et al., 1972; KALUZA et al., 1972). KLENK et al. (1972b) have presented evidence for the accumulation of a non-glycosylated HA precursor in the presence of high concentrations of glucosamine. This procedure should prove generally valuable in studying the glycoproteins not only of influenza viruses but also of paramyxo viruses, rhabdoviruses, togaviruses, and other enveloped viruses. It may be

possible to accumulate the precursor polypeptide for analysis or to trace the subsequent steps in the glycosylation process following reversal of the glucosamine block.

6. Budding

In a comprehensive electron-microscopic study of the budding process COMPANS et al. (1970a) presented pictures from which the probable sequence of events can be deduced. Only plasma membrane and vacuoles derived from it are involved. The first viral protein to enter plasma membrane is doubtless HA, since areas of membrane showing no visible change can adsorb erythrocytes (COMPANS and DIMMOCK, 1969). The membrane protein M then associates with the lipid to form an electron-dense layer not visible in normal membrane (BÄCHI et al., 1969; COMPANS and DIMMOCK, 1969; APOSTOLOV et al., 1970). Budding seems to be linked with the appearance of spikes over these thickened areas of membrane; it is not observed in regions lacking spikes, nor are spikes seen without buds (DUC-NGUYEN et al., 1966; BÄCHI et al., 1969; COMPANS and DIMMOCK, 1969; APOSTOLOV et al., 1970). Usually budding also coincides in time with the arrival of RNP beneath the altered membrane (DUC-NGUYEN et al., 1966) but this is unlikely to represent the triggering event because empty particles commonly occur.

The role of neuraminidase in the budding process is still unclear. Neuraminidase and hemagglutinin seem to move concurrently into the same area of membrane, beginning at one pole of cells spread in a monolayer (MAENO and KILBOURNE, 1970). Presumably the enzyme is responsible for the observed absence of neuraminic-acid from the glycoproteins of those areas of plasma membrane where budding is occurring (KLENK et al., 1970). Whether this is a necessary preliminary to insertion of hemagglutinin, spike formation, or budding is not clear, but it must be remembered that rhabdoviruses, togaviruses and leukoviruses, which also mature by budding, have no neuraminidase. It used to be considered that neuraminidase played a vital role in viral release from the cell surface because there was much evidence to indicate that antineuraminidase antiserum inhibits the release of virions. However, BECHT et al. (1971) have shown that univalent Fab fragments of IgG, obtained by digesting antineuraminidase antibodies with pepsin, no longer diminish the yield of virus even though they are as effective as normal bivalent IgG in blocking the enzyme activity of free virions or of isolated neuraminidase. The authors propose that the inhibition of viral release regularly observed with divalent antineuraminidase is not due to the blockage of enzyme activity but perhaps to the formation of bridges between neuraminidase peplomers, one result of which could be to restrict the flexibility the membrane requires for budding. This proposal gains support from the recent observation from the same laboratory that concanavalin A, which binds more strongly to infected cells than to normal ones and probably more strongly to neuraminidase than to hemagglutinin, inhibits the production of influenza virus (ROTT et al., 1972). BECHT et al. (1971) put forward an alternative explanation of the inhibitory effect of bi-

valent anti-neuraminidase antibody, namely that budding is not prevented but that released virions are trapped on the outside of the cell by being held to NA peplomers in the adjacent membrane and to other virions. A similar conclusion was reached by COMPANS et al. (1970) in an electron-microscopic study of the phenomenon. However, this hypothesis must be considered in the light of the fact that antihemagglutinin has not been reported to inhibit viral release.

At the heart of the mystery of budding is the question of how the viral proteins come to supplant cellular proteins from the membrane. Contrary to earlier impressions, it is a fact that the viral envelope contains little or no host protein (HOLLAND and KIEHN, 1970). The much discussed "host antigen" is cellular carbohydrate covalently linked to virus-coded protein (LAVER and WEBSTER, 1966). The lipids also closely resemble those of the plasma membrane of the particular host cell species, though minor differences have been noted between different viruses grown in the same cell type (BLOUGH and MERLIE, 1970; KLENK and CHOPPIN, 1970; BLOUGH, 1971).

There are at least four distinct mechanisms whereby host proteins could be supplanted in plasma membrane destined to become viral envelope. Firstly, viral proteins might be inserted only into newly synthesized membrane. Following shutdown of host protein synthesis viral glycoproteins, perhaps after glycosylation in the Golgi apparatus, would be the dominant protein available for incorporation into new membranes as they were produced. Some support for this idea comes from the elegant demonstration by MARCUS (1962) that red blood cells adhere only to the "poles" of cells in monolayer culture in the early stages of virus production. However, such a hypothesis requires that the plasma membrane of an infected cell be substantially replaced within a space of about 3 hours, because the whole surface of the cell develops the capacity for hemadsorption during that interval. Similar objections can be raised against the second hypothesis, namely that membrane proteins are continuously being turned over at this rate, hence viral glycoproteins can be substituted in their place. It is more likely that viral membrane proteins are initially incorporated into areas of cytoplasmic membrane noted for their vigorous synthetic activity, namely Golgi vesicles and adjacent areas of smooth endoplasmic reticulum, which only later become part of the plasma membrane and bud off as viral envelopes. In this connection it should be noted that both HA (HOLLAND and KIEHN, 1970; WHITE et al., 1970; COMPANS, 1973; STANLEY et al., 1973b) and M (COMPANS, 1973) proteins are found not only in plasma membrane but in other cytoplasmic smooth membranes as well.

Perhaps the most attractive hypothesis is that, as viral glycoproteins are inserted into the plasma membrane, existing cellular proteins are simply displaced laterally. This proposal makes the assumption that membranes are much more "fluid" than used once to be thought and that proteins are free to move around in a "sea of lipid" (TAYLOR et al., 1971). The fact that the displacement of cellular proteins from the viral envelope is so total may be due to saturation of the region of the membrane from which the virion is budding with large numbers of locally synthesized viral glycoprotein molecules, or

aggregation of viral glycoproteins which are also capable of lateral movement through the lipid matrix.

7. Ribonucleoprotein

RNP extracted from infected cells, like that from virions, is always found in fragments containing RNA of several size classes (DUESBERG, 1969; KINGSBURY and WEBSTER, 1969; PONS et al., 1969; KRUG, 1971). We do not know whether RNP occurs in this state in the cells or whether it has broken during extraction at specific weak points between the individual RNA fragments.

How do viruses with segmented genomes ensure the ordered synthesis and packaging into the virion of the correct combination of RNA molecules? COMPANS et al. (1970a) postulated that the RNP fragments are permanently separate and are selected at random for incorporation into the budding virion. They calculated that a reasonable minority of the resulting virus particles would receive a complete set of genes (thought at the time to be 5, but more probably at least 7) provided that the average particle contained an excess of RNP pieces. It must be said, however, that such a mechanism of assembly would be extraordinarily uneconomical. Furthermore, without complex control mechanisms to regulate the rate of transcription and translation of separate RNA (RNP) fragments the smaller genes would be read much more frequently than the larger ones. It is true that the smallest protein NS is synthesized in large numbers and the largest, P, only in trace amounts, but the generalization can be extended no further than that.

PONS (1970) proposed that the several viral RNA molecules remain permanently associated with a continuous length of RNP throughout the replication cycle, the protein serving as a backbone to ensure that the RNA molecules are correctly ordered and synthesized in constant ratio. This is an ingenious suggestion, though there are difficulties in envisaging precisely how the RNA replicates in this form. PONS finds support for his model in his discovery that 10% of the RNA isolated from intracellular RNP is cRNA, whereas all that found in virion RNP is vRNA (PONS, 1971). The strong affinity of NP for both vRNA and cRNA *in vitro* (SCHOLTISSEK and BECHT, 1971) could also be seen as consistent with the theory, but could equally mean that RNP free of contamination with cRNA cannot easily be recovered from cells. Certainly some mechanism must operate to select only RNP containing vRNA for incorporation into virions. If the RNA is exposed on the surface of RNP, the membrane protein M might conceivably have the capacity to discriminate between the two.

If the various RNA molecules are somehow linked together during budding and/or RNA replication, what is the mechanism of linkage? NP is the most logical candidate, but the transcriptase or protein P are also possibilities. The finding that the transcriptase is present in each individual RNP fragment (BISHOP et al., 1972; COMPANS and CALIGUIRI, 1973) is interesting in this regard. The possibility that the several RNA molecules may be held together by short regions of base-pairing (PONS, 1970) is contradicted by the consistent

recovery of individual RNA pieces in unimolecular ratio without recourse to melting by dimethyl sulfoxide or heat (DUESBERG and ROBINSON, 1967; DUESBERG, 1968; PONS and HIRST, 1968; BISHOP et al., 1971a; CONTENT and DUESBERG, 1971; SKEHEL, 1971c) and the more recent end-group analyses which demonstrate the 5'-terminus of most of the fragments to be adenosine triphosphate (YOUNG and CONTENT, 1971) and the 3'-terminus nonphosphorylated uridine (LEWANDOWSKI et al., 1971). The latter data also provide the best evidence published to date that the individual RNA molecules are separately synthesized and occur as separate molecules in the virion.

NP is synthesized in the cytoplasm and migrates to the nucleus (TAYLOR et al., 1969, 1970) yet is eventually incorporated into virions which bud from cytoplasmic membrane. The question springs to mind: Was the journey really necessary? RNP can be recovered from cytoplasm as well as from nucleus (KRUG, 1971, 1972) and electron microscopy reveals large cytoplasmic inclusions containing "clusters of strands 55 Å in diameter" (COMPANS and DIMMOCK, 1969; COMPANS et al., 1970a) or "fibrillar material 60 Å in diameter" (APOSTOLOV et al., 1970) which may well have been RNP. It is still completely unknown whether viral RNA replicates in the nucleus or in the cytoplasm. SCHOLTISSEK and ROTT (1970) demonstrated that "minus"-strand (messenger) RNA is synthesized first and is found predominantly in the cytoplasm as is also the viral transcriptase. KRUG (1972) has recently shown that the RNP found in the nucleus contains mainly "plus"-strand RNA, whereas that in the cytoplasm contains both "plus" and "minus" RNA. This raises the possibility that "minus"-strand RNA (messenger RNA) may be synthesized in the cytoplasm, whereas "plus"-strand (viral RNA) may be made in the nucleus and assembled there into RNP.

8. Polymerases

Several types of polymerase activity have been reported to be induced in influenza-infected cells. Early papers, well reviewed by BLAIR and DUESBERG (1970), were contradictory. The position is still far from clear, but it now seems that there may be up to three such enzymes altogether, but that only two are likely to be virus-coded.

The first of the virus-induced RNA-dependent RNA polymerases was found in the cytoplasm of infected cells by HO and WALTERS (1966). They determined that the enzyme is dependent on Mg^{++} ions and that actinomycin D has no effect on its activity, although it prevents its production *in vivo*. Their findings have been amply confirmed (SCHOLTISSEK, 1969; SCHOLTISSEK and ROTT, 1969; RUCK et al., 1969; SKEHEL and BURKE, 1969; MAHY and BROMLEY, 1970). Although the polymerase is detectable as early as one hour post-infection, when RNA synthesis is first apparent (SCHOLTISSEK and ROTT, 1969), its concentration builds up only late in the cycle, reaching a peak in the cytoplasm at around 6 hours (SCHOLTISSEK and ROTT, 1970). SCHOLTISSEK et al. (1971) also showed that convalescent serum from fowl plague-infected chicks neutralizes the activity of the polymerase from cells infected with any of the

strains of influenza A they tested, but not influenza B or NDV; in other words it is type-specific, not strain-specific. The product of the enzyme consists mainly of short lengths of RNA (10–18 S) (SKEHEL and BURKE, 1969) which are complementary in base sequence to viral RNA (SCHOLTISSEK, 1969). In short, this enzyme has all the characteristics of the virion transcriptase and is probably identical with it. Indeed COMPANS and CALIGUIRI (1973) have recently reported that the 70 S cytoplasmic particle with which this polymerase is associated (SCHOLTISSEK and ROTT, 1969; SKEHEL and BURKE, 1969) is in fact RNP and is indistinguishable from that found in the virion.

There must also be a "replicase" to direct the synthesis of viral RNA (vRNA) from complementary RNA (cRNA), though no such enzyme has yet been conclusively identified. HASTIE and MAHY (personal communication) have evidence for the existence of an RNA-dependent RNA polymerase which appears in the nucleus quite early in infection, reaching peak concentration by about 3 hours. It is not yet clear whether this enzyme is distinct from the cytoplasmic transcriptase or not. Several of the early papers on the cytoplasmic ("microsomal") polymerase indicated that a significant proportion of the enzyme activity was also found in the nucleus (SCHOLTISSEK and ROTT, 1969; SKEHEL and BURKE, 1969). Reports from other laboratories that the product of the cytoplasmic polymerase is largely (RUCK et al., 1969) or partially (MAHY and BROMLEY, 1970) "plus" strands (vRNA) may mean that the enzyme preparations used in these experiments were mixtures of transcriptase and replicase. Since all these studies were conducted before the availability of assays discriminating between vRNA and cRNA, it could be profitable to repeat them now. The results might indicate whether there is one viral polymerase or two, which one makes vRNA and which its complement, how they are distributed between nucleus and cytoplasm, and their activity at various stages of the replication cycle.

There is the interesting possibility already briefly mentioned, that the synthesis of vRNA (replication) may occur in the nucleus and the synthesis of cRNA (transcription) in the cytoplasm. Superficially, such an arrangement presents certain advantages. Newly transcribed mRNA (cRNA) would find itself in proximity to cytoplasmic ribosomes where it is needed for translation, while newly replicated vRNA would associate with NP in the nucleoplasm. However, coordination of the two processes would be quite complex and would depend on a continuing "leakage" of RNA in both directions.

A third RNA polymerase has been reported to increase in influenza virus-infected cells, namely a DNA-dependent RNA polymerase, presumably cellular. The activity of this enzyme rises early in infection to reach a peak at 2 hours about 60% above background (BORLAND and MAHY, 1968; MAHY et al., 1972); this is hardly a striking increase and its significance is at present uncertain. MAHY et al. (1972) believe this enzyme to be the α-amanitin-sensitive RNA polymerase II of the nucleoplasm and postulate that it is responsible for an early burst of transcription of cellular mRNA that includes the actinomycin D- and UVL-susceptible function vital to influenza replication.

9. Non-Structural Proteins

The NS of most influenza strains comigrates with the membrane protein M in neutral SDS gel electrophoresis and was not identified in the early gels of infected cells (JOSS et al., 1969; TAYLOR et al., 1969; HOLLAND and KIEHN, 1970). LAZAROWITZ et al. (1971) first resolved the protein and demonstrated it to be quite distinct from M by peptide mapping of both following separation on an agarose column. They also showed that, while M is synthesized in limiting amounts and is found mainly in plasma membranes, NS is made in very large quantities and is found mainly in the nucleus. Clearly NS is the protein of 21,000 MW which TAYLOR et al. (1969, 1970) reported to accumulate rapidly in the nucleolus of infected cells. It is almost certainly also identical with the nonstructural antigen previously serologically identified in the nucleolus (DIMMOCK, 1969; DIMMOCK and WATSON, 1969). DIMMOCK (1969) demonstrated nucleolar immunofluorescence in infected chick cells using mouse convalescent peritoneal fluid which had been absorbed with disrupted virions to remove antibodies to structural antigens. Since the same reagent gave fluorescence in the nucleoli of cells infected with A_0, A_1 or A_2 strains, it was concluded that the antigen was virus-coded and type-specific rather than strain-specific. Electron microscopy also revealed that the nucleolus in influenza-infected cells becomes grossly swollen, distorted and finally destroyed (COMPANS and DIMMOCK, 1969; APOSTOLOV et al., 1970; COMPANS et al., 1970a).

The function of NS is one of the more pressing unsolved problems of influenza research. One could postulate that it is a subunit of the viral replicase, assuming that enzyme to be a polymer of high MW. TAYLOR et al. (1970) proposed that NS may be a "sigma factor" having the capacity to bind to a cellular RNA polymerase and to alter its specificity so that it recognizes viral rather than cellular nucleic acid as template. It may not be irrelevant that the activity of cellular RNA polymerase I of the nucleolus falls off dramatically during influenza infection, reaching 50% of control levels by $1^1/_2$–2 hours (while the activity of the nucleoplasm polymerase II is increasing) and 10% by 4 hours (MAHY et al., 1972). Alternatively, NS could be a sigma factor which binds to viral transcriptase, so changing its template specificity from vRNA to cRNA. In either case the new enzyme would be a replicase transcribing vRNA from cRNA in the nucleus of the infected cell.

The fact that the nucleolus is the site of synthesis and assembly of ribosomes raises another very interesting possibility, namely that NS associates with ribosomes to make them able to bind viral mRNA rather than cellular mRNA. Recently PONS (1972) and COMPANS (1973) reported finding NS in association with cytoplasmic ribosomes.

While influenza workers are casting around for a role for NS, it may be rewarding for others to search for low-MW, arginine-rich, nonstructural, virus-coded proteins migrating to the nucleolus of cells infected with other viruses. Whatever NS turns out to be — ribosome-modifying protein, polymerase subunit, or cell-shutdown protein — it could be the prototype of a class of viral proteins of quite widespread occurrence.

SKEHEL (1972) has recently presented evidence for the existence of a second nonstructural protein (MW 11,000) which he finds in small amounts in virus-infected cells. The designation of HA as a "nonstructural" protein is a question of semantics; it is a structural protein in virions of most of the influenza viral strains analyzed so far, but not in FPV.

IX. The Versatility of Viral Proteins

We tend to think of each virus-coded protein as having a particular function; indeed it has been one of the major objectives of most of the work discussed here to identify that function for the products of each of the influenza genes. However, we should also consider the possibility that most viral proteins have more than one function. They may originally have evolved to carry out a single task, which is perhaps still their major function, but have since acquired secondary capacities as well. Several of the influenza viral proteins are polymers of high MW (hemagglutinin, neuraminidase, and quite probably transcriptase), while even the individual polypeptides are quite large, containing 200–800 amino acids each. Only a very small proportion of these amino acids contribute to the active site of hemagglutinin or of an enzyme; the remainder presumably confer on the protein the tertiary structure needed to do several additional jobs. Consideration of likely supplementary functions of each viral protein may help to direct our thinking about its chemistry as well as its biology.

For example, the primary function of the membrane protein (M), is probably to confer some rigidity of structure on the viral envelope in the absence of any cellular protein; it must have an affinity for itself because it packs closely to form a continuous monolayer (SCHULZE, 1972) but it must also have a special capacity to associate with lipid, presumably via hydrophobic amino acids. The other side of the molecule probably has an affinity for NP or viral RNA or both, for in this way the M protein — which can be viewed as the outer layer of the viral core as well as the inner layer of the envelope — could serve as the main point of attachment for RNP. If the helical coil visible in some virions (ALMEIDA and WATERSON, 1967, 1970; SCHULZE et al., 1970; NERMUT and FRANK, 1971; NERMUT, 1972) is in any way typical, the RNP is arranged in the virion in a very ordered fashion, more like a coiled spring than a tangled mass of thread. The surplus space in this large plastic virion is probably occupied by fluid since the particle collapses on drying (NERMUT and FRANK, 1971), and there is potential danger in allowing the RNP to float free. Even if the RNP is fragmented and possesses no helical symmetry (SCHULZE et al., 1970; SCHULZE, 1972; COMPANS et al., 1972), there are reasons for believing that it is bound to M. Physical attachment of RNP to the layer of M laid down beneath the spikes in a segment of altered plasma membrane would be the most certain way of ensuring the budding of a complete virion.

The nucleoprotein NP most certainly has an affinity for viral RNA, both + and − (SCHOLTISSEK and BECHT, 1971), and may link the several RNA molecules together (PONS, 1970). The hypothesis has also been put forward that protein(s) P, which may be a component of the virion transcriptase, is

firmly bound to a polymerase-attachment site at the 3′-end of each separate piece of RNA and might even link them together.

The glycoproteins HA and NA have biological functions which are well known. The hemagglutinin peplomer specifically adheres via its distal end to erythrocytes of many species and to similar glycoprotein receptors present in the plasma membranes of a wide range of infectible cells. The neuraminidase cleaves neuraminic acid off a variety of glycoprotein inhibitors present in mucus, serum and other body fluids that would otherwise compete for virus with the receptors on cells. In addition, both peplomers carry exposed antigenic determinants that elicit the production of immunoglobulins in the animals they infect; antibodies directed against HA block the attachment of virus to cells and hence neutralize infectivity and inhibit hemagglutination. The constituent glycoproteins, HA_1, HA_2 and NA, all have a strong tendency to aggregate via hydrogen or ionic bonds with molecules of the homologous class (LAVER, 1971; STANLEY and HASLAM, 1971; LAZDINS et al., 1972; ECKERT, 1973), while HA_1 and HA_2, or NA and NA (NA_1 and NA_2) are tenaciously linked by disulfide bonds (LAVER, 1971; SKEHEL and SCHILD, 1971; STANLEY and HASLAM, 1971; BUCHER and KILBOURNE, 1972; KENDAL and ECKERT, 1972; LAZDINS et al., 1972). It is presumably no accident that these proteins are rich in the particular amino acids through which carbohydrate side chains are known to be attached (LAVER and WEBSTER, 1966; LAVER, 1971; KENDAL and ECKERT, 1972; LAVER and BAKER, 1972). The function of this carbohydrate is unknown. It is becoming increasingly apparent that most membrane proteins, particularly those secreted through membranes, are glycoproteins. Carbohydrate is especially abundant in HA_1 and at least some of it contributes to the hydrophilic tip of the spike (LAVER and WEBSTER, 1966; BECHT et al., 1972; KLENK et al., 1972a). On the other hand, much of the carbohydrate in NA is situated near the base of the peplomer (LAZDINS et al., 1972; ROTT et al., 1972) which suggests that NA (and HA_2) may attach to the lipid of the envelope via carbohydrate-carbohydrate interaction. Glycolipid is certainly present in influenza (KLENK and CHOPPIN, 1970; KLENK et al., 1972a). Whether in this way or by virtue of a hydrophobic region of the protein at the base of HA and NA peplomers, it appears that these viral proteins dictate the choice of host lipids for construction of the viral envelope (BLOUGH and MERLIE, 1970; KLENK and CHOPPIN, 1970; BLOUGH, 1971).

X. Gene: Gene-Product Relationships

The very existence of viruses with divided RNA genomes indicates that the system must offer some evolutionary advantage. The necessity for punctuation points in RNA or post-translational cleavage of proteins would be abrogated only if each RNA fragment corresponded to a single gene; larger fragments would seem to be an unsatisfactory compromise.

On the basis of a comparison of the MWs of influenza RNAs and proteins it has been proposed that each molecule of vRNA does indeed represent a single

gene (WHITE et al., 1970). Definitive proof for such a hypothesis must await the *in vitro* synthesis of viral proteins using as templates monocistronic mRNA molecules transcribed from individual vRNA fragments. In the meantime we can examine the available data on the number and MWs of the individual viral RNA and protein molecules.

Table 3 indicates that the MWs of the known viral proteins range from about 90,000 to about 25,000 (and perhaps a nonstructural protein of 11,000). The exact number of virus-coded proteins is still somewhat uncertain. If NS is virus-coded, which is highly probable but still unproven, the minimum current estimate of the number of primary gene products would be six (P, HA, NA, NP, M, NS) and the maximum nine (the six plus a second P, a second NA, and a second NS (MW 11,000). HA_1 and HA_2 are not primary gene products, since they arise by post-translational cleavage of HA; it has not been excluded that NA could also be a cleavage product. Needless to say, the carbohydrate moieties of the viral glycoproteins, HA and NA, are not coded by viral RNA.

Turning to the nucleic acids, uncertainty persists about the precise number and MWs of the individual fragments. In recent years estimates of the number have gradually crept up from three (DUESBERG and ROBINSON, 1967), to five (DUESBERG, 1968), to six (PONS and HIRST, 1968; CONTENT and DUESBERG, 1971; SKEHEL, 1971c), to seven (LERNER and HODGE, 1969; BISHOP et al., 1971a) or even more, perhaps as many as ten (ETCHISON et al., 1971; LEWANDOWSKI et al., 1971). It seems reasonable to assume that all the viral RNA molecules have the same polarity and that each of the complementary RNA molecules transcribed *in vivo* contains meaningful information, since there is no self-annealing of vRNA on extraction from the virion (PONS, 1971; SCHOLTISSEK and BECHT, 1971), cRNA extracted from polyribosomes includes molecules capable of hybridizing to all classes of vRNA (PONS, 1972), and each class is unique (CONTENT and DUESBERG, 1971). Estimates of the MWs of the individual RNA fragments have also crept up slightly over the years. The earliest of the determinations based on SDS-acrylamide gel electrophoresis gave the MW range as $2–7 \times 10^5$ daltons (DUESBERG, 1968; PONS and HIRST, 1968), whereas more recent estimates are higher: $3.5–10.5 \times 10^5$ (BISHOP et al., 1971a), $3.4–9.8 \times 10^5$ (SKEHEL, 1971c), $4.5–10.0 \times 10^5$ plus small amounts of lower MW material (LEWANDOWSKI et al., 1971). Direct length measurements from electron micrographs gave values convertible to a MW range of about $2–8 \times 10^5$ for A/WSN and $3–10 \times 10^5$ for A/X-7 (LI and SETO, 1971).

If we accept that an RNA codon comprising three nucleotides totalling just over 10^3 daltons codes for an amino acid of MW just over 10^2 daltons, it can be seen that the MWs of the set of RNA molecules are in the right range to code for the known viral proteins. If anything, the viral RNA molecules seem slightly larger than might be expected; this tendency is best illustrated by comparing the MWs of the two smallest RNA molecules 3.5 and 3.5×10^5 (BISHOP et al., 1971a) or 3.4 and 3.9×10^5 (SKEHEL, 1971c) with those of the small viral polypetides, M and NS (2.5×10^4 [Table 3]). Perhaps future

refinements in methods of determining the MW of nucleic acids and proteins may indicate whether the currently available estimates of the former are too high or the latter too low, but it should also be appreciated that a proportion of the bases in each gene presumably code for a ribosomal binding site, a polymerase attachment site, ando ther nucleotide sequences quite unrelated to the message itself.

XI. Questions for the Future

Influenza research has come a long way in the last three years, yet many questions remain unanswered. Some of them may be briefly listed. Let us begin with the one that is infinitely the most important from the practical point of view.

What is the nature of the HA antigenic site? Does the tip of the peplomer carry only one type of antigenic determinant accessible to neutralizing antibody or a wide spectrum of overlapping sites?

What is the precise sequence of steps leading to the glycosylation of HA and NA? What is the role of this carbohydrate?

How are the viral-envelope proteins HA, NA and M inserted into the plasma membrane? How are existing cellular proteins supplanted? How are the lipids selected and rearranged? What holds the peplomers to the lipid bilayer? What triggers the process of budding?

What is the real structure of the RNP? Are the several RNA molecules linked in the virion, and if so, how? Do they remain associated with RNP during transcription and/or replication?

How many polymerases are there? How are they coded? Is protein(s) P a polymerase, or a polymerase subunit or sigma factor? What is the role of NS and why does it accumulate in the nucleolus? How are viral and cellular transcription and translation controlled?

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Immunology and the Melanomas

MARTIN. G. LEWIS

With 13 Figures

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I. Introduction

Tumours arising from the pigmented components of skin, mucous membranes and the choroid of the eye, collectively known as the melanomas, have over the years stimulated a tremendous amount of interest in biologists, clinicians and pathologists for a variety of reasons. These rather striking and usually well recognizable lesions have caused controversy from the point of view of the

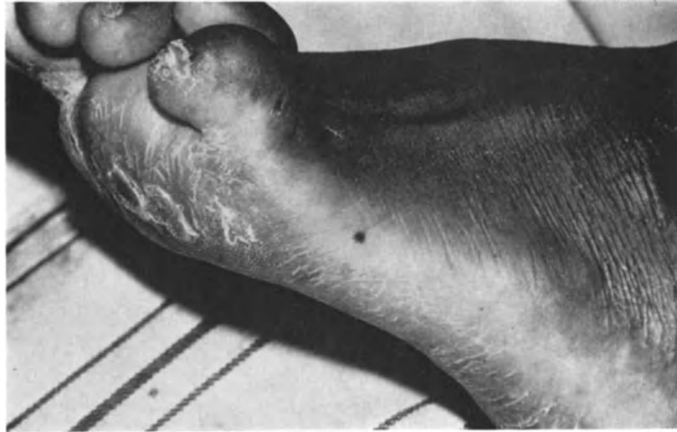


Fig. 1. Benign pigmented mole (junctional naevus). The precursor of malignant melanoma

origin of the cells in question embryologically and histogenetically and in the very nature of the neoplasms that arise in them. On one hand they represent one of the commonest neoplastic processes known to man, namely the benign pigmented moles and naevi (Fig. 1), and on the other a highly variable and often extremely malignant tumour whose ability to metastasize widely throughout the body has few rivals in the field of oncology (PACK, 1948; EWING, 1922). It is, therefore, an ideal group of tumours in which to study the relationships between immune mechanisms and neoplasia, since the advantages of having such a wide spectrum of biological activity are considerable. Added to this one can study such superficial and easily observed tumours from their earliest appearance to the very wide spread metastatic disease that they often represent ultimately.

II. Historical Perspectives

Although this group of tumours has been known since antiquity (URTEAGA and PACK, 1966) the recognition of them as a particular group of neoplasms has often been attributed to the French physician, Laennec (1806), who is said to have given then the name la melanose. Even at this stage in the history of this tumour, considerable and sometimes quite heated controversy arose since Dupuytren also clamis to have given this particular name to the tumour (COCHRAN, 1965). Despite this, the famous English anatomist, John Hunter, clearly recognized this as a specific and separate group of tumours some considerable time before the two Frenchmen were engaged in this argument (LEWIS, 1968). Certainly in the middle 19th century several observers noted with considerable interest the variable natural history of this pigmented tumour (WARDROP, 1830; FERGUSSON, 1851) and several speculated as to the possible explanations (CARSWELL, 1838; COOTE, 1846; EVE, 1903; EISELT, 1861). It was early recognized that the tumour could apparently present as

nodules in lymph nodes. In most cases this was erroneously attributed to de-Novo origin of melanoma from lymphoid tissue (EBERMAN, 1896). DUBREUILH (1892–1912) and HUTCHINSON (1892–1894) almost simultaneously became aware of the fact that at least one variant of this group of tumors, occuring usually on the exposed surfaces of more elderly patients, had much less of a tendency to metastatic spread and in many instances a local or complete involutional regression occurred. This condition, known variably as melanosis or Hutchinson's melanotic freckle, has recently been extensively reviewed by Clark and his colleagues (CLARK and MIHM, 1969) and will be discussed, particularly in its relevance to immune mechanisms, later in this article.

III. Host Factors

Probably one of the earliest informative attempts at establishing the role of the host in the development and natural history of the melanomas was brought about by the very careful observations of Sampson-Handley. He carefully showed lymphoid infiltration in melanomas and beautifully worked out the lymphatic spread and showed evidence of regression of melanomas, (SAMPSON-HANDLEY, 1907) in fact, bringing together the evidence of the 19th century and pointing towards the development which will form the major part of this article. Since these early writings, various other observers have noted the variable natural history of melanoma (COLEY and HOGUET, 1916) with particular reference to two important observations; the first and most dramatic of these being the phenomenon of spontaneous regression (EVERSON and COLE, 1966; TODD et al., 1966; SMITH and STEHLIN, 1965; SUMNER and FORAKAR, 1960). This has, of course, been observed in many other tumours apart from melanoma but it is of considerable significance that this tumour has such a relatively high rate of spontaneous regression and is in this respect in a group of tumours including choriocarcinoma; hypernephroma; Burkitt's lymphoma; and neuroblastoma, all of which have subsequently had very good evidence of tumour-specific antigens and immune response of the host directed against them. For instance, in melanoma it has been shown that the spontaneous regression rate accounts for 15 % of all cases of spontaneous regression in human tumours, whereas this particular form of cancer represents only 1–3 % of all malignancy (KOPF, 1971). The second phenomenon is the ability of the tumour to remain localized for considerable periods of time in some individuals (MACDONALD, 1963; ALLEN and SPITZ, 1953; WILBUR and HARTMANN, 1931). In many instances, tumours of a particular limb wax and wane so that new tumours arise as previous ones locally regress. This has been largely brought to light by the work of Bodenham who refers to this as smouldering disease (BODENHAM, 1968, 1972). This , in fact, has been a very important stimulus to the whole approach of tumour immunology in the field of human malignancy. Studies in Ugandan Africans, in whom melanoma arises from the plantar surfaces of the feet in most cases (Fig. 2), clearly showed three types of host-tumour relationship: Group 1, where the tumour remained localized for many

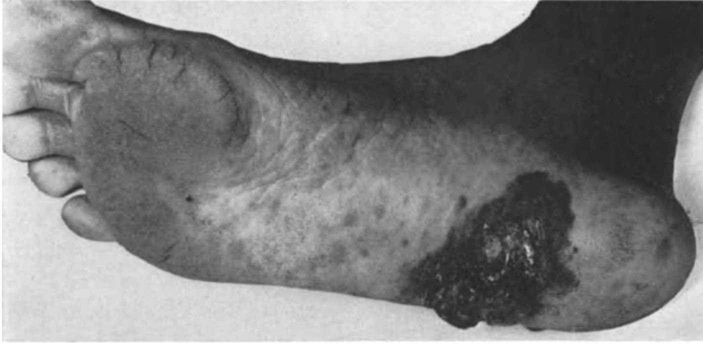


Fig. 2. Malignant melanoma on the plantar surface of an African. (From LEWIS 1973, with permission of Springer-Verlag Heidelberg)

months or years, group 2, with rapid dissemination within weeks or months, and group 3, with regression of the primary tumour with ramaining lymph node metastases (LEWIS and KIRYABWIRE, 1968). The age, sex, tribal distribution and histopathological appearances did not explain these differences in biological behaviour of the tumours, and the suggestion was made that some form of host reaction existed. Following this period of time, in which such a vast body of clinical and pathological data had accumulated, strongly suggesting that there was more to the natural history of melanoma than the mere inherent variability of the tumour itself. The discovery of tumour-specific antigens in animals (BALDWIN, 1955; FOLEY, 1953; PREHN and MAIN, 1957) led to the early attempts at demonstrating some form of immune response of individual patients and groups of patients against their tumour cells. This has now developed into a considerable accumulation of data wherein evidence has indicated antigenicity of melanoma cells different from the host normal cells, various types of circulating antibody, and collectively what has been referred to as a cellular immunity.

Although there are many ways of reviewing and discussing the rapidly accumulating data on immune reactions in patients with malignant melanoma, the way in which this is most likely to produce valuable, important, and meaningful results will be if it can relate to the natural history of the disease, explain some of the variables and anomalies and, of course, ultimately lead to an understanding of the tumour itself and particularly ways in which the host can modify the tumour. This, it is hoped, will set the ultimate scene for manipulation of the immune response even in those cases where previously this has been overcome and metastases caused the death of the patients. In addition, various immune mechanisms can be important tools in understanding other aspects of the biology of the melanomas and these will be referred to very briefly.

Although there are many references in the literature (HAMILTON FAIRLEY, 1969) to some form of immunity, either antibody or cellular, in malignant

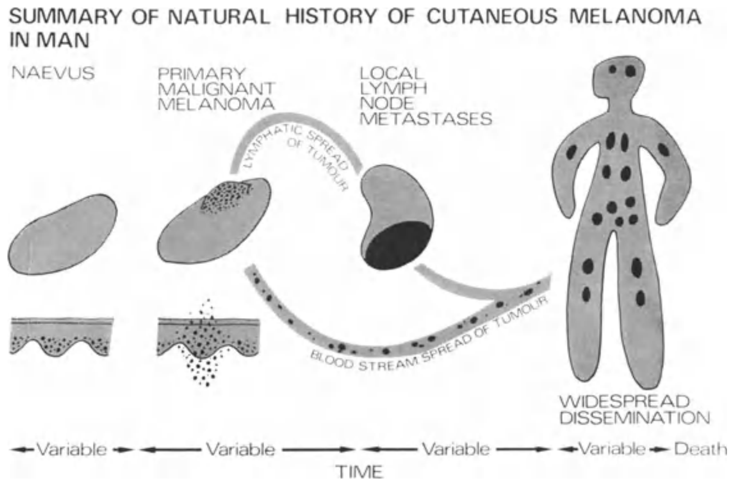


Fig. 3. Schematic representation of the natural history of human cutaneous malignant melanoma

melanoma (PIESSONS, 1970; KOPF, 1971), there is often no reference made to the particular stage of the disease at the time at which the tests were done. The need is not only to demonstrate antibodies or lymphocytes against tumour cells, but to relate particular antibodies and particular cellular immunity to the stage in the natural history of the disease. I would, therfore, like to commence by reviewing the evidence that immunity is involved from the very commencement of this disease and follow it through to those patients with advanced metastatic malignancy. For convenience, the natural history of this tumour, which of course is similar to many malignant tumours, is summarized in Fig. 3, with key recognizable moments marked. It is upon this format that the evidence of immunological mechanisms will be positioned, where appropriate.

IV. The Earliest Stages of Malignant Melanoma or Mole to Melanoma

The most difficult stage in this disease and yet probably one of the most fundamentally important is the situation where the tumour is first manifest as malignant. In many instances preceeding this stage, there is a benign abnormal collection of melanocytes known either as a naevus or a mole. There is considerable controversy still as to how many melanomas necessarily arise in such naevi (DAWSON, 1925; ALLEN and SPITZ, 1953; LENNOX, 1960; MCGOVERN, 1972). This is more than of passing interest, in that future development in this field may well be assisted by the tools developed in the need to study the immunology of melanomas. A certain start has been made in this area by studying histological evidence of cellular immune mechanisms in the relationship between benign pigmented moles and early malignant melanomas (COCHRAN,

Table 1. Test for antibodies in the sera of patients with a number of skin lesions, showing only positive reactions in melanoma. (From LEWIS 1973a, with permission of J. B. LIPPINCOTT Co.)

HISTOLOGICAL CLASSIFICATION OF SKIN LESION	TOTAL NO TESTED	IMMUNOFLUORESCENCE RESULTS	
		NO. +VE	NO. −VE
BASAL CELL CARCINOMA	62	1 pigmented B. C. C.	61
BASAL CELL PAPILLOMA	17		17
SQUAMOUS CELL CARCINOMA	6		6
KERATOACANTHOMA	6		6
EPIDERMOID - SEBACOUS CYSTS	15		15
SENILE KERATOSIS	10		10
PIGMENTED NAEVI (various)	14		14
JUVENILE MELANOMA	3		3
PRIMARY MELANOMA	4	4	
MISCELLANEOUS	26		26
TOTAL	163	5	158

1972; LITTLE, 1972). In another study to establish the degree of specificity of antibodies detectable against the cytoplasmic components of malignant melanoma cells, (described in more detail later) sera from patients with other pigmented lesions, including several examples of active or very cellular moles and naevi, the vast majority had no antibody detectable against melanoma antigens (Table 1). There was however, one interesting exception which may be of considerable importance in the understanding of early malignant melanoma; namely the condition of the halo or Sutton naevus syndrome. This was described before the turn of the century and is characterized by the fact that a particular pigmented mole may develop a symetrically arranged halo of depigmentation and subsequently disappear completely (SUTTON, 1916). In some situations more than one halo naevus may be present in any individual patient and numerous crops of such have been reported (Fig. 4). Histologically, the appearances are that of an active melanocytic tumour in which dense lymphocytic infiltration occurs and subsequently the entire lesion may regress leaving only histiocytes containing melanin. In a recent report serum from such patients with halo naevi were shown to contain antibody which cross-reacts with the cytoplasm of allogenic malignant melanoma cells and suggests that the phenomenon of the halo naevus may well be related to or may mimic spontaneous regression of established malignant melanoma (LEWIS and COPEMAN, 1972). In fact, it may well be that this is an exaggerated form of a more

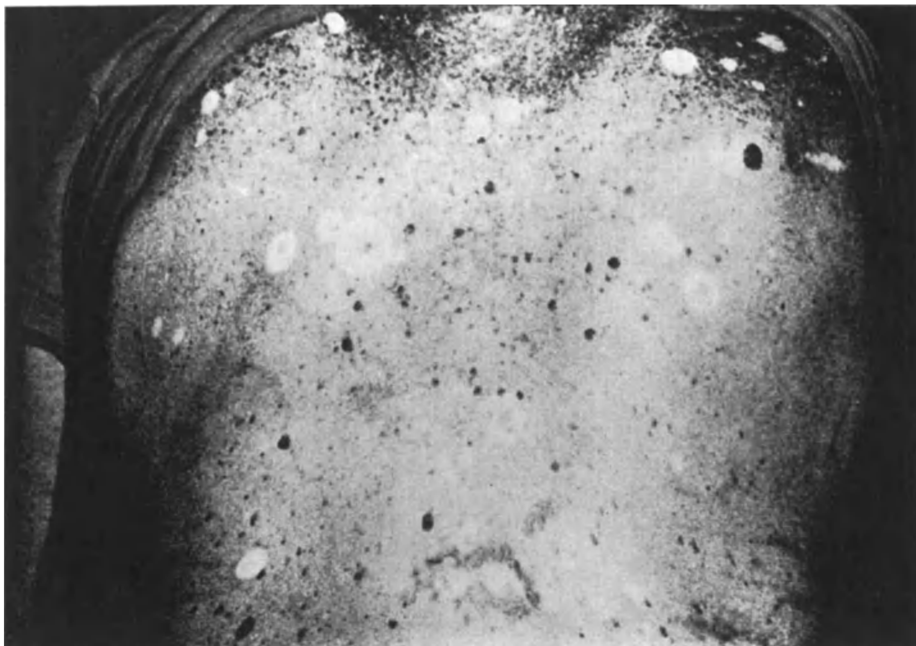


Fig. 4. Multiple "Halo-Naevi", showing pigmented moles surrounded by zones of depigmentation. (Photograph kindly supplied by Dr. W. H. CLARK of Temple University, Philadelphia)

frequently occuring phenomenon for it has still not yet been explained why moles are extremely common and melanomas relatively rare and still what determines the malignant change and the establishment of a melanoma. Therefore it is of importance that such immunological methods should be applied in this area, since it is the original establishment of this tumour which will determine the subsequent natural history. The early recognization of malignancy in a naevus and early malignant melanoma has always been a difficult problem diagnostically. In recent years, however, CLARK and McGOVERN and others (CLARK, 1967; CLARK and MIHM, 1969; McGOVERN, 1970; LITTLE and DAVIS, 1969) have established very reasonable criteria for recognition of early malignancy and in a recent Conference in Sydney, Australia, (International Cancer Conference), an agreed classification was adopted. This classification which clearly separates three types of melanoma with different biological behaviour patterns and different histological appearances opens up the possibility of studying the immune reactions in these types of tumour at this very early stage. Techniques are at present being adapted so that tests of antibody and cellular immunity can be applied to these very early lesions. There are, however, well established way of observing host immunity which do not require living cells, or the technicial problems of fluorescent antibody techniques and lymphocyte cultures and it would be clearly foolish to ignore observations which can be made with such simplicity. One of these is the histological

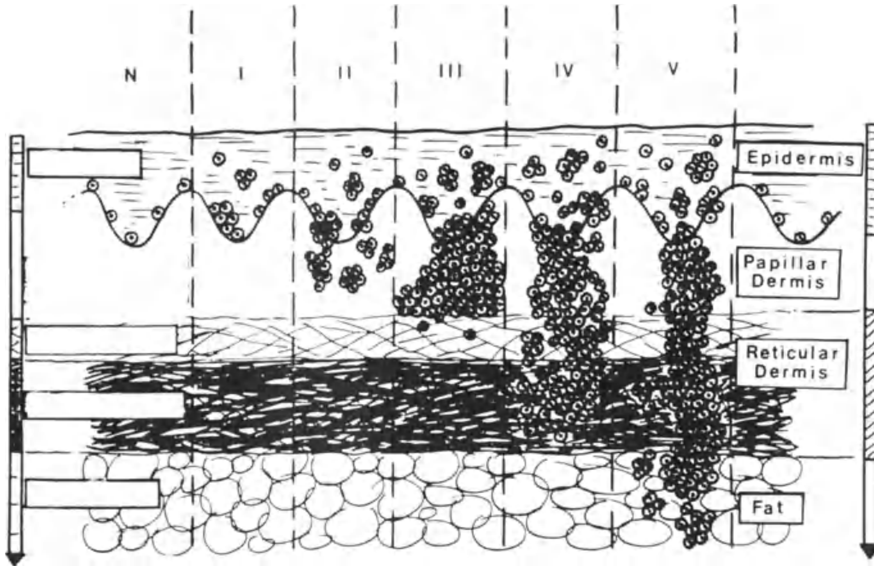


Fig. 5. Diagrammatic summary of the stages of invasion of cutaneous melanoma after CLARK and MIHM. (From BAILLY, 1972)

appearance of early tumours and the recognition of the host responding cells present in them. The round cell infiltration of various types of lesions has been recognized by pathologists for over 100 years, in many cases without realizing what the round cells were doing or what they were. There is, of course, even now with all the sophistication of modern research into the lymphocytes still considerable doubt as to exactly the function of these cells under varying conditions. Nevertheless, in malignant melanoma it has been recognized for many years that some lesions, particularly early ones, have dense lymphocytic and monocytic infiltrations around and within the tumour (COUPERUS and RUCKER, 1954). In some recent studies carried out by a number of observers an attempt has been made to semiquantitate and therefore to correlate the lymphocytic infiltration at this early stage of the disease with subsequent effect on the natural history, either in the form of delay of metastases or in terms of ultimate survival (LITTLE, 1972). Although a relatively crude method, and containing pitfalls, it has nevertheless clearly indicated that early malignant melanomas often have dense host cell responses and that if the tumour is excised at this stage the prognosis is good. If the tumour remains, often only a little larger to a later stage, the lymphocytes appear to melt away and the prognosis is considerably worse. These studies have recently been elaborated upon by CLARKE (1973) and his colleagues where even the difference between early infiltration of the epidermis and the appearance of tumour cells in the reticular dermis, fractions of a centimetre away, make all the difference, and can be clearly shown to influence the subsequent development of this tumour. This same correlation with cellular immunity is much more difficult to establish later on

in the disease, particularly if the complications of ulceration and infection are present (LEWIS and KIRYABWIRE, 1967). *In vivo* tests of cellular and tumour immunity, described later, have also been performed in patients with early melanoma by using allogenic melanoma cells in studies where no autologus cells were available (LEWIS et al., 1973). The stages of invasion in early malignant melanoma are summarized in Fig. 5.

V. Established Invasive Primary Melanoma

The next stage in the development of malignant melanoma usually consists of some nodular or at least appreciably enlarged tumour with invasion of the dermis and in some cases surface ulceration and extension locally (Fig. 6).

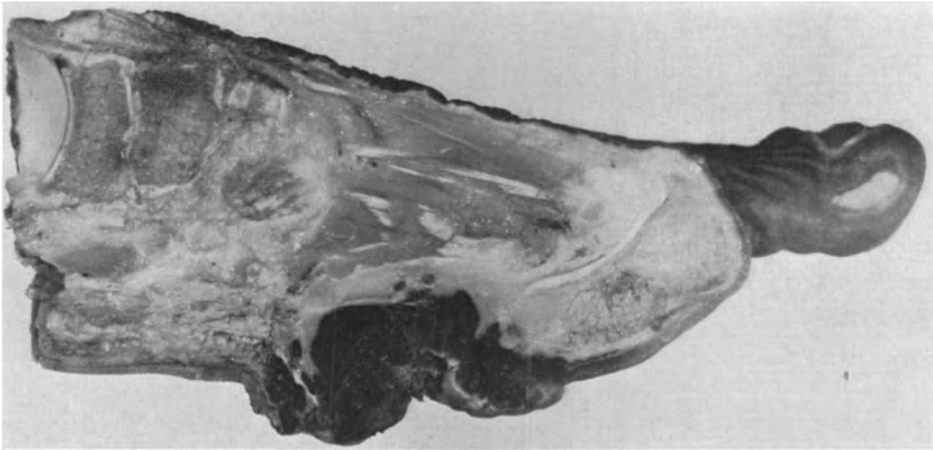


Fig. 6. Section of foot showing invasive heavily pigmented malignant melanoma

Under these circumstances it is possible to obtain autologous tumour cells and with these various types of immunological investigation have been carried out. These have included the use of either extracts of such tumour or tumour cell preparations as a source of antigen with cutaneous injection and the presence of delayed (HUGHES and LYTTON, 1964) or immediate hypersensitivity reactions noted in the skin of individual patients (STEWART, 1969). In some cases homogenates of the tumour have been used and in others irradiated or formalin treated cell preparations or subcellular fractions of such tumours (FASS et al., 1970). The results have, in fact been variable but in many cases, particularly in this still relatively early stage of the disease, positive skin reactions have been obtained (ZIEGLER et al., 1969). Normal skin as a control (KOPF, 1971) in such experiments has not always been used, but is obviously essential if one is to be sure of the specificity of such reactions (BLUMING et al., 1972). This topic will be further discussed in a later section, in the context of more advanced stages of malignancy and immunotherapy.

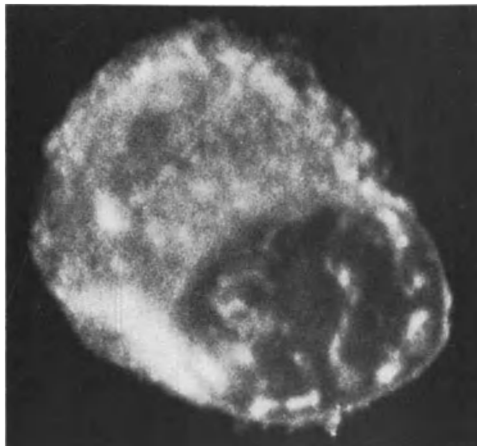


Fig. 7. Immunofluorescence of the cytoplasmic contents of snapfrozen malignant melanoma cell

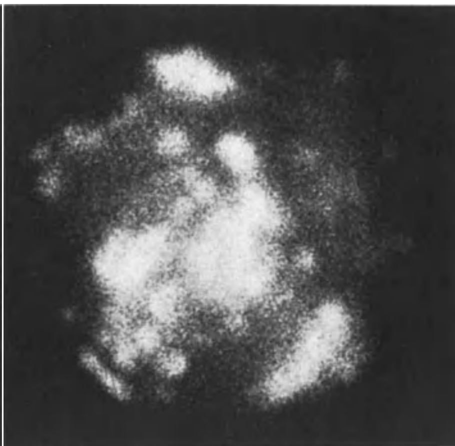


Fig. 8. Immunofluorescence of the surface of a live melanoma cell viewed by epi-illumination

VI. Humoral Immune Reactions in Melanoma

Tumour cells as an antigen have also been used to demonstrate the presence of circulating antibody. Various types of antibody antigen reaction have been demonstrated in this particular tumour; one such method was to demonstrate by means of the fluorescent antibody technique (COONS and KAPLAN, 1950) localization of immunoglobulin components of serum from such patients against fixed cell preparations (Fig. 7) of autologous cells. (NAIRN, 1969; LEWIS et al., 1969; PHILLIPS and LEWIS, 1970; MORTON et al., 1968; MUNA et al., 1969). In addition, viable tumour cells have been used as substrate, using the technique of surface membrane immunofluorescence (Fig. 8) where the antibody antigen reaction occurs at the cell surface (KLEIN et al., 1966; PHILLIPS and LEWIS, 1970). In order to carry out any of these procedures, sufficient viable tumour cells are needed and presents a variable problem, particularly if the tumour is still so early that only a small number of cells can be obtained. Some investigators have used trypsin and other enzyme preparations to prepare the cell suspensions needed for these procedures, whereas others have avoided the use of such enzymes in view of the possibility of alteration of surface antigens which may be critical to the test concerned (LEWIS and PHILLIPS, 1972a; PHILLIPS, 1971). Many of these variable methods of preparing cells may well account for some of the rather conflicting results reported. Another method of demonstrating antibody has been to demonstrate in some individuals what appears to be complement-dependent cytotoxic antibodies (LEWIS, 1967; NAIRN et al., 1971). These have been shown in a number of ways using cells in tissue culture, either freshly removed from the patient or initially grown in such cultured conditions. Again, the variety of methods of doing such cytotoxicity tests have produced variable results

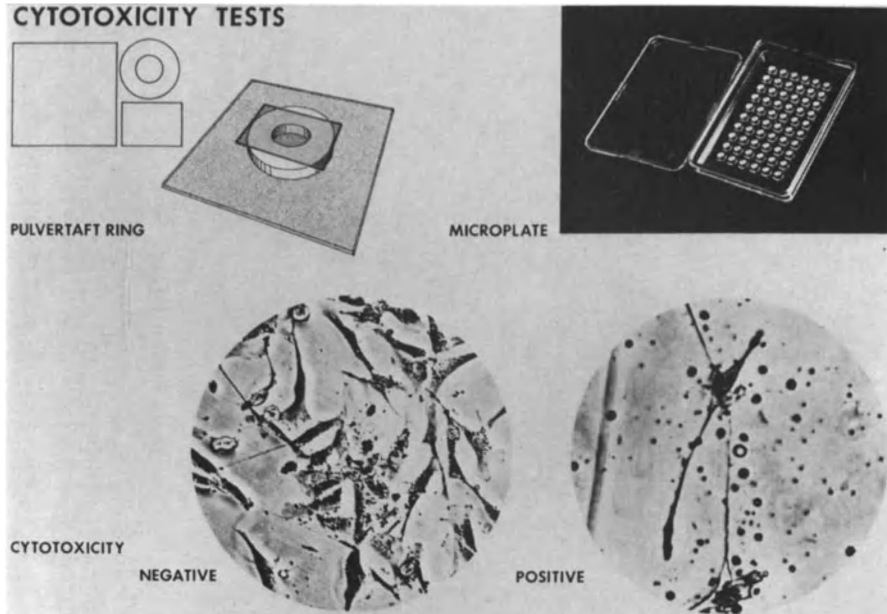


Fig. 9. Some examples of in-vitro cytotoxic tests used in the immunological studies of melanoma. (From LEWIS, 1973a, with permission of J. B. LIPPINCOTT Co.)

(ARPELS and SOUTHAM, 1969). In some cases the cells are mixed with the antibody-containing serum and a source of complement after being taken freshly from the patient and then placed in culture dishes made of teflon rings with a coverslip placed on top (PULVERTAFT, 1959). The ability of the cells under these conditions to attach to glass coverslips and to remain attached compared with controls, constitute the main feature of the test (LEWIS et al., 1969). Under these conditions, the effect on the cells, results in them falling from the glass into the culture medium, sometimes two or three days after the addition of antibody and complement. In other test systems, cells are allowed first to attach either to glass coverslips or to the wells of micro-test plates and then serum and complement added and after washing, the number of cells remaining attached to the wells are measured compared with the controls (HELLSTROM et al., 1968). Some of the methods described are summarized in Fig. 9. There are many variations in between these techniques, but it is obvious that one may well be measuring several different phenomena. In many ways the word cytotoxic may indicate more than one process, the important feature being that cells which are already attached to glass may well have the important areas of their surface already inaccessible to both antibody and complement. The concentration of antibody and complement under various culture conditions, particularly in view of the amount of culture fluid available to the cells may also be very critical. The use of dye-exclusion procedure such as the trypan-blue test to indicate cell death also has important problems in view of the fact that cells which are trypan-blue positive are not necessarily dead but

damaged and may well recover if kept in culture for long enough. In one particular series where individual sera were tested against autologous cells by both membrane immunofluorescence and cytotoxicity a very close correlation occurred so that an individual serum usually demonstrated antibodies by both techniques or was negative by both techniques (LEWIS et al., 1969). There is a certain degree of controversy regarding the specificity of the reactions under these circumstances. Some authors have indicated what appears to be multiple cross-reactivity, using membrane fluorescence particularly (MORTON et al., 1968), whereas in another series the reactions were considered to be largely patient-specific any cross-reactivity being seen at very much lower levels of concentration (LEWIS et al., 1969; LEWIS and PHILLIPS, 1972a). In the cytoplasmic antibody antigen reaction, cross-reactivity occurred on a multiple scale and at a high titre throughout (PHILLIPS and LEWIS, 1970). The cytotoxicity test using the Pulvertaft ring technique as described, where the cells fall from glass, indicates a remarkable degree of patient-specificity. Other cytotoxicity tests have occasionally indicated however, a different degree of specificity. It is obvious that the standardization of the method of testing is critical to the interpretation of these results. The other possibility that different antigen are involved, must also be considered (LEWIS et al., 1973).

VII. Evidence for Tumour Specificity of Antibody-antigen Reactions in Melanoma

Regarding the evidence that these antibody antigen reactions really are tumour-specific, various critical experiments have indicated that the antibodies, for instance, can be absorbed out effectively with homogenates of the tumour whereas homogenates made of the patient's own skin, melanin granules and other tissue component could not reduce the antibody reaction either by means of cytotoxicity or by means of immunofluorescence (Table 2). (LEWIS et al., 1969). In addition, the positive sera tested against other tumour cells and other human cells of a variety of types indicate that the reactions only occur significantly with the cells of malignant melanoma (MUNA et al., 1969; LEWIS, 1973; RHOMSDAHL and COX, 1970). The same type of absorption techniques have also been used to demonstrate further evidence for the patients unique specificity of some of these reactions. A series of autologous melanoma cells and corresponding sera were used and only the autologous cells could reduce the positive serum reaction which was still positive when absorbed with the cells of other patients and reapplied to the original autologous cells (Table 3) (LEWIS and PHILLIPS, 1972a). It has been possible on the basis of this and recent preliminary experiments to separate antibodies in an individual sera against the surface component antigens compared with the antigens of the cytoplasmic contents (LEWIS and PHILLIPS, 1972b). It is possible if not likely that there are more than one group of antigens representing both surface and the cytoplasmic content. This has recently been suggested even more so by the finding of negative reactions in the serum of patients with very early malignant

Table 2. The specificity of antibody antigen reactions in melanoma by absorption with a variety of tissues, as well as tumour tissue. (From LEWIS 1973a, with permission of J. B. LIPPINCOTT Co.)

MATERIALS USED IN ABSORPTION	MAXIMUM TITRES OF ANTIBODY FOLLOWING ABSORPTION				
	MEL 1	MEL 2	MEL 3	MEL 4	MEL 5
BOVINE LIVER	1/32	1/32	1/32	1/32	1/32
RAT LIVER	1/32	1/32	1/32	1/32	1/32
MELANIN GRANULES	1/32	1/64	1/32	1/32	1/32
HUMAN SKIN (NON PIGMENTED)	1/32	1/64	1/32	1/32	1/32
HUMAN SKIN (HEAVILY PIGMENTED)	1/32	1/32	1/32	1/32	1/32
HUMAN SKIN (POOLED)	1/32	1/64	1/32	1/32	1/32
AUTOLOGOUS SKIN	1/32	1/64	1/32	1/32	1/32
ALLOGENEIC MELANOMA EXTRACT	1/8	1/8	1/16	1/8	1/16
AUTOLOGOUS MELANOMA EXTRACT	1/2	0/0	0/0	0/0	0/0

Table 3. Cross absorption of positive antibodies containing sera detected by membrane immunofluorescence with autologous and allogeneic melanoma cells, showing that only the autologous cells can significantly reduce the reaction. (From LEWIS and PHILLIPS 1972a, with permission Editor International Journal of Cancer)

CROSS ABSORPTION EXPERIMENT

MEMBRANE IMMUNOFLUORESCENCE

Patients Cells	Corresponding Patients Serum						
	Law	Sh	Jas	Dow	Rug	Co	Lie
Law	1/4	1/64	1/64	1/32	1/32	1/64	1/32
Sh	1/32	1/4	1/32	1/32	1/16	1/32	1/64
Jas	1/32	1/32	1/8	1/32	1/32	1/64	1/64
Dow	1/8	1/8	1/16	1/4	1/16	1/8	1/16
Rug	1/32	1/32	1/64	1/32	1/4	1/64	1/32
Co	1/32	1/32	1/32	1/32	1/32	1/8	1/32
Lie	1/64	1/32	1/32	1/64	1/32	1/32	1/4

melanomas against allogeneic rather than autologous tumour cell preparations. Since in many of these cases it was not possible to test the autologous serum and cells one cannot be certain that this lack of antibody against the allogenic cells is particularly significant. It might suggest that more than one antibody

antigen system are in action as the tumour progresses from this very early stage to its well established stage. For instance, in situations where there is sufficient tumour to test the autologous serum against autologous cells and where positive results are obtained the positive serum also appears to be positive against a variety of allogeneic melanoma cells from other patients. Recent results indicate persistence of antibodies against allogeneic antigen — even in patients with more widespread tumour. One series showed the presence of antibody in 20% of sera from normal patients (MORTON, 1970). Our own findings are not in complete agreement, possibly due to the dilutions of sera used in the immunofluorescence test.

VIII. In vitro Tests of Cellular Immunity

It has already been stated that observations of lymphocytic infiltration are of some significance in this very early stage of melanoma and that this is the situation where most of the positive results of skin testing have occurred. The in vitro tests, however, have produced a certain amount of conflicting and confusing information. This again is probably related to the variety of technical methods used and to the type of cellular immunity which is being measured. In some cases, the tumour cells have simply been used as an antigen source to produce blasto-genic response in lymphocytes taken from the patient, so called lymphocyte transformation (SAVEL, 1969; NAGEL et al., 1971; JEHN et al., 1970). In some recent experiments this has been challenged to some extent by the finding that normal tissue also will have some appreciable degree of blastogenic ability against autologous lymphocytes (KLAVINS et al., 1971). The use of the lymphocyte migration-inhibition technique whereby the ability of lymphocytes to migrate through capillary tubes is altered by the presence of antigen to which they are sensitive, has also given some conflicting results though in fact in most situations the positive results were obtained again in the patients with these relatively early stages of melanoma. For instance some workers have, however, pointed out at least one danger in this particular test system in that patients recovering from anaesthetic procedures also have a rather nonspecific suppression of the MIF and other tests and that, therefore negative results have to be considered very much in the light of the detailed knowledge of the other procedures carried out on the patient (MACKIE et al., 1972). COCHRAN (1972) showed positive inhibition of leucocyte migration in 10 out of 16 patients with tumour localized in the primary site, but only 1 out of 6 positive in patients with dissemination. Little, for instance, is known about the effect of drugs, cytotoxic drugs particularly, in this respect.

IX. Lymphocyte Cytotoxicity

One of the most direct methods of measuring cellular immunity is to demonstrate that lymphocytes taken from a patient can cause cytotoxicity against the tumour target cells and not against control cells. This has been achieved in a number of ways in malignant melanoma. The majority however

depending on first, plating out tumour cells in either plastic microtest plates or the smaller Terasaki microtest plates, then adding the lymphocytes and after a variable period of time washing away the lymphocytes and either demonstrating cytotoxicity by means of trypan blue uptake or the target cell removal from the wells with washing (HELLSTROM et al., 1971; FOSSATI et al., 1971; DE-VRIES et al., 1972). Others have used fresh cells with noculture selection prior to the test (CURRIE, et al., 1971). There appears to be some controversy as to the relationship between positive results obtained by methods of lymphocyte cytotoxicity and the stage of the disease. In some cases there appears to be no relationship between cytotoxic lymphocytes and early or disseminated melanoma (HELLSTROM et al., 1971; DE-VRIES et al., 1972), whereas other authors have clearly shown that this type of cell mediated immunity is easily demonstrated in the early or localized stage of disease or where the mass of tumour is small but that in disseminated or large tumours, lymphoctye cytotoxicity is much more difficult to demonstrate or absent (CURRIE et al., 1971). As is so often the case with laboratory techniques, one has to make a qualitative judgement on quantitative data which is often difficult to reproduce. The studies by DE-VRIES and his colleagues although showing no correlation with stages at any one moment in time, did show in individual patients a change with progression of disease and resultant fall in lymphocyte cytotoxicity (DE-VRIES et al., 1972). Some light has recently been thrown on this by some work reported by the Hellstroms where they showed as before, that no difference could be found in the cytotoxic effect of lymphocytes from patients with growing melanoma or from patients who had become clinically tumour free following therapy when a high ratio between lymphocytes and target tumour cells was used. When, however, titrations were made with various lymphocyte doses, it was clearly shown that patients with advanced melanoma demonstrated less reactivity than patients with small tumour load or with localized melanoma (HELLSTROM and HELLSTROM, 1973). The problem of suitable controls in lymphocyte cytotoxicity is still a difficult area and from time to time normal tumour free individuals clearly demonstrate markedly effective lymphocyte cytotoxicity against the target tumour cells (DE-VRIES et al., 1972). It is also of some interest that lymphocyte cytotoxicity as measured by the usual techniques has often been reported as showing much less unique patient specificity than that described in antibody studies (HELLSTROM et al., 1971; DE-VRIES et al., 1972; FOSSATI et al., 1971). There have been studies however again with fresh tumour cells where a lack of cross reaction was seen (CURRIE et al., 1971). This again may be due to the fact that lymphocytes are recognizing a number of antigens on the surface of cells and are not descriminating between those which are patient specific and those which are common antigens. An interesting aspect of lymphocyte cytotoxicity by normal tumour free individuals has recently being reported. It has been shown that 7 out of 9 healthy black North American negros had lymphocytes which were cytotoxic to cultivated malignant melanoma cells but not to tumour cells of other types or to normal skin fibroblasts (HELLSTROM et al., 1973). The possi-

bility that this represents a situation where lymphocytes are recognizing closely related antigens involved in pigmentation needs to be clarified and of course the possibility that these patients were, in fact, or had in fact, some form of autoimmune disorder. No such non tumour immune reactions were seen in the humoral antibody studies in Ugandan Africans (Lewis, 1967), but autoantibodies of other types were seen in a number of African sera, causing confusion in a study of cross reaction patterns in melanoma (Lewis et al., 1969).

X. Macrophages in Human Melanoma

There is a growing awareness that macrophages may play an important role in some aspects of tumour immunity and this subject from the animal experimentation point of view has been reviewed in detail recently. It is well known that pigment containing macrophages, the so called melanophores, are often a prominent feature in many examples of melanoma both in man and in animals. Recently evidence has been presented that tumour phagocytosis may also be a feature of the natural history of human and animal melanomas (Currie et al., 1971; Lejeune, 1973). In a recent publication a patient was described who experienced spontaneous remission of a malignant melanoma lasting three years and a biopsy, an imprint preparation from lymphnode metastases, showed a remarkable degree of tumour phagocytosis. Material examined from 37 other melanoma patients without a history of tumour regression, showed only 4 patients with some degree of tumour phagocytosis (The et al., 1972). An interesting animal model to be mentioned in more detail later, the Hardy-Passey melanoma has also been shown to be the useful method for examining the relationship between macrophages and tumour cells in melanoma. The exact relationship between macrophages, tumour cells, circulating antibody and lymphocytes has yet to be determined, although there is some evidence from the work of Evans and Alexander (1972) that a specific macrophage arming factor is present, produced by spleen cells in animals immunized with their own tumour cells. Antibody which could be eluted from tumour cells in the lymphnode metastasis, was shown to induce phagocytosis of cultured melanoma cells. This observation has certain resemblance to the specific macrophage arming factor postulated by Evans and Alexander. It is of interest that in some recently reported experiments lymphocytes or lyphoid cells obtained from a lymph node draining a localized malignant melanoma had no cytotoxic effect when added to the tumour cells in culture chambers whereas, the peripheral blood lymphocyte taken from the same patient did show some degree of cytotoxicity (Nairn, 1972). The critical experiment of whether lymphoid cells from lymph node not draining the tumour were also abnormal was not commented on in this series.

XI. Colony Inhibition Techniques of Cellular Immunity

Another method has been to measure the effect of lymphocytes and serum on the ability of cells to clone in culture and produce colonies. This so-called

colony inhibition technique has been used in a variety of human tumour situations including malignant melanoma (HELLSTROM, 1967) and in some cases the results obtained appear to show some correlation with the early stage of the disease and in others none at all. One of the technical problems, however, involved in this is that the cells have first to be established in culture. Unfortunately not all melanomas will, in fact, produce colonies and the really early localized tumours, particularly of the skin, are notoriously difficult to establish in culture.

XII. Stage 2 of Malignant Melanoma

The next stage of development of malignant melanoma usually results in either a recurrence in or around the site of the original tumour following surgical excision or a progression of the tumour locally in the form of satellite nodules and during this period of time the appearance of metastases in the draining lymph nodes (Fig. 10). In most series where this intermediate stage is recognized the various tests for immunity have been probably the most variable. This is not in many ways surprising since it is during this change from localized tumour prior to widespread dissemination that the least is known concerning the step by step progression. In many instances, the serum still contains positive antibodies detectable against the melanoma cells both by membrane fluorescence, cytoplasmic fluorescence, and cytotoxicity (LEWIS et al., 1969; MORTON, 1971). In addition there is now, often, relatively large amounts of tumour so that methods such as extraction of tumour antigens (JEHN et al., 1970; NATHANSON et al., 1971), complement fixation (MORTON et al., 1970), immunodiffusion (MCKENNA et al., 1964; CZAJOWSKI et al., 1967), immunoelectophoresis, halmagglutination (FINNEY et al., 1960) and various other techniques can be used. In some instances, investigators have still reported positive cellular immunity of a variety of types, including lymphocyte cytotoxicity, though to a much lesser degree than those earlier stage 1 cases as mentioned previously. In some individuals during this stage of the disease vast amounts of tumour may appear and grow and yet remain localized. It is during this time that the phenomenon described by BODENHAM (1968) of "smouldering" or chronic malignant melanoma becomes apparent. It is possible for a whole limb, for instance, to be filled with melanomatous deposits and yet remain in such a state for months or years before even the first sign of disseminated tumour elsewhere is apparent. In several such patients detailed and more closely timed immunological studies have indicated the following: The presence of circulating antibody is not related to the volume or mass of the tumour present but to the degree of localization or dissemination (LEWIS, MCCLOY et al., 1973). Patients with these vast amounts of localized tumour may still have antibody in contradistinction to the negative sera seen during the disseminated phase, even though this represents smaller total mass of tumour. Patients in this stage 2 have antibody levels which fall as the disease progresses and secondly the fall in antibody may well in some cases precede the appearance

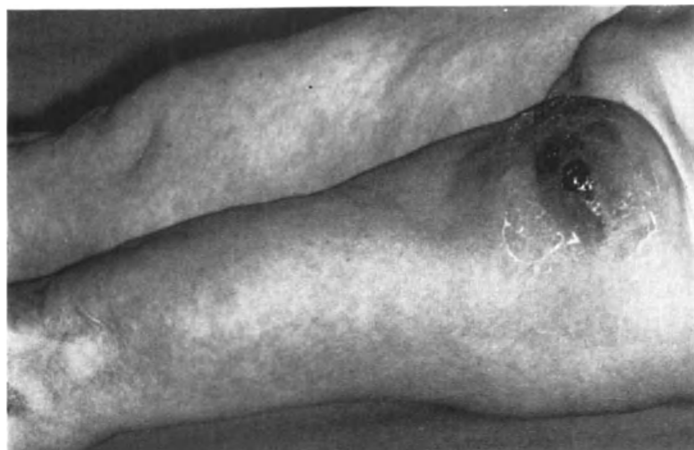


Fig. 10. Secondary spread of malignant melanoma to the lymph node of the groin

of more distant spread of tumour by weeks or months. It is, of course, very difficult to be absolutely sure that these individuals had not already clinically undectable metastases, but in many cases this has been satisfactorily shown not to be the case. One area of speculation here would be that although tumour cells have already been circulating during the entire natural history of this disorder right from the early stage, the settling out of such tumour cells and the formation of clinical metastases is related to the presence or absence of circulating antibody. There is some supportive evidence from experimental animal systems, in that an immunologically specific factor in lymph and blood, appears to prevent lung metastases in rats with sarcomas (PROCTOR et al., 1973). In addition humoral antibodies have also been shown to exert a controlling effect on progression of feline sarcomas (ESSEX et al., 1971). In most instances, during this stage of the disease the cellular immunity in most of its measured forms has been variable as described earlier. There have been reports, however of re-emergence of both cellular and humoral immunity — resulting from immunization procedures which will be described in more detail later in the review. In addition a recent study has revealed what appears to be an increase in lymphocyte cytotoxicity — following extensive "washing" of peripheral blood lymphocytes (CURRIE, 1972).

XIII. Delayed Hypersensitivity Reactions

Investigations of other aspects of patients immune responses, particularly during this more advanced stage of malignant melanoma, have produced some confusing and in some cases, conflicting result. The ability, for instance, of the patient to react to non-specific stimuli such as BCG, DNCB and other skin reacting allergens may well be supressed or remain intact (SIEGLER et al., 1972). It is obvious that at very advanced cachectic individuals may well

have non-specific immunological supression but there is also indication that patients carrying tumour loads may in some respects be abnormal (MORTON et al., 1970). This, however, is by no means universal and there have been clear indications that patients in stage 2 of malignant melanoma or even in stage 3 may well have normal and intact immunity, not only non-specifically but also against extracts of their own tumour cells (ZIEGLER et al., 1969; FASS et al., 1970).

XIV. Stage 3 or Disseminated Malignant Melanoma

The ability of this particular group of tumours to widely desseminate throughout the organs and tinssues of the body is almost without parallel. It is possible for the patient to have so much metastatic disease that melanin liberated into the bloodstream causes such intense pigmentation that the entire patient takes on a dusky coloured hue. Melanin may be in the urine is such quantities in these cases that the urine turns black. The study of the various metastatic patterns of melanoma has been extensive over the years and there is almost no tissue or organ of the body that has not been recorded as having metastatic malignant melanoma (DASGUPTA and BRASEFIELD, 1964). It is of some interst that organs which are relatively uncommonly involved in metastatic desease of other types frequently show these metastases in the 'black cancer'. Examples such as the gall bladder, small intestine, the mucosa of the stomach, the spleen are typical (LEWIS, 1968). Whether there is some form of immunological relationship between the metastatic pattern in this tumour compared with others is open to speculation but is an area in which some research is obviously needed. As in the stage 2, patients vary tremendously in the amount of tumour deposits which may be present and still not produce death. Multiple cutaneous metastases, particularly in Europeans, are extremely common. In this respect, again, it is a most convenient tumour in which to study biological behaviour and various aspects of host resistence, since in many cases readily available tissue is obtainable. In most studies, however, in these advanced stages, tumour associated immunity is almost universally decreased to the point of total unrecognition. In most of the larger series, antibodies are usually not detectable at all at this stage (MORTON et al., 1970; LEWIS et al., 1969), with exception of a study reported by MCBRIDE (1972) where antibodies against Nucleolar structures were seen in patients with disseminated melanoma. Although some reports have indicated cellular immunity may still be present, usually this is also suppressed (HELLSTOM et al., 1973; CURRIE et al., 1971). In the very advanced stages many other non-specific immune reactions, as previously described, may also be suppressed in addition. Despite this, complete spontaneous regression and subsequent recovery may even occur in these very advanced stages and these have been well documented (EVERSON and COLE, 1966). One of the most classical examples of all of delayed metastases in human oncology is the malignant melanoma, usually of the intra-occular type, which presents with metastatic disease, usually in the liver anything from ten to thirty years following enucleation of

the eye with no evidence in the interim period of tumour present. Studies on human intra-occular melanoma have revealed tumour specific antibodies against those tumours — and not seen when tested with normal choroidal melanocytes (FEDERMAN et al., 1973). Again antibody has so far only been detected in localised and not disseminated tumours.

Summarizing the situation, with regard to the relationship between various types of host tumour immunity and the natural history of the disease, the following generalizations can be made from the collective literature, as pointed out there being exceptions in many cases. The presence of both cellular and humoral immunity appears to be strongest in the early stage of the disease. In the case of circulating antibody, this has been followed in many patients over the course of the progression of the disease and antibody levels appear to fall as the disease progresses. In more detailed studies the suggestion has been made that the antibody levels fall prior to dissemination and overt clinical metastases beyond the region of origin. It appears, therefore, to be very likely that there is a ralationship between both humoral and cellular immunity, probably at different stages of the disease, to the actual staging and natural history of the melanoma in man. This, of course, raises many important questions, some of which will be considered in the next sections.

XV. Other Tumour Associated Antigens in Melanoma

Antibodies and antigens in malignant melanoma, not necessary associated with tumour-specific antigens have also been described. In one series of high level of autoantibody against smooth muscle has been reported in patients with malignant melanoma and indeed in patients with other tumours as well (WHITEHOUSE et al., 1971). The suggestion that this is related in some way to membranes of the tumour antigens has not entirely been confirmed by other series and the final interpretation of this phenomenon awaits further evidence and experiment. The other situation of some interest is the relationship between so-called fetal or embryonal antigens and tumour antigens, a rapidly increasing subject which will only be mentioned very briefly here in its relevence to melanoma. The question of what appears to be the common cytoplasmic antigen in malignant melanoma which can cross react with a number of different patients suggested that this groupspecific type antigen might be related to fetal antigens in a similar way perhaps to the carcioembryonic antigen described for tumours for the G.I. tract (GOLD and FREEDMAN, 1965; GOLD, 1967). In an attempt to investigate this procedure, some preliminary data has shown that an antiserum raised against human (fetal components) in rabbit subsequently highly purified by absorption with adult tissues showed a reaction with fetal cells and a reaction with the membrane of some melanoma cells. This same serum had no reaction against adult cells and equally interesting, the positive patients' serum, although producing positive reaction against autologous melanoma membrane, had no reaction against fetal cells. In addition, it was possible to block by specific immune blocking, the positive autolo-

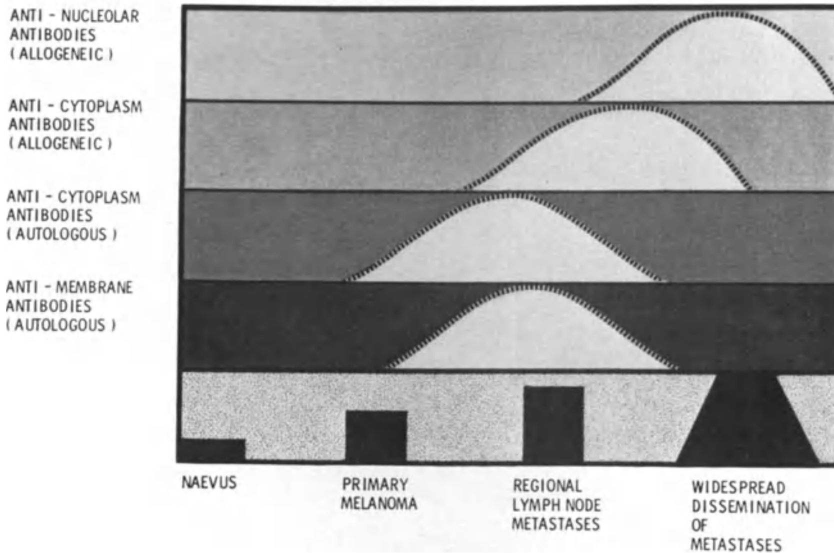


Fig. 11. A possible time relationship between the stages of the disease and antibodies against some of the melanoma antigens. (From LEWIS et al., 1973b, with permission Ed. YALE J. of Biol. and Med.)

gous melanoma sera and still produce a reaction with the anti-fetal serum and vice versa, indicating that although fetal antigens may under certain circumstances be expressed on the surface of these cells they were not necessarily to be confused with tumour specific antigens (AVIS and LEWIS, 1973). So far, however, this anti-fetal serum has produced no reaction with the cytoplasmic components of melanoma cells, although it has with a number of teratomas and embryomal tumours. The various groups of antigens and their possible time sequences in terms of antibodies response has recently been summarized (LEWIS et al., 1973) and are shown in Fig. 11.

XVI. Experimental Immunotherapy of Malignant Melanoma

(Immunization of Patients with Malignant Melanoma)

Although the word "therapy" here might well be regarded with certain skepticism, the need to produce some additional support in the treatment of patients with advanced malignant melanoma is obvious and since surgery, radiotherapy and chemotherapy are of limited value in this respect it was natural that as soon as any evidence pointed towards some form of host resistence that an attempt to boost this resistence would be forthcoming. Experimental immunotherapy of malignancy is by no means new and was introduced earlier on in this century, and has been extensively reviewed (HADDOW, 1965; HADDOW and ALEXANDER, 1964; FEFER, 1971). In this review I will attempt to present from the main reports the approach to experimental immunotherapy

in malignant melanoma with particular reference to the way in which this also adds further light on the host tumour relationship and attempts to answer some of the questions posed in early parts of this survey. It is usual to divide immunotherapy to specific and non-specific, although combinations of the two are now much more in vogue.

XVII. Specific Active Immunization

This procedure has usually involved the use of autologous melanoma cells, usually given by subcutaneous or intracutaneous injection. In most cases the cells are rendered incapable of division by some means, the most effective being irradiation (IKONOPISOV et al., 1970; KREMENTZ et al., 1972) or neuraminidase trated melanoma cells (SIMMONS, 1972). Various means of measuring the effect of such procedure have been reported. The most difficult of all and yet, ultimately, the one that, from the point of view of therapy, is most important, has been the survival rates of patients treated in this manner and careful clinical correlation. There have been varying reports, some extremely optomistic, some extremely pessemistic, on the effectiveness of these various attempts at immunotherapy (HUGHES et al., 1970; KREMENTZ et al., 1972; HUMPHREY et al., 1971; VAN-DEN BRENK, 1969). This is hardly surprising in view of the variability of the natural history of melanoma, even in this advanced stage. In many cases, no selection of patients was used, in others the patients were highly selected and the amount of tumour present at the time varied from small or no residual tumour to vast amounts. More consistent results have, however, been obtained using some of the laboratory procedures previously described. In several series antibodies have been detected in the blood stream following such autoimmunization procedure, measured either by immunofluorescence (IKONOPISOV et al., 1970; LEWIS et al., 1971; CURRIE et al., 1971), cytotoxicity or by means of complement fixation (MORTON, 1970). In most cases, however, the antibodies were short lived, often only lasting a matter of two or three weeks (LEWIS, 1972) (Fig. 12). In one series an individual, patient was given multiple autoimmunizations with irradiated autologous melanoma cells with antibody response following every time more than 200 million cells were given but if the immunizing dose was dropped to less than 100 million no response was obtained. Further repeating with 500 million again showed a clear rise in antibody (IKONOPISOV et al., 1970). Cytotoxic lymphocytes have also been detected following the same sort of procedure, although no close parallels were seen with antibody in the same individuals (CURRIE et al., 1971). Despite these results, there have been very few indications that remaining solid deposits of tumour have appreciably altered. In fact, in one particular deliberate study the tumour nodules were examined before, during and following autoimmunization with irradiated tumour cells. Although antibody would be detected in the bloodstream or even cytotoxic lymphocytes, the solid tumour deposit had no evidence of coating of immunoglobulin on the surface of the cells (LEWIS et al., to be Published).

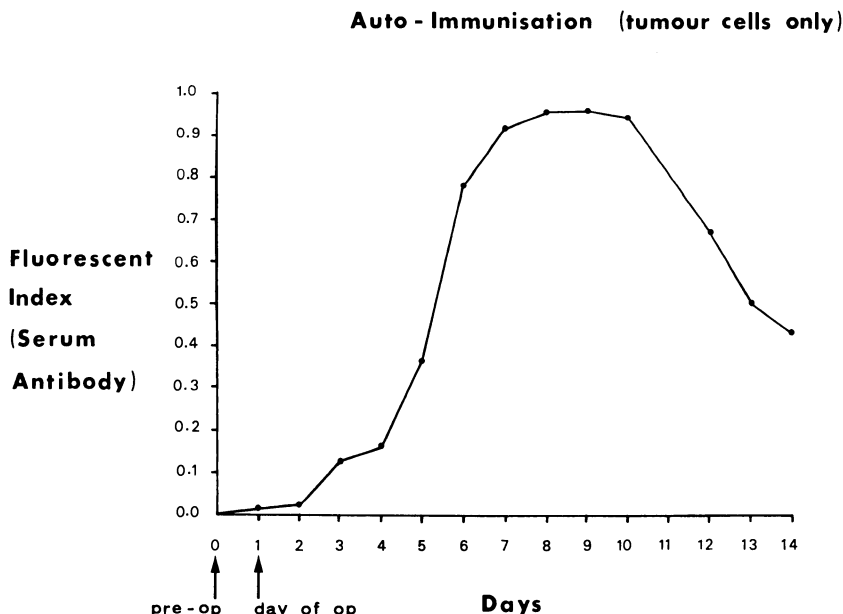
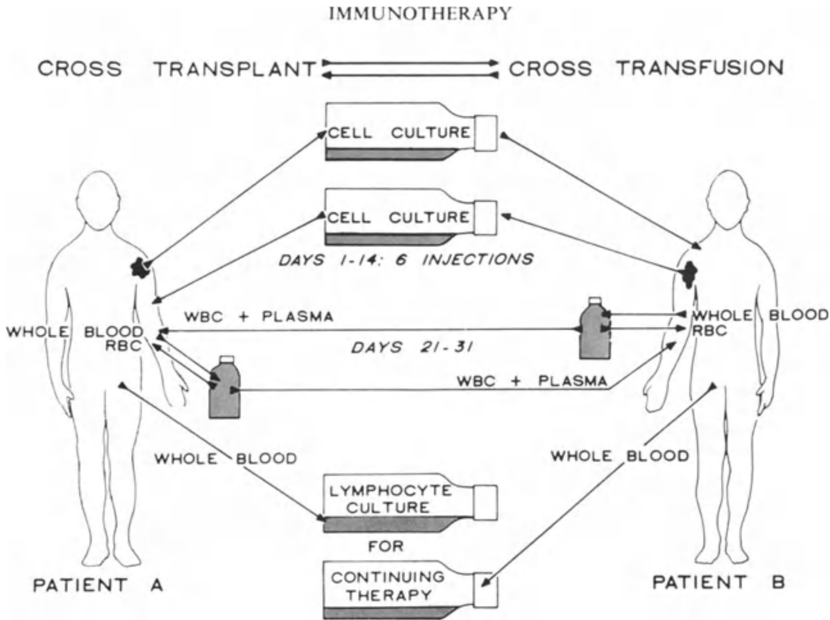


Fig. 12. Autoimmunization with irradiated tumour cells of a patient with stage 3 malignant melanoma, showing transient rise in serum antibody

XVIII. Passive Specific Immunization

The cross-transfusion of serum from patients with regressing melanoma to those with active melanoma has been reported on few occasions in the past (SUMNER and FORAKER, 1960; TEIMOURIAN and McCUNE, 1963), but no large scale series has been attempted in this respect and little evidence is available to commend it. An interesting variation on these themes has recently been highlighted by GHOSE and his colleagues (GHOSE et al., 1967; GHOSE et al., 1972) in the use of heterologous antibody raised against melanoma cells in goat and coupled with chlorambucil. Only one patient with melanoma has so far been reported in these studies, but some lesions apparently regressed. Many theoretical objections including the possibilities of enhancement, blocking of immunity and the possibility that if antibody antigen reactions at such high intensity did occur, deposition in the kidney with subsequent renal damage, have also to be considered (LOUGHRIDGE and LEWIS 1971; LEWIS et al., 1971). Another approach has been to use lymphocytes, in one patient following homologous immunization with another patient's melanoma cells then reinfusion of lymphocytes back into the original tumour donor (NADLER and MOORE, 1969; KREMENTZ et al., 1971; CURTIS, 1971) (Fig. 13). Again, clinical evidence has been quoted for partial and sometimes complete regression, but a large scale study on melanoma over a period of years has not been reported. Attempts have been made to stimulate lymphocytes under culture conditions with antigen from melanoma cells and then vast quantities of such lymphocytes grown in culture transfused into patients with malignant melanoma (KREMENTZ et al.,



Technique of immunotherapy by cross-transplant cross-transfusion using cultured cells.

Fig. 13. Diagrammatic summary of the cross transplant — cross transfusion type of immunotherapy attempts in melanoma patients. (From Krementz et al., 1972)

1971). In some cases, dramatic responses have been reported but additional problems, such as graft verses host reactions and other unpleasant side effects and complications have rendered this approach of some limited value (Moore and Gerner, 1970). The use of reciprocal tumour grafts between two patients with melanoma and subsequent injections of extracts of buffy coat of peripheral blood resulted in regression of tumour in one of the patients (Brandes et al., 1971).

XIX. Non Specific Immunization in Melanoma

The concept of using non specific stimulation of the immune system is by no means new and it was investigated extensively early this century. More recently several investigators have used a variety of adjuvants including BCG, corynebacterium parvum and chemical stimulants such as dinitrochlorobenzene (DNCB) and in addition vaccinia virus in the treatment of local cutaneous melanoma. This whole subject has been extensively reviewed, and I will briefly mention only a few studies which are relevent to the subject of human malignant melanoma.

XX. BCG Vaccination

Various preparations of bacillus calmette guerin (BCG) have been used in an attempt to stimulate immunity of animal and human tumours. The majority

of investigators have used BCG either in the form of scarification of the skin repeated at intervals (MATHE et al., 1969), or by application directly into cutaneous lesions (MORTON et al., 1970; KREMENTZ et al., 1972). There have been some attempts recently in addition to use BCG orally although no large series is yet available for comparison. In a recent series by MORTON and his colleagues, BCG was given into the lesions and there was complete regression in 90% of these lesions. Some of their patients were reported to have regression of nodules which were not injected with BCG. Circulating antibodies to malignant melanoma cells were demonstrated in 5 of the patients studied (MORTON et al., 1970). KREMENTZ and his colleagues also used a very similar technique and again have reported lesions regressing even when they were not directly inoculated with the vaccine (KREMENTZ et al., 1972). In another series 7 out of 9 partients showed objective response to BCG inoculation (NATHANSON, 1971). In the series reported by MORTON (1970) not only were circulating antibodies found in the patients whose tumours regressed but all of them demonstrated delayed tuberculin sensitivity and could be allergically sensitized to DNCB. The patients who failed to respond had no response to either tuberculin or DNCB. Both GOSHE (1972) and KREMENTZ (1972) however report further recurrence of lesions either following the successful BCG vaccination or in the case of GOSHE(1972), new sattelite nodules occurring during the procedure. On some occasions however, these further lesions also responded to subsequent injections with BCG. The use of this vaccine in the stimulation of immunity against malignant melanoma obviously has certain firm foundation but the variation described and the timing of the use of BCG is obviously critical and furthermore systematic studies at various stages of the disease are needed.

Another approach, largely championed by DR. E. KLEIN, has been the topical application of both cytotoxic drugs and sensitization stimulants such as DNCB on melanomas and other skin tumours (KLEIN, 1969; KLEIN, 1973). Many of the patients studied in this way have shown quite dramatic local regressions of their tumours and in some instances evidence of more widespread regression in addition. The principle has been to first sensitize the patient to DNCB and then apply the DNCB to the cutaneous nodules, the result being a markedly exaggerated hypersensitivity reaction involving the tumour with its subsequent destruction.

The virus of vaccinia has been shown to have a particular destructive effect on cutaneous deposits of malignant melanoma (BURDICK and HAWK, 1964). In one series reported by MILTON and LANE-BROWN (1966), not only did the lesions inoculated with vaccinia regress, but other lesions in addition showed some degree of regression. The series reported by HUNTER-CRAIG and his colleagues showed a marked regression of lesions inoculated but they did not confirm that other lesions responded (HUNTER-CRAIG et al., 1970). The question that naturally arises from these reports is whether the vaccinia is acting in some direct way by causing cytolysis of the melanoma cells under the local conditions or whether there is the possibility that vaccinia in its effect of immunizing the patient is causing again a local immunologically mediated reaction. The

virus of Rabies has in the past also been utilized in much the same way as vaccinia, with claim of partial tumour regression of up to 10 years duration (PACK, 1950; HIGGINS and PACK, 1951).

Another form of non specific stimulation of immunity in patients with malignant melanoma has recently been investigated, namely the use of phyto-hemagglutinin. This was given intravenously to a young boy with multiple cutaneous and subcutaneous metastases, and resulted in many of the nodules regressing, with pain, heat and swelling, preceeding this phenomenon. The tumour cells were shown on biopsy to be coated with IgG and there was marked lymphocytic infiltration of the Nodules (LEWIS et al., 1971). This is at present under further investigation.

Whatever techniques of autoimmunization have been used, the same basic problem remains and that is that it does not explain why the immunity in the first place was ineffective. The fact that both antibodies and lymphocytes can show a re-emergence of activity following autoimmunization does, of course, indicate certain important facets.

XXI. Some Unanswered Questions

In view of the evidence for tumour specific antigens in human malignant melanoma, and the awareness and response by the individual patients against such antigens, the inevitable question arises as to why in the first place a small number of tumour cells appear to overcome what in some instances is a vastly superior number of cells of the lymphoid tissue. This same question, of course, is fundamental to all of tumour immunology but in malignant melanoma we may well have a good model on which to study this question since, as previously mentioned, this is one of the few human tumours in which recognition of very early stage is possible and the ability to examine serial sections of such early lesions with the application of some of the more recently developed laboratory procedures for immunology investigations obviously is of some considerable importance. In recent publications a great deal of emphsis has been placed on at least one mechanism which accounts for failure of cellular immunity, particular in this early stage of disease, namely the production of what appears to be blocking substances (HELLSTROM et al., 1971; SJOGREN et al., 1971). Whether these are antibody or antibody antigen complexes is still a question at issue. These blocking phenomena have also been investigated by a number of people in malignant melanoma. The second question is why do the antibodies during the subsequent stages of the disease fall when evidence points towards at least some of the antibodies being of considerable importance in delaying metastases. There are several possible explanations, including the concept that a vast amount of antigen present in disseminated disease can soak-up, as it were, antibody. There are many theoretical reasons why this is less likely to be the case and there is little evidence that the tumour deposits, particularly the cutaneous ones, which are easily available for study, show any such soaking-up or coating of antibody. Tumours removed from pleural effusions, ascitic

fluid or lymphnodes have, however, been shown by elution methods to have immunoglobulin on their surfaces (THE et al., 1972). In addition, the relationship between the presence of antibody and the volume or mass of the tumour is against this in that the only relationship clearly defined has been between the degree of localization rather than the amount of tumour (LEWIS, MCCLOY et al., 1973). Finally, the evidence suggesting that the antibody falls prior to dissemination adds further support to the concept that the soaking-up of antibody by antigen is not the only explanation. The problem, however, of circulating antigen in a soluble form liberated by the tumour in its development, is a much more difficult one. It is very likely and, indeed, has been shown that various types of antigen or product of the melanoma cell may be liberated (JEHN et al., 1970). Indeed, this is the basis on which melanemia and melanuria occur. The presence of such circulating antigen has recently been demonstrated very clearly and the ability of this antigen to transform the patient's lymphocyte and the lymphocytes of some other patients is of some significance (JEHN et al., 1970; COCHRAN et al., 1972). In some other investigations, however, what appears to be an anti-idiotype antibody has also been demonstrated against the tumour specific antibody, also explaining the fall in antibody as the disease progresses (LEWIS et al., 1971). It is still quite possible, however, that this antibody could be an antibody antigen complex in which the antigenic component is very firmly attached and very small. This last proviso is important in that the blocking substance demonstrated in these experiments could not be separated from IgG by all the techniques available. It is very likely that there is more than one mechanism involved in this rather complex situation in which a malignant tumour overcomes the immunological defenses of a host which are otherwise perfectly adequate and perfectly intact and capable of recognizing the tumour antigens. For instance, to assume that all can be explained on the basis of circulating antigen, it is necessary to determine the type of antigens which are liberated into the blood stream by a growing tumour at various stages in the natural history of the tumour. In melanoma, for instance, it is now known that at least six groups of antigen occur (LEWIS et al., 1973); namely, (1) autologous membrane, (2) autologous cytoplasm, (3) allogeneic membrane, (4) allogeneic cytoplasm, (5) autologous nucleolar (MCBRIDE et al., 1972), (6) fetal or carcinoembryonic (AVIS and LEWIS, 1973). The liberation of the internal or cytoplasmic antigen would obviously be more likely to be related to volume or mass of tumour or degree of necrosis, this liberating more of such antigens. The membrane or tumour specific surface antigen, however, is the one most likely to be involved in the neutralization of circulating tumour-specific antibody. It is obvious that one of the fruitful lines of future research is to be able to demonstrate and to categorize the different antigens present and to relate them in much more detail to the patient's staging of the disease in the same way as antibody and cellular immunity have been tackled. The third and perhaps most important question of all is, can an understanding of the immune mechanisms in this particular tumour assist in the switching on or reemergence of a complete and satisfactory immunity against the tumour? If

one, for instance, were to consider the possible immune mechanism involved in spontaneous regression, if this could be mimicked then obviously a very dramatic approach to cancer therapy would be developed. Very few cases of spontaneous regression have been studied in enough detail for this mechanism to be clearly defined. It is worthwhile here noting again that spontaneous regression of very early cases is probably much more common than we have previously realized and that this may be a very fruitful area of research into these mechanisms. The halo naevus syndrome, mentioned earlier, being one possible important area. Although less dramatic, probably a more practical approach would be at least to attempt to control metastases. It is, after all, the metastases in most cases of malignancy which causes the disease to be a problem and the local excision of tumours, even melanoma, is largely a surgical problem. If it were feasible to prevent metastases, possibly by maintaining circulating cytotoxic antibodies in the blood stream, then a combined surgical and immunotherapeutic approach might ultimately be the answer. For this to be achieved, the exact timing of both the surgery and the immunological procedures must be very critical. It has been stated on several occasions that immunotherapy is more likely to succeed in cases where there is virtually no residual tumour remaining. If this is the situation, then in melanoma it is important that more and more of the earlier cases are studied and methods determined whereby a boosting of immunity can be achieved earlier or in the stage of disease. For this approach to be successful, detailed cooperation between clinicians and expermentailists is absolutely essential.

XXII. Possible Diagnostic and Prognostic Uses of Immunological Techniques in Human Malignant Melanoma

There is a considerable need in this particular tumour to make accurate and meaningful diagnoses and, since the diagnoses are now being made earlier in the natural history of the disease, the problem becomes even more difficult. If malignant melanomas are excised adequately with wide excision early enough, the prognosis can be very good indeed (BODENHAM, 1968; OLSEN, 1966). Therefore, much is to be gained by early diagnosis and, as a result, more pressure is put upon the pathologists, for instance, for such diagnostic procedure, at a stage usually when there are so few obvious signs of malignancy that he is tested to his limit. Therefore, any form of diagnostic aid would be extremely welcome. One attempt in this direction is to utilize the facts derived from studies of the common type of antigens such as that mentioned in the cytoplasm of melanoma cells. In fact, there are large studies in progress to determine how reliable antibodies detectable against these allogeneic cytoplasmic component are in terms of future development of a diagnostic test for melanoma. In many instances, where the study has so far been carried out, antibodies have not been shown in the serum of patients with a veriety of other pigmented skin disorders, including junctional and compound naevi and the so called juvenile melanoma (COPEMAN et al., 1972). The difficulty is that anti-

bodies may not be present, however, in very early melanomas if not enough tumour cells have liberated enough antigen to stimulate the production of antibody. So that it is quite possible that at the stage when the need for such a diagnosis is most acute, the absence of antibody cannot be used reliably. Therefore, only a positive result can be useful. It is, however, important that such studies are carried out to determine the exact feasibility of such an approach. The detection of small amounts of circulating antigenic components of malignant cells liberated from early tumours is obviously of some considerable importance. Something along the lines of the Carcino embryonic antigen (CEA) (GOLD, 1967) work for gastro-intestinal disease is strongly indicated in skin tumours. Here, of course, the quantity of tumour in the very early stages is again a critical problem and may present insurmountable difficulties in this respect.

XXIII. Animal Models for Human Malignant Melanoma

The need for a suitable animal model to answer many of the questions posed by the studies so far reviewed is beyond question. There are a number of transplantable and spontaneous melanomatic tumours in laboratory animals. For example, the HARDING PASSEY melanoma, (HARDING and PASSEY, 1930), the amelanotic and melanotic melanoma of hampsters (FORTNER, 1961), the B16 mouse melanoma, Melanoma S91 on mice (CLOUDMAN, 1941), and the Mexican Kilifish (GORDON, 1948). These have been well documented on numerous occasions, and examples are available in many laboratories. Most of them, however, suffer from the disadvantages that they either do not arise from epidermal melanocytes and, therefore, do not exactly mimic the progression and natural history of the disease in man or they are non-metastatic under normal circumstances (ALGIRE and LEGALLAIS, 1948). In either event, this limits the use for various aspects of the understanding of melanoma in man. In many instances, tumours are merely pigment containing sarcomas and resemble much more the malignant blue naevus of man which is an extremely rare tumour in the human and behaves quite differently from cutaneous melanoma (DORSEY and MONTGOMERY, 1954). There are, however, certain domestic animal with a relatively high spontaneous rate of malignant melanoma, some of which resemble the human tumour in that they are derived from epidermal melanotic cells. These have been extensively studied and reviewed by LEVENE and include the melanomas of dogs, cats, and the grey horse (LEVENE, 1972). The latter is a rather special example of a melanotic tumour in that it is slowly growing, occurs usually in older grey horses, often arising in the perineal region, and may become extensively distributed throughout the body; but LEVENE questions whether these are really metastatic tumours or a form of storage disorder. They certainly behave differently from human melanoma and resemble cytologically and in tissue culture the melanophores (Melanin Laden Macrophages) rather than malignant melanoma cells. There are relatively few studies on the immune response to tumours in these domestic animals

largely due to the difficulty of obtaining suitable animals and studyiug them in depth. More recently melanotic tumours have been described in a variety of swine (Hjerpe and Theilen, 1964; Flatt et al., 1972). One particular varient, occurring in the Sinclair breed of Hormel Miniature Swine, which is also being studied in the author's laboratory, certainly shows histological patterns very similar to that in the melanomas of man (Flatt et al., 1972; Millikan et al., 1973). The tumour arises in a pre-existing pigmente patch which has most of the criteria of a junctional naevus. In some of these animals there is a high rate of malignant transformation in these junctional naevi, but even more interesting and important, the tumours have a high rate of local spontaneous regression. Despite this, however, there have been reports, although limited in number and in scope, indicating that these tumours can become widespread and metastatic and follow a metastatic pattern, again very similar to that seen in man. Kopf and his colleagues have recently reported studies using the B16 melanoma in mice transplanted into C57 black mice (Kopf, 1971). Under these circumstances inhibition of subsequent challenge of tumour was noted and the author suggests that this might be a useful model for the study of at least some aspects of tumour immunity. The limitation in terms of the difference in the natural history of this tumour, particularly in view of its somewhat different ability to metastise, is obviously to be taken into consideration. In a very recent, and as yet unpublished observation in our own studies on the melanoma of the miniature swine, one of these animals during the regression of primary melanoma developed a widespread vitiligo so that the pig, although pigmented originally, is now almost completely white This phenomenon has also been seen in three swine by Millikan (Personal. Communication). This relationship between regression of melanoma and vitiligo has been seen from time to time in human cases (Copeman et al., 1972) and, therefore, presents a very interesting and potential model for the interaction between these tumours and the immune system.

For the study of some aspects of immunity in an animal melanoma, the limitations in terms of the difference in the natural history of this tumour compared with man must, however, be remembered.

XXIV. Summary and Comclusions

Although this intriguing group of tumours has stimulated much in the way of research, from a variety of points of view, with certain leads in the field of tumour immunology, inevitably even more questions have been raised and posed. Nevertheless, a human tumour such as malignant melanoma, with a suitable animal model which can be followed carefully in a detailed fashion and from which there is a vast body of clinical and pathological information to back it, can be of great importance in the understanding of immune mechanisms in malignancy. Indeed the 'black cancer' continues to present investigators with a variety of challenging, and interesting, problems referable to many fields of biological activity including the immunology of tumours.

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Coronaviruses: A Comparative Review

KENNETH MCINTOSH

With 13 Figures

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I. Introduction

The coronaviruses have been recently classified as a separate virus genus on the basis of several fundamental characteristics, which include their nucleic acid type, the presence of a lipid envelope, and, in particular, their distinctive morphology (Tyrrell et al., 1968a). Members of the genus infect a number of different animal species, and until their reclassification were considered to belong to the myxovirus group although they possessed many atypical features. It was through detailed studies of their morphology in negatively stained preparations that they were finally differentiated and set out as a separate genus. When properly prepared, coronavirus particles appear medium-sized, round, and moderately pleomorphic, and bear characteristic widely-spaced club-shaped surface projections. Coronaviruses naturally infect man, chickens, pigs, mice and rats, causing a wide variety of disorders involving a number of different organ systems. Indeed, new species are being added at frequent intervals as the techniques of electron microscopy and modern virology are applied to diseases which have often been clinically recognized for decades. A tentative scheme of the coronavirus genus is shown in Table 1, with a listing of serotypes and strains. The list of types is not complete, but those of importance to this review are shown.

In spite of the wide distribution of coronaviruses in nature, information about their structure, their composition, and the events occurring in infected cells is limited. Until very recently, research into the nature of these organism has of necessity emphasized the highly pragmatic aspects of disease control, rather than more basic concepts of virus structure and function. On the other hand, it now appears clear that more fundamental knowledge is necessary not only for the advancement of science itself, but also for the achievement of many of the desired goals of treatment and control.

It will be the aim of this review to introduce the clinical aspects of coronavirus infections, to discuss the comparative biology of all recognized members of the coronavirus genus and to suggest, by both inference and direct allusion, areas where future investigation may yield useful information. The review is by no means exhaustive, and the reader is referred at intervals to more detailed publications concerning individual species.

II. Brief History and Clinical Aspects of Coronavirus Infections

A. Infectious Bronchitis Virus

Infectious bronchitis virus (IBV) is the cause of a common and highly contagious acute respiratory disease of chickens known as avian infectious bronchitis. The disease was recognized as a separate clinical entity as early as 1931 by Schalk and Hawn (1931), and the responsible agent was first grown in embryonated hens' eggs by Beaudette and Hudson in 1937 (1937). The disease in chickens is characterized by acute respiratory distress, tracheal rales and cough, a precipitous drop in egg production, a deterioration of egg

Table 1. A tentative scheme of the coronavirus genus with a partial list of types and strains

Coronavirus species	Animal host	Coronavirus serotypes	Coronavirus subtypes or strains (if applicable)	Original description
Infectious bronchitis virus (IBV)	chicken	Massachusetts	IBV-41	VAN ROEKEL et al., 1950
			Beaudette	BEAUDETTE and HUDSON, 1937
			IBV-F	ESTOLA, 1966
		Connecticut	IBV-46	JUNGHERR et al., 1956
		Iowa-97		HOFSTAD, 1961
		Iowa-609		HOFSTAD, 1961
		Gray		WINTERFIELD and HITCHNER, 1962
		Holte		WINTERFIELD and HITCHNER, 1962
		Clark-333		COWEN et al., 1971a
		others		
Mouse hepatitis virus (MHV)	mouse	JHM	(subtypes and strains not described)	CHEEVER et al., 1949
		MHV-1		GLEDHILL et al., 1951
		MHV (PRI)		NELSON, 1952
		MHV-3		DICK et al., 1956
		A-59		MANAKER et al., 1961
		others		
Human coronavirus (HCV)	man	229E	229E	HAMRE and PROCKNOW, 1966
			LP	TYRRELL et al., 1968b
			489	KAPIKIAN et al., 1969
		B814		KENDALL et al., 1962; TYRRELL and BYNOE, 1965
		OC43	OC43	McINTOSH et al., 1967b
			OC38	McINTOSH et al., 1967b
		others		
Transmissible gastroenteritis virus (TGEV)	pig	(all are antigenically inseparable)	Purdue	DOYLE and HUTCHINGS, 1946
			New York II	LEE et al., 1954
			SH	SASAHARA et al., 1958
			FS 216/64	CARTWRIGHT et al., 1964
			TO	HARADA et al., 1967
			others	
Hemagglutinating encephalomyelitis virus (vomiting and wasting disease virus) (HEV)	pig	(all are probably antigenically inseparable)	HEV-1	GREIG et al., 1962
			2063/68	CARTWRIGHT and LUCAS, 1970
			67N	MENGELING et al., 1972
Rat coronavirus (RCV)	rat	only one strain characterized	8190	PARKER et al., 1970
Sialodacryoadenitis virus (SDAV)	rat	only one strain characterized	681	BHATT et al., 1972

quality by laying flocks (McDOUGALL, 1968), and a high mortality in young chicks. More recently, strains of IBV have been recovered from chickens suffering from nephrosis and uremia (WINTERFIELD and HITCHNER, 1962;

Hirai and Shimakura, 1971). It thus appears that disease affects many different organs, including the upper and lower respiratory tract, the genital tract, and the urinary tract. At various times during infection, virus can be recovered from lung, spleen, cecal tonsil and kidney (Cook, 1968; Hofstad and Yoder, 1966). In well isolated chickens, infection persists in the trachea for 4 weeks (Cook, 1968). In flocks under field conditions, virus may persist for longer periods (Hofstad, 1947; Cook, 1968). Immunity is probably passed to the young through antibodies in the yolk sac, and offspring of immune hens can be expected to retain some resistance to disease for several weeks (Jungherr and Terrell. 1948),

The diagnosis may be made serologically, using the neutralization test (Fabricant, 1951), a test for precipitating antibody (Witter, 1962; Woernle, 1966), or an indirect hemagglutination test (Brown et al., 1962; Mohanty et al., 1964b). The presence of virus in infected chickens can be determined by direct fluorescent antibody staining of cells in the tracheal epithelium (Bito et al., 1971; Braune and Gentry, 1965), and by recovery of the virus in embryonated eggs. The characteristic effect of IBV on chicken embryos, stunting and urate deposition in the kidneys and ureters, is often not seen for several egg passages. On the other hand, staining of cells in the allantoic fluid with fluorescent antibody (Clarke et al., 1972) and direct electron microscopy of allantoic fluids to detect characteristic coronavirus particles after one or two passages (Marsolais et al., 1971; McFerran et al., 1971) have been recently used for the rapid and specific diagnosis of IBV infection.

Significant disease in poultry flocks occurs worldwide (Kumar, 1971; Estola, 1966; Berry and Stokes, 1968; Stephens and Simmons, 1968), and attempts at control by means of vaccines have met with only limited success. The existence of multipie serotypes has been at least one factor obstructing adequate prophylaxis.

Much of the research in avian infectious bronchitis has been performed with the Beaudette strain (IBV–42), which is serologically similar to the Massachusetts serotype but has been adapted through multiple passages in the embryonated egg to the point that it rapidly kills chick embryos (which other strains of IBV do not) and has lost its infectivity for older chickens. It is not clear whether this virus strain has altered in other more fundamental properties, but this possibility must be kept in mind by those using it for research.

A comprehensive review of avian infectious bronchitis and infectious bronchitis virus has been recently published (Cunningham, 1970). For clinical aspects of the disease, the reader is referred to the chapter by Hofstad in *Diseases of Poultry* by Biester and Schwarte (Hofstad, 1965).

B. Mouse Hepatitis Virus

Modern small animal laboratory research has necessitated the breeding of large numbers of rodents in closed colonies. Murine viruses of low or moderate

pathogenicity have proliferated under these epizootiologic circumstances, often going unnoticed because they could be perpetuated as asymptomatic infections in colonies with a high prevalence of antibody. When "virgin" mouse strains were introduced into such colonies, or when some other stress was added, mortality rates suddenly became high. Murine hepatitis due to mouse hepatitis virus (MHV) was first recognized in a closed mouse colony by GLEDHILL and ANDREWES (1951). In this instance, combined infection with an otherwise harmless murine protozoon, *Eperythrozoon coccoides*, and MHV led to fatal hepatitis in mice (GLEDHILL et al., 1952; NIVEN et al., 1952).

Several years earlier a virus which caused encephalitis in mice had been isolated by CHEEVER et al., (1949). This virus, JHM strain, was subsequently shown to be antigenically related to the viruses discovered by GLEDHILL (MORRIS, 1959; CALISHER and ROWE, 1966). Many other strains of MHV have now been described (see Table 1). All have been found to cause both hepatitis and encephalitis, although most are either primarily encephalitogenic or hepatitogenic. Intravenous infection of susceptible mice with strain MHV-3 leads to the appearance of infectious virus in spleen, liver, brain, kidney, lung, heart, testicles and numerous other organs (PIAZZA, 1967). The pathology of the hepatic disease by both light and electron microscopy has been described in detail (BAILEY et al., 1949; GLEDHILL and ANDREWES, 1951; NELSON, 1952; MIYAI et al., 1963; DICK et al., 1956; RUEBNER and BRAMHALL, 1960). Necrosis is focal in nature, and both Kupffer and parenchymal cells appear to be involved. The virus shows in addition a striking ability to destroy lymphoid cells both in the spleen (BIGGART and RUEBNER, 1970; HIRANO and RUEBNER, 1965) and the bone marrow (BARINSKY and DEMENTIEV, 1968; HUNSTEIN, 1969).

The major problem in MHV control has been the riddance from mouse colonies of subclinical endemic infection (ROWE et al., 1963). This problem has been effectively combatted by means of breeding techniques which have made possible the maintenance of Cesarean-derived, barrier-sustained colonies (TRENTIN et al., 1966). Such colonies, under proper care and surveillance, can be guaranteed free of all known viruses except those of the mouse leukemia group.

At the present time the importance of MHV lies in its usefulness as a model for various problems in the pathogenesis of hepatitis or encephalitis and in studies of virus-cell interaction. It is a curious characteristic of infection by several strains of MHV that pathogenicity is markedly enhanced by various forms of "stress": infestation with *Eperythrozoon coccoides* (GLEDHILL, 1962), simultaneous infection with murine leukemia viruses (NELSON, 1952; CHANY, 1969; GLEDHILL, 1961), polyoma virus (STURMAN and TAKEMOTO, 1972), or K-virus of mice (TISDALE, 1963), and pretreatment of mice with corticosteroids (DATTA and ISSELBACHER, 1969; VELLA and STARR, 1965; MANSO et al., 1959; HIRANO and RUEBNER, 1965; BANG and WARWICK, 1960; GALLILY et al., 1964; LAVELLE and STARR, 1969). Liver infection in mice by MHV has been used extensively as a model for human viral hepatitis

(Piazza, 1969). Strain JHM causes wide-spread destruction of myelin in the central nervous system (Bailey et al., 1949) and has been recently suggested as a possible model for the human demyelinating encephalitides. There has been considerable interest in variations of the genetic susceptibility to MHV infection in cells obtained from different strains of mice, both *in vivo* and *in vitro* (Bang and Warwick, 1960; Gallily et al., 1967; Shif and Bang, 1970a; Kantoch and Bang, 1962; Shif and Bang, 1970b).

A detailed and well-referenced review of MHV research has been published recently, and the reader is referred to it for information about the pathogenesis of murine hepatitis and its value as a model for human disease (Piazza, 1969).

C. Human Coronavirus Strains

The existence of ether-labile viruses which caused colds in human volunteers but were unrelated to known myxo- or paramyxoviruses was first suspected by Tyrrell and Bynoe (1965). These workers were able to passage a virus recovered from the human upper respiratory tract in organ cultures of human embryonic trachea. The virus, named B 814 strain, was subsequently examined by electron microscopy (Almeida and Tyrrell, 1967) and found to resemble the previously described particles obtained from cultures of IBV (Berry et al., 1964).

Hamre and Procknow (1966), working independently, recovered five virus strains from medical students with colds which were grown, with some difficulty, in secondary human embryonic kidney monolayers or in human diploid fibroblasts. The prototype virus, strain 229E, was shown to be ether-labile, medium-sized, and RNA-containing, and was later found to resemble IBV and strain B 814 in electron microscopic appearance (Almeida and Tyrrell, 1967).

The organ culture technique was subsequently used to recover 9 further human strains with identical morphology (McIntosh et al., 1967b; Tyrrell and Bynoe, 1965; Bradburne and Tyrrell, 1971), and still other strains have been recovered in tissue culture (Kapikian et al., 1969; Oshiro et al., 1971; McIntosh, unpublished). All these human coronavirus (HCV) strains, with a single exception, were originally isolated from specimens obtained during upper respiratory disease. Five strains (B 814, LP, EVS, 229E and OC43) have been administered to human volunteers and cause colds which differ in minor respects from the colds produced by rhinoviruses (Tyrrell and Bynoe, 1965; Bradburne et al., 1967). Seroepidemiologic studies of HCV have shown that infection occurs at all ages and is widespread in populations in the United States and the United Kingdom (McIntosh et al., 1970; Kaye and Dowdle, 1969; Kaye et al., 1971). It is a reasonable estimate of their importance that they are responsible for about 15 % of colds in human adults. Of interest has been the consistent finding that HCV strains are primarily epidemic in the winter and early spring (McIntosh et al., 1970; Cavallaro and Monto, 1970). One recent study has implicated HCV in

exacerbations of symptoms in adults with chronic bronchitis (PHILLIPS et al., unpublished studies). Efforts to implicate coronaviruses in lower respiratory tract diseases of children have been disappointing (MCINTOSH et al., 1970) except in young children with asthma (MCINTOSH et al., 1973) and in infants under 18 months where pneumonia and bronchiolitis during infection with these agents may occur (MUFSON et al., 1972).

A comprehensive review of the human coronaviruses has appeared recently (BRADBURNE and TYRRELL, 1971).

D. Transmissible Gastroenteritis Virus of Swine

A distinct disease characterized by a short incubation period (18–24 hours), high infectivity, diarrhea and vomiting, and a high mortality in piglets under 2 weeks of age was first described and attributed to a virus by DOYLE and HUTCHINGS (1946). The disease characteristically occurs in the winter time (FERRIS, 1971; HAELTERMAN, 1962) and is most striking when an outbreak occurs in a farrowing herd, since the symptoms tend to be less severe in the adult animal than in the newborn, where the mortality approaches 100% (CARTWRIGHT et al., 1964; FERRIS, 1971).

The virus causing transmissible gastroenteritis (TGE) was first grown in primary pig kidney monolayers by LEE (1956). Many strains have been isolated, either in piglets or in cell culture, and the disease appears to be widely distributed in many parts of the world (BÄHR, 1969; GOODWIN and JENNINGS, 1958). Although the virus has been recovered from many organs (CARTWRIGHT, 1966; KONISHI and BANKOWSKI, 1967; LEE et al., 1954), the highest virus titers are found in the duodenum and jejunum (HOOPER and HAELTERMAN, 1966), and clinical disease is confined primarily to the gastrointestinal tract, where the characteristic pathologic lesion of severe blunting of the jejunal and ileal villi is seen (PENSAERT et al., 1970a; PENSEART et al., 1970b; THAKE, 1968). Virus is shed from the gastrointestinal tract for as long as 8 weeks after infection (LEE et al., 1954). TGE has been suggested as a model for both adult (HAELTERMAN and HOOPER, 1967) and childhood (KELLY et al., 1972) diarrhea in man.

Because of the observation that epidemics tend to occur following snow falls, when starlings have a habit of congregating around pens, it has been found that virus fed to starlings appears in bird droppings for 32 hours after feeding (PILCHARD, 1965). It is also likely that dogs and foxes can be infected with the virus (HAELTERMAN, 1962).

The most crucial aspect of control involves effective protection of newborn piglets. Since all known strains of TGE virus are serologically identical (CARTWRIGHT, 1966; LEE et al., 1954; BOHL, 1967) the problem of antigenic variation, so familiar to workers in the field of IBV, does not occur. However, effective vaccines must take into consideration the observation that immune mothers confer immunity to their offspring through colostrum and milk. Thus, vaccination of mothers must stimulate adequate levels of secretory

antibody (Djurickovic and Thorsen, 1970; Thorsen and Djurickovic, 1970; Thorsen and Djurickovic, 1971).

There has recently been some dispute about the nature of the virus responsible for TGE which deserves comment. One group of workers (McClurkin and Norman, 1966) claimed to have detected two agents in material passaged from infected pig intestines into tissue culture: one agent caused a cytopathic effect and the other did not. This dispute arose partly because convalescent pig sera incubated with preparations of TGE virus failed to neutralize the capacity of these preparations to produce disease (Lee et al., 1954; McClurkin and Norman, 1966). This failure was, however, effectively explained by antigen-antibody dissociation in the stomach (Witte and Easterday, 1967).

Recently, Norman et al. (1968) claimed that they could separate two agents by phenol extraction of a tissue culture-grown virus mixture. A ribonuclease-sensitive pathogenic agent was derived from this extraction, which appeared to produce TGE in susceptible piglets. However, the data presented did not show convincingly that the agent derived from the phenol-extracted material was different from intact TGEV. Indeed, several other workers (Caletti et al., 1970; Witte and Easterday, 1967) have presented evidence that TGEV is a single agent with the characteristics of a coronavirus. The effect of phenol extraction on purified coronavirus preparations deserves further study.

Clinical aspects of TGE are thoroughly reviewed by E. H. Bohl in his chapter of H. W. Dunne's *Diseases of Swine*, 3rd Edition (Bohl, 1970).

E. Hemagglutinating Encephalomyelitis Virus

Of the several viruses implicated in encephalomyelitis syndromes of pigs, one appears to be a coronavirus. The exact pathogenic range of this virus, hemagglutinating encephalomyelitis virus (HEV), is not altogether clear. Some strains appear to cause a severe encephalitis in suckling pigs (Roe and Alexander, 1958; Mitchell, 1963). This disease, which is fatal in close to 100% of piglets up to one week of age, is characterized by squealing, loss of appetite, vomiting, constipation, occasional diarrhea, progressive paralysis (often accompanied by paddling movements of the legs) and death in 3–4 days (Mitchell, 1963). Slightly older piglets may survive, but they remain sickly and grow poorly for several months. Adult animals apparently develop vomiting and anorexia but usually recover. Strains recovered in Great Britain, apparently serologically identical to the encephalitogenic strains, have been shown to cause "vomiting and wasting disease" of piglets under 2 weeks of age, whose primary symptoms are those of gastrointestinal infection rather than encephalomyelitis (Cartwright et al., 1969; Cartwright and Lucas, 1970). Virus was, however, recovered from the brain, and there were histologic signs of encephalitis. Still another strain was isolated incidentally from an asymptomatic pig (Mengeling et al., 1972).

It appears that all strains of HEV are serologically closed related or identical. Likewise, all strains hemagglutinate and cause a syncytial cytopathic

effect in primary pig kidney cells. The appearance of typical coronavirus particles by electron microscopy was first demonstrated by PHILLIP et al. (1971) and subsequently confirmed by MENGELING et al. (1972). The virus was also reported to be antigenically related to the virus of TGE (PHILLIP et al., 1971), although the relation appeared not to be a close one.

F. Rat Coronavirus

In their studies of antibodies to MHV in species other than the mouse, HARTLEY et al. (1964) found that a high proportion of laboratory rats had apparently experienced infection with that or a related virus. A systematic effort to detect such a virus was made by PARKER et al. (1970) who recovered a coronavirus serologically related to MHV from the lungs of asymptomatic rats. The virus, named rat coronavirus (RCV), was pneumotropic, causing a fatal pneumonitis in newborn suckling rats and an asymptomatic pulmonary infection in adults. Apparently infection was widespread, being found in 3 of 5 specific pathogen free colonies, 100% of conventional colonies and 40% of individual rats trapped in the wild.

G. Sialodacryoadenitis Virus

A virus serologically related to RCV has been recently recovered from rats with sialodacryoadenitis (SDA) (BHATT et al., 1972). The disease was first described in 1961 (INNES and STANTON, 1961) and was studied pathologically by JONAS et al. (1969). These authors found virus-like particles in thin sections of submaxillary salivary glands which, in retrospect, resemble coronaviruses as described by others. BHATT et al. (1972) studied the properties of this virus and discovered its close resemblance to both MHV and RCV. There is now preliminary evidence for the existence of still other rat coronaviruses (BHATT, personal communication), but these have not been described in detail.

H. Summary

It thus appears that members of the coronavirus group are widely distributed in nature and show a variety of tissue and species tropisms. Man, chickens, pigs, mice and rats are naturally infected, and disease is caused by virus multiplication and cytopathogenicity in the upper and lower respiratory, tract, the kidneys, the genital tract, the small and large intestines, the brain, the salivary glands and elsewhere. It appears likely that coronaviruses infecting other species will be found. As was mentioned previously, antibody to TGE virus has been found in dogs (NORMAN et al., 1970), and this could represent either infection with TGE virus or, possibly, evidence of a related canine virus. Moreover, a virus associated with infectious feline peritonitis has been described which has a morphology in thin sections making it a likely candidate for a cat coronavirus (WARD, 1970; WARD et al., 1968).

It is the purpose of the following section to discuss common issues of importance among all the known members of the coronavirus group. It will

be seen that, although the coronaviruses share many features in common, they differ significantly in certain ways, and the problems facing virologists in their study and control vary widely.

III. Properties of Coronaviruses

A. Morphology of the Virion

1. Negative staining

Although the particles in suspensions of IBV were studied by electron microscopy as long ago as 1948 (Reagan and Brueckner, 1952; Reagan et al., 1948), it was not until the techniques of negative staining were applied that adequate surface morphology was outlined. On the basis of its physical characteristics, IBV had always been classified in the myxovirus group. Berry et al. (1964) were the first to draw attention to the unique "bulbous appearance of the surface projections" of IBV particles and to the obscure nature of the internal portion of the virus. Both these characteristics were in contrast to influenza virus, which they studied by the same techniques.

Some time later, in attempts to outline the properties of the human coronavirus B 814, Almeida and Tyrrell (1967) described clearly the distinct morphology of that virus strain and strain 229E (Hamre and Procknow, 1966) and their resemblance to IBV. McIntosh et al. (1967b) soon thereafter found that the ether labile viruses they had recovered from man in human embryonic tracheal organ culture had a similar morphology, and in related

Table 2. The size of coronavirus virions as measured by electron microscopy of negatively stained preparations

Virus species	Type or strain	Investigator (s)	Over-all diameter (nm)	Length of projections (nm)
IBV	A163, H17, Conn. & Mass.	Berry et al., 1964	80–120	—
IBV	42	McIntosh et al., 1967b	120–200	20
IBV	F	Estola and Weckstrom, 1967	100–120	—
IBV	Mass.	Nazerian and Cunningham, 1967	110 (av.)	—
IBV	GN-2	Hirai and Shimakura, 1971	60–220	20
IBV	Clark-333	Cowen et al., 1971b	50–100	—
HCV	B814, 229E	Almeida and Tyrrell, 1967	80–120	20
HCV	OC16, 229E	McIntosh et al., 1967b	120–200	20
TGE	TO	Okaniwa et al., 1968a	97–120	12
TGE	TO	Tajima, 1970	100–150	24
TGE	?	Phillip et al., 1971	133–168	20
HEV	?	Phillip et al., 1971	140–177	19
RCV	8190	Parker et al., 1970	79–118	17

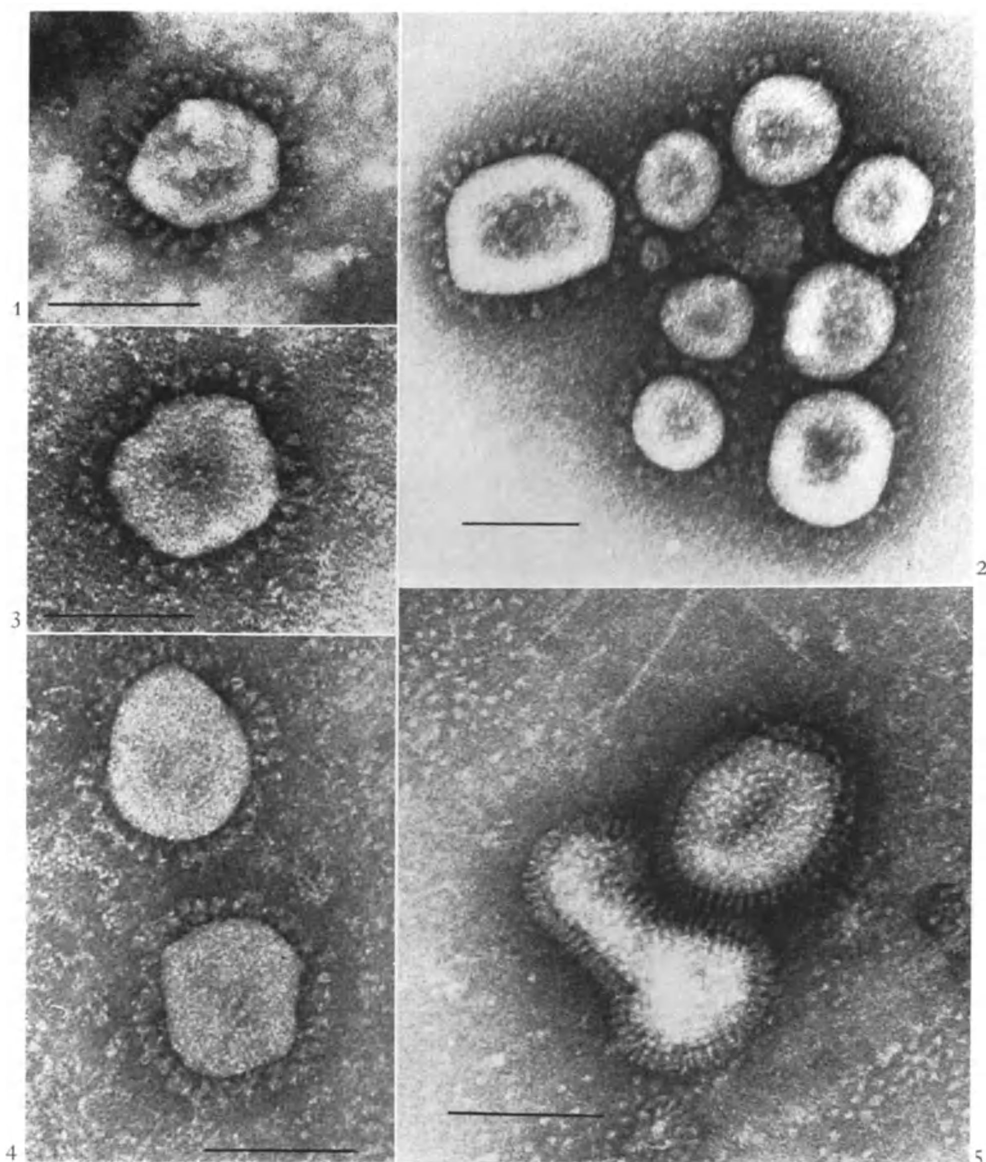

Fig. 1. Human coronavirus, strain 229E, prepared from untreated infected WI-38 cell lysate and stained with 2% phosphotungstic acid at pH 7.0. Bar represents 100 nm. Figs. 1, 2, 3, and 5 are printed with permission of the Proceedings of the National Academy of Sciences and appeared originally in McIntosh et al., 1967b

Fig. 2. Human coronavirus. strain OC16, prepared from infected human embryonic tracheal organ culture fluids, concentrated by ultracentrifugation and stained as in Fig. 1. Bar represents 100 nm.

Fig. 3. Infectious bronchitis virus, strain Beaudette, prepared from infected 10-day embryonated eggs as untreated allantoic fluid. Staining as in Fig. 1. Bar represents 100 nm

Fig. 4. Mouse hepatitis virus, strain A-59, prepared from infected NCTC-1469 cells, untreated. Stained as in Fig. 1. Bar represents 100 nm

Fig. 5. Influenza A2, negatively stained with phosphotungstic acid, shown for comparison with coronaviruses. Bar represents 100 nm

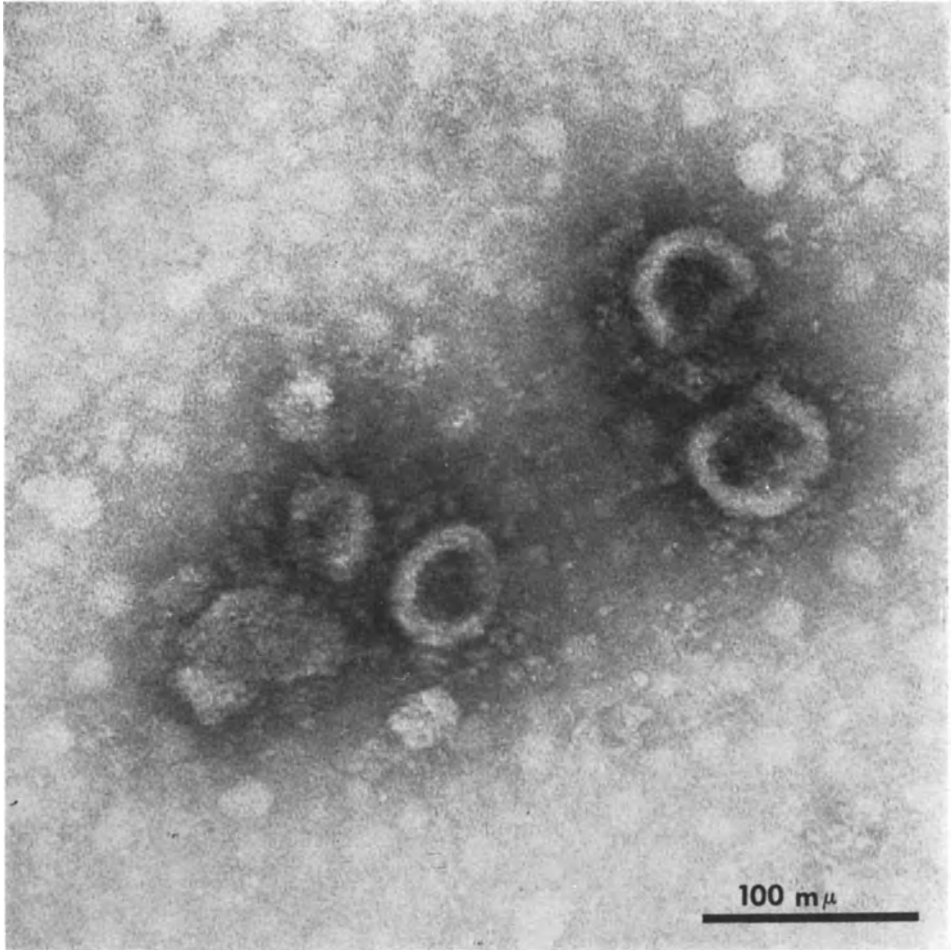

Fig. 6. HCV, strain 229E, treated for 10 minutes at room temperature with tween 80-ether. Projections are distorted or destroyed, and no internal component is visible. Magnification before publication, ×288,000

studies added MHV to this morphologic group (McIntosh et al., 1967a). It was at this point that the name "coronavirus" was coined by an *ad hoc* international committee (Tyrrell et al., 1968a), the name being descriptive of the "corona" of projections seen in negatively stained preparations. Other members were then, one by one, added to the group on the basis of this characteristic appearance.

Although there is some disagreement between investigators on the exact size range of various coronaviruses, the common properties and the size of the projections of all coronaviruses are clear (see Table 2). Filtration studies (Beach and Schalm, 1936; Beaudette and Hudson, 1937; Estola, 1966; Tevethia and Cunningham, 1968; Cartwright et al., 1965; Sheffy, 1965; McClurkin and Norman, 1966; Hamre and Procknow, 1966; Tyrrell and Bynoe, 1965; Kapikian et al., 1969; Bhatt et al., 1972) tend to confirm the

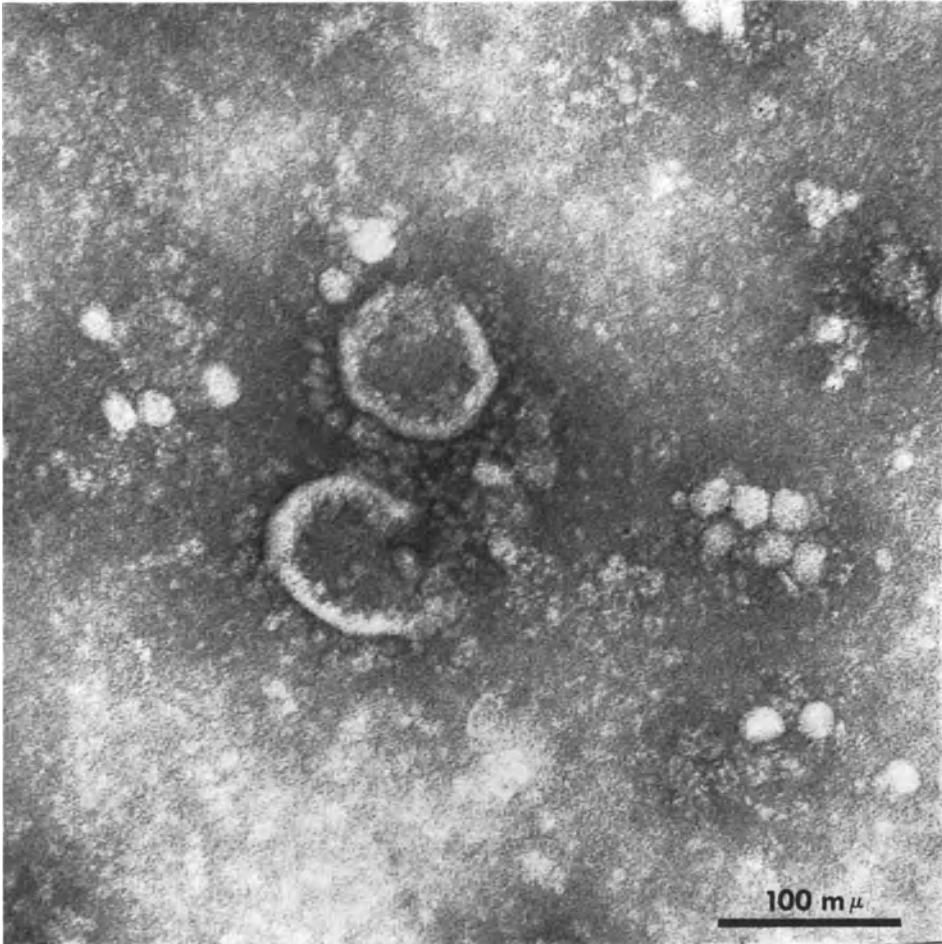

Fig. 7. HCV, strain 229E, treated with 1.0% sodium dodecylsulfate for 10 minutes at room temperature. The projections are relatively spared, but, again, no internal component is visible. Magnification before publication, ×240,000

measurements obtained on electron microscopy, and will not be outlined in detail. Moderate pleomorphism has been a feature of all coronavirus strains, as have the peculiar club-shaped projections and the characteristic wide spacing of these projections (Fig. 1–5).

Attempts to obtain a consistent picture of the internal structure of coronaviruses by means of negative staining have met with only qualified success. Ether, tween 80-ether, sodium dodecyl sulfate, or desoxycholate treatment of coronavirus suspensions destroys the morphologic integrity of the virus particles and leaves amorphous material which is presumably altered membrane and "internal component" (KAYE et al., 1970; BERRY and CRUICKSHANK, 1964) (Fig. 6 and 7). Formalinization of strain 229E produces particles which seem to extrude a possible internal component. This "loop" (see Fig. 8) appears to remain attached to the external membrane of the virus.

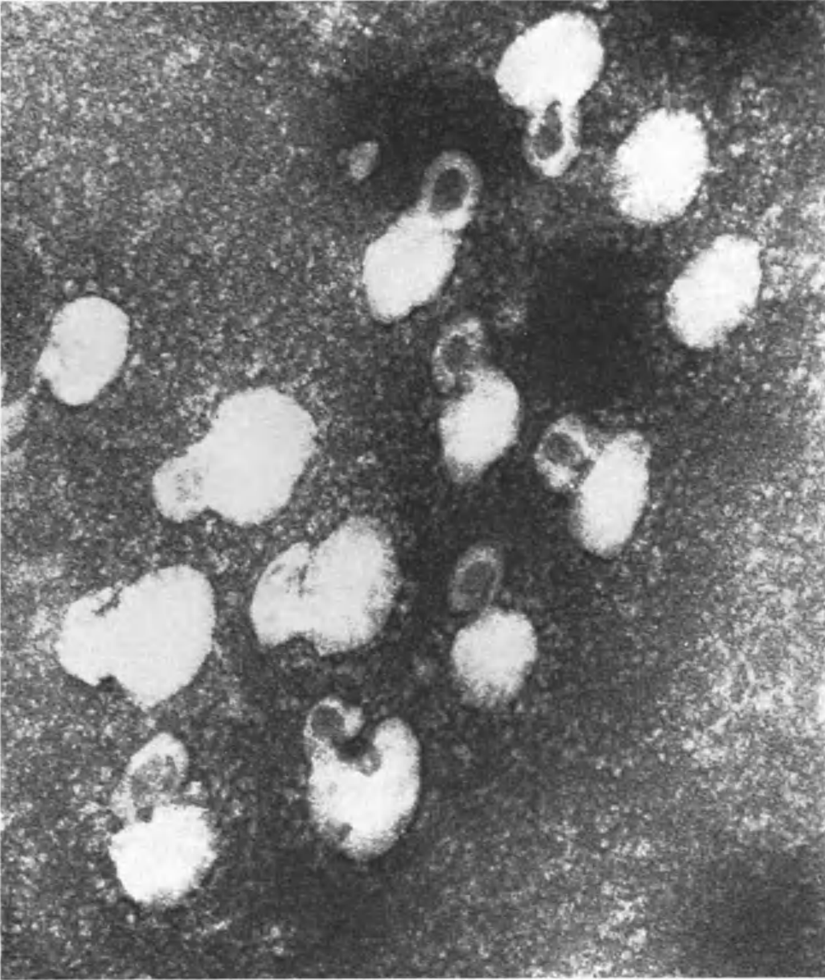

Fig. 8. HCV, strain 229E, treated with 10% formalin at room temperature for one hour. Many particles appear to be extruding a "loop", which may be related to the "tongues" seen in Figs. 9 and 10. Magnification before publication, ×180,000

Perhaps the most convincing view of coronavirus internal structure has emerged from studies of J. D. Almeida. She has applied a technique which uses antibody and complement to produce small holes in the outer envelope of IBV (Berry and Almeida, 1968). By this means she introduced phosphotungstic acid inside with little distortion of over-all morphology. Internally projecting "tongues", continuous with the outer membrane of the virus, were observed in virus particles treated this way (Fig. 9 and 10). The relation of these "tongues" to the nucleic acid of the virus is unknown. The "tongues" may be identical to the "loops" seen in formalinized virus preparations. Almeida's observations remain unconfirmed at this time, although they represent a plausible concept of internal structure which is consistent with the findings in fixed ultra-thin sections.

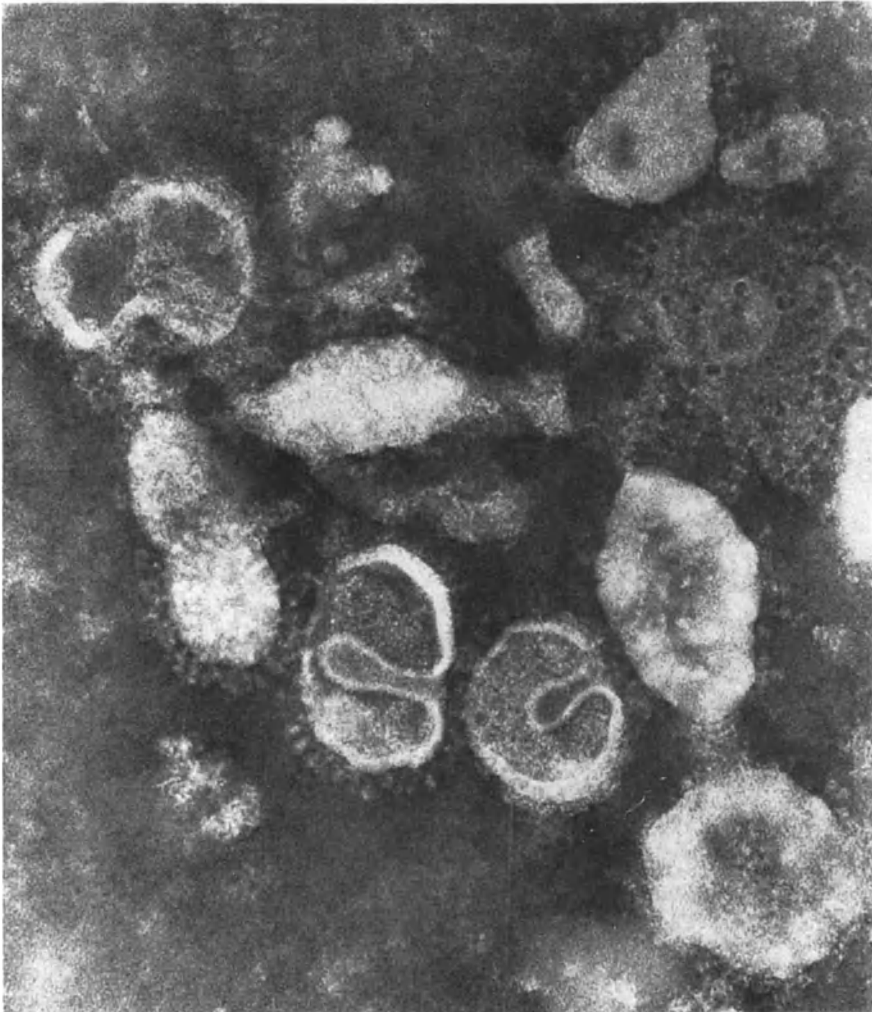

Fig. 9. IBV, incubated before staining with fresh specific (heterologous) rabbit antiserum, unheated. Stained with 3% phosphotungstic acid. The "holes" are visible in some of the virus envelopes and internally projecting "tongues" are visible. Magnification before publication, ×200,000. Figs. 9 and 10 were kindly supplied by Mrs, J. D. ALMEIDA

2. Ultra-thin sections

Just as in the direct examination of virus particles, early investigators described coronaviruses in sections of infected tissue, but no details were seen (DOMERMUTH and EDWARDS, 1957). Before it was recognized as a coronavirus, the particles of MHV were described in thin sections of mouse liver (STARR et al., 1960). They were thought to be 90 ± 20 nm in diameter, round, and clustered in cytoplasmic vesicles. A later study of MHV, strains MHV-2 and MHV-3 (SVOBODA et al., 1962), confirmed and extended these findings. Particles thought to be MHV virions were 90 nm in diameter and contained

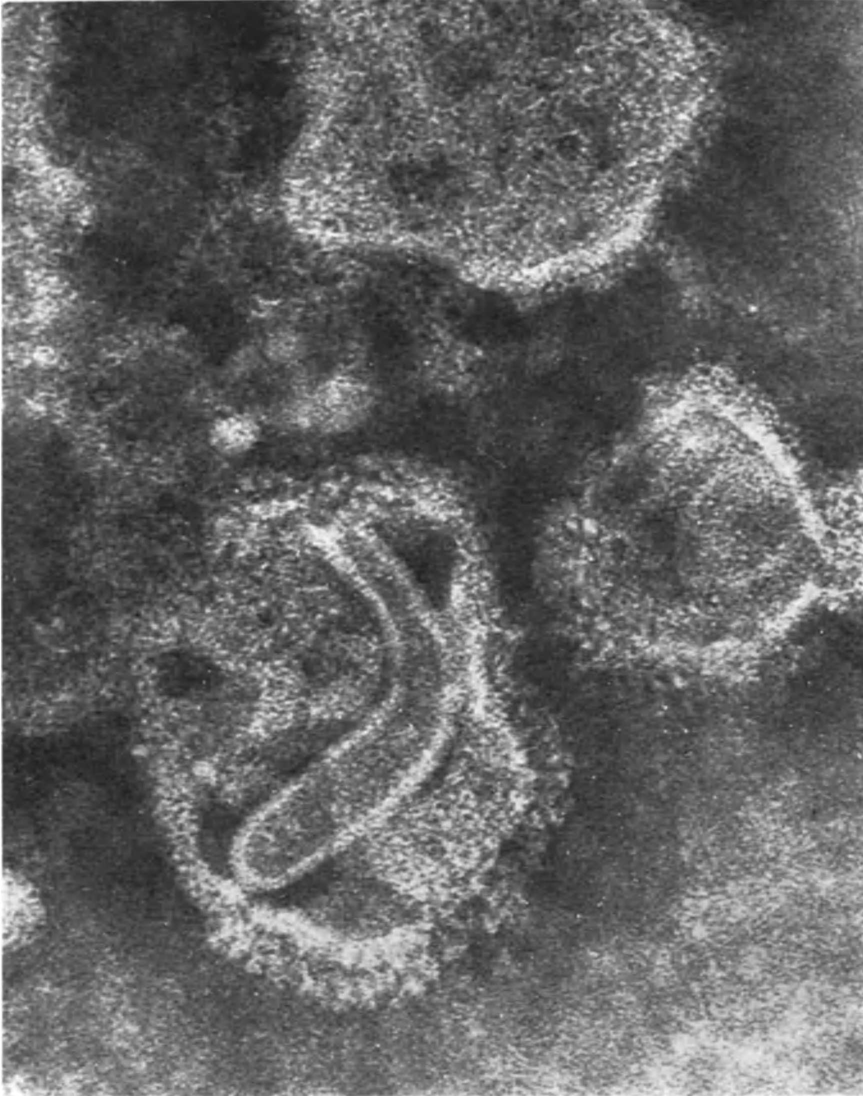

Fig. 10. IBV, incubated before staining with fresh human serum containing antibodies to IBV. Stained as in Fig. 9. A long "tongue" is visible. Magnification before publication, ×300,000

a "nucleoid" separated from the outer membrane by an electron-lucent space. A further study of MHV, strain A-59, described particles in tissue culture cells (David-Ferreira and Manaker, 1965). In this instance, the average diameter of the particles was 75 nm, and an electron-dense inner shell 55 nm in diameter was observed, which was separated from the outer "double" membrane by an electron-lucent space 8 nm wide. The studies of Becker et al. (1967) showed that strain 229E, a human coronavirus grown in WI38 cells, was very similar in morphology to MHV. Fig. 11 shows a detailed section

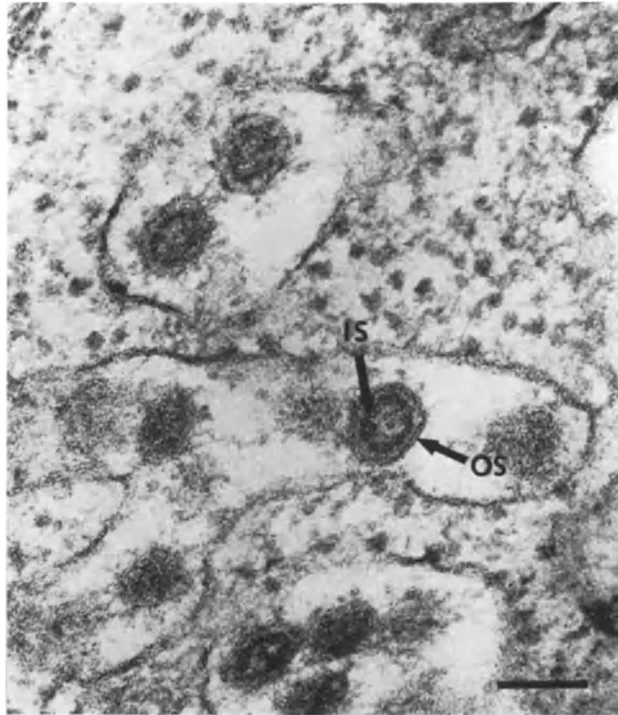

Fig. 11. Cytoplasm of a 229E-infected WI38 cell. Complete virus particles are shown, with the double outer membrane, inner core and space in between. Cells were fixed in 6.5% glutaraldehyde for 1 hour, post-fixed in 1% osmium tetroxide and stained with uranyl acetate and lead citrate. IS: inner shell. OS: outer shell. Magnification before publication, ×120,000. Bar represents 100 nm. Figs. 11, 12, and 13 are printed with permission of the American Society for Microbiology and appeared originally in BECKER et al., 1967

of strain 229E particles, including the inner and outer shells, reproduced from this published paper. In the same study, IBV was described as the same size as 229E (67–110 nm, mean 82 nm), but the avian virus showed no evidence of an electron-lucent space between the outer and inner shells (Fig. 12). It is of interest that this feature of IBV, namely the close apposition of the outer and inner shells, was described also by NAZERIAN and CUNNINGHAM (1968) and COWEN et al. (1971b). However, the recent micrographs of UPPAL and CHU (1970) show the space present. Thus, it appears that there is no consistent difference in morphology between IBV and the human or murine coronaviruses.

APOSTOLOV et al. (1970) have recently examined thin sections of purified IBV particles. They described an "internal thread-like structure 7–8 nm in diameter". The morphologic definition of that structure, however, was not absolutely clear from their published micrographs. It may represent the structure described as the "inner shell" by BECKER et al. or as the "core" by others.

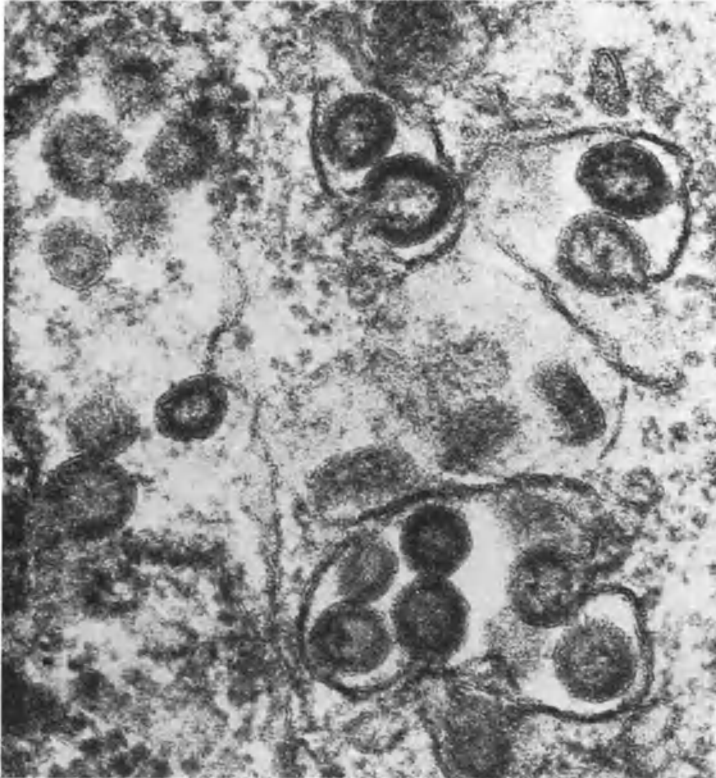

Fig. 12. Chorioallantoic membrane cell infected with IBV, Beaudette strain. Complete virus particles show no space between the core, or inner shell, and outer membrane. Fixation and staining as in Fig. 11. Magnification before publication, ×120,000

Virus particles with a size and morphology similar to those of strain 229E have been described for TGE virus (Bradfute et al., 1970; Chandler et al., 1969; Okaniwa et al., 1968b; Thake, 1968; Vetterlein and Liebermann, 1970; Witte et al., 1968), HEV (Mengeling et al., 1972), RCV (Parker et al., 1970) and SDA virus (Jonas et al., 1969). Numerous studies of MHV in mouse liver and spleen cells have been published (Watanabe, 1969; Ruebner and Bramhall, 1960; Cuccurullo and Piazza, 1964; Ruebner et al., 1967; Biggart and Ruebner, 1970), and all are in essential agreement with the findings of others. It is of interest that infectious feline peritonitis virus shows a very similar morphology and morphogenesis, although micrographs confirming a coronavirus-like appearance in negatively stained preparations have not been published (Ward, 1970).

B. Physical and Chemical Characteristics of Coronaviruses

1. Nature of the nucleic acid

In all instances where they have been so studied the growth of coronaviruses has been shown to be insensitive to the action of inhibitors of DNA

metabolism (BOHL and KUMAGAI, 1965; HARADA et al., 1968; McCLURKIN and NORMAN, 1967; PENSAERT, 1970; SHEFFY, 1965; WITTE et al., 1968; CALETTI et al., 1968; GREIG and GIRARD, 1969; ESTOLA, 1966; TEVETHIA and CUNNINGHAM, 1968; MALLUCCI, 1965; HAMRE and PROCKNOW, 1966; KAPIKIAN et al., 1969; McINTOSH et al., 1967b; BRADBURNE, 1969). The inhibitors which have been tested have been principally 5'-iododeoxyuridine and 5'-bromodeoxyuridine, but cytosine arabinoside (PARKER et al., 1970), 5'-fluorodeoxyuridine (ESTOLA, 1966; MALLUCCI, 1965) and aminopterin (AKERS and CUNNINGHAM, 1968) have also been used. These inhibitors do not affect either the induction of cytopathic effect (CPE) or the production of new viruses. Likewise, actinomycin D (0.01 – 1 mg/ml) has no effect on the CPE produced by several coronavirus strains (McCLURKIN and NORMAN, 1967; PARKER et al., 1970; MALLUCCI, 1965; McINTOSH, unpublished).

Because of these studies, the nature of the coronavirus nucleic acid has been thought to be RNA. Direct extraction and analysis of the nucleic acid from highly purified virions, including determination of single- or double-strandedness, have not been reported.

2. Sensitivity to Lipid Solvents

Both ether and chloroform destroy or markedly reduce the infectivity of coronaviruses. It is likely, in view of their morphology and morphogenesis in thin sections, that the outer shell of the virus consists of cell-derived membrane which contains lipid and is necessary for infectivity.

There have been many attempts to visualize the interior of the virion by examining ether or ether-tween treated virus in the electron microscope. As seen in Fig. 6, one can observe the disintegration of the virion by this method, but there remains little or no organized internal structure (KAYE et al., 1970; BERRY et al., 1964; NAZERIAN and CUNNINGHAM, 1967). Treatment with detergents such as sodium dodecyl sulfate and sodium desoxycholate has a similar destructive effect (Fig. 7), following which little infectivity or organized structure remains (CARTWRIGHT et al., 1965; KAYE et al., 1970; HIERHOLZER et al., 1972; BERRY et al., 1964; ESTOLA, 1966; TEVETHIA and CUNNINGHAM, 1968; GREIG and GIRARD, 1969).

3. Sensitivity to Physical Agents: Heat and Acid

The details of the stability of coronaviruses at various temperatures depend so markedly on the exact nature of the experimental conditions, the purity of the virus preparations and the presence of ions and colloids in the medium that a summary of the results of studies in this area seems unwarranted. It is clear that most coronaviruses are inactivated at 56° C in 10–15 minutes, at 37° C in several days, and at 4° C in several months (HOFSTAD, 1956; ESTOLA, 1966; WITTE and EASTERDAY, 1967; SHEFFY, 1965; HARADA et al., 1968; VON BÜLOW, 1967; HIRONAO et al., 1970; HIRAI and SHIMAKURA, 1971; COWEN et al., 1971b; BUCKNALL et al., 1972b; PAGE and CUNNINGHAM,

Table 3. pH Stability of Coronaviruses

Virus species	Serotype or strain	pH	Temperature °C	Time at designated pH	Change in titer (PFU, $TCID_{50}$, EID_{50} or LD_{50})	Reference
IBV	Beaudette	3.03	4	14 days	"fully active"	Cunningham and Stuart, 1946
IBV	?	2.0	20	1 hr.	"no loss"	Quiroz and Hanson, 1958
IBV	Beaudette	3.0	4	30 min.	"92–96% survival"	Stinski and Cunningham, 1969
IBV	IBV_F	2.9	20	4 hrs.	$10^{4.5} \rightarrow 10^{2.5}$	Estola, 1966
IBV	Mass.	2.9	20	30 min.	$10^{1.9}$ loss	Cowen et al., 1971b
IBV	Conn.	2.9	20	30 min.	$10^{2.7}$ loss	Cowen et al., 1971b
IBV	Clark 333	2.9	20	30 min.	$10^{1.3}$ loss	Cowen et al., 1971b
IBV	GN-2	3.05	4	30 min.	$10^{7.2} \rightarrow 10^{2.5}$	Hirai and Shimakura, 1971
TGE	SH	3.0	20	45 min.	$10^{2.0}$ loss	Sheffy, 1965
TGE	FS 216/64	3.0	37	3 hrs.	"no change in titer"	Cartwright et al., 1965
TGE	Purdue, Iowa, SH, Illinois, N. Y.	3.0	37	1 hr.	$10^{2.0}$ to $10^{3.0}$ loss	McClurkin and Norman, 1966
TGE	Purdue	3.0	?	$3^3/_4$ hrs.	$10^{1.4}$ loss	Witte and Easterday, 1967
TGE	SH	3.0	20	3 hrs.	$10^{5.5} \rightarrow 10^{5.3}$	Harada et al., 1968
HCV	489 and other 229E-like	2.7	25	3 hrs.	"at least 100-fold reduction"	Kapikian et al., 1969
SDAV	681	3.0	25	3 hrs.	$10^{0.8}$ loss	Bhatt et al., 1972

1962). The viruses are generally quite stable through several freeze-thaw cycles and may be kept at temperatures of $-60°$ C or lower for years without loss of infectivity. There may be considerable variation in thermolability between different egg passages of the same strain (VON BÜLOW, 1967). On the other hand, more detailed studies of two human coronavirus strains demonstrated that differences in susceptibility to heat were almost entirely accounted for by differences in aggregation (BUCKNALL et al., 1972b).

Investigators disagree regarding the stability of various coronaviruses at acid pH. It is not clear whether these disagreements reflect differences in methods or in strains of coronaviruses tested. Some of the published results of stability tests are shown in Table 3. It is clear that a systematic and standardized study of the pH stability of coronaviruses should be performed.

4. Chemical Composition of the Virion

The presence of lipids essential to infectivity has been amply demonstrated by the action of detergents and lipid solvents on coronavirus particles. Similar early attempts to demonstrate essential proteins concentrated on the effect of trypsin on virus infectivity. Although there is some disagreement on the point (CORBO and CUNNINGHAM, 1959; NAZERIAN and CUNNINGHAM, 1967), the bulk of evidence indicates that the biological activity of coronaviruses IBV and TGEV is not sensitive to trypsin (ESTOLA, 1966; STEELE and LUGINBUHL, 1964; VON BÜLOW, 1967; COWEN et al., 1971b; SHEFFY, 1965; CARTWRIGHT et al., 1965; HARADA et al., 1968; MULDOON and CUNNINGHAM, 1961) and that, indeed, there is no morphologic effect of this or other proteolytic enzymes on IBV (BERRY et al., 1964; APOSTOLOV et al., 1970). On the other hand, purified human coronavirus OC43 loses its infectivity, its complement fixing activity, and much of its hemagglutinating ability when treated with 1% trypsin for 2 hours (KAYE et al., 1970).

More recently, coronavirus OC43 has been extensively purified by batch CaHPO4 chromatography, and the solubilized polypeptides have been analyzed by polyacrylamide gel electrophoresis (HIERHOLZER et al., 1972). In these recent studies six or seven separate polypeptides were identified in purified virions with molecular weights varying from 15000 to 191,000 daltons. Four polypeptides contained carbohydrates and one contained lipid. Bromelin treatment (COMPANS et al., 1970) removed the projections, and purified "bald" particles lacked two of the four glycopeptides, numbers 2 and 6.

HIERHOLZER et al. (1972) tested purified OC43 virions for neuraminidase by the sensitive technique of LAVER and KILBOURNE (1969) and found none. Earlier, BERRY et al. likewise found no detectable neuraminidase activity in partially purified preparations of IBV (1964). Some neuraminidase activity has been reported associated with the hemagglutinating activity of allantoic fluid harvested from IBV-infected eggs (BISWAL et al., 1966). It appears likely, however, that this activity is not part of the infectious IBV particle itself (see below).

5. Buoyant Density of the Virion

There are several reports of the buoyant densities of coronaviruses in gradients of various compositions (Table 4). Infectivity of coronaviruses is preserved both in sucrose and potassium tartrate, but is much reduced in CsCl (Tevethia and Cunningham, 1968).

Table 4. Buoyant Density of Coronaviruses by Equilibrium Centrifugation

Coronavirus species	Strain	Gradient substance	Buoyant density (gm/ml)	References
HCV	OC 43	Sucrose	1.19	Kaye et al., 1970
	OC 43	K Tartrate	1.18	Hierholzer et al., 1972
	229E	Sucrose	1.18	Bradburne and Tyrrell, 1971
IBV	?	Sucrose	1.19	Cunningham, 1970
	Beaudette	CsCl	1.23	Tevethia and Cunningham, 1968
TGE	Purdue	Sucrose saline	1.19	Witte et al., 1968

Hierholzer et al. (1972) reported that treatment of the virion of strain OC43 with bromelin decreased the buoyant density in potassium tartrate gradients from 1.18 to 1.15 gm/ml with concomitant removal of the surface projections.

IV. Coronaviruses in Infected Cells

A. Growth of Coronaviruses in Cell Monolayers and Laboratory Animals

The major impediment to progress in research on certain members of the coronavirus group has been the fastidious nature of their growth requirements in the laboratory. This problem is particularly acute in the cultivation of the human strains but also exists to some extent in research on IBV, TGEV, RCV, SDAV and MHV (Barinsky, 1967).

Table 5 shows a summary of the available information on the growth of coronaviruses in animals and cell monolayers. The table is in certain instances somewhat misleading: at times although virus growth in one or another cell type has been conclusively demonstrated, this growth has been of limited extent and poorly reproducible in different laboratories. This characteristic has rendered this particular cell or tissue unsuitable for general research.

Of particular importance has been the difficulty in cultivating field strains of IBV in any tissue except embryonated eggs. This feature has led to confusion in the areas of serologic typing, the definition of antigenic relationships between strains, and the choice of suitable strains for use in vaccines. Extensive adaptation, first to eggs and then to chick embryo kidney or chicken kidney monolayers, is necessary before sophisticated antigenic comparisons can be made. This adaptation has been performed only for a limited number of field strains.

Table 5. Growth of coronaviruses in various animals, tissue cultures and organ cultures

Corona-virus species	Serotype or strain	Animal or tissue	Reference
A. Primary Isolation			
IBV	all	IBV has been isolated primarily only in chickens or in embryonated hens' eggs	
MHV	all	all strains of MHV have been isolated in suckling mice	
MHV	MHV-1	NCTC-1469 (tc[a])	HARTLEY and ROWE, 1963
		1°[b] mouse kidney (tc)	STARR and POLLARD, 1960
		mouse embryo (tc)	GOMPELS and NIVEN, 1953
MHV	MHV-3	NCTC-1469	HARTLEY and ROWE, 1963
		1° mouse liver (tc)	VAINIO, 1961; PARADISI, 1968
		mouse macrophage (tc)	MALLUCCI, 1965; VAINIO and JUDAH, 1962
		mouse embryo	HAFF, 1962
MHV	A-59	NCTC-1469, 1° mouse spleen (tc)	MANAKER et al., 1961
MHV	MHV-2	NCTC-1469	HARTLEY and ROWE, 1963
		mouse liver macrophage (tc)	BANG and WARWICK, 1960
MHV	MHV-B	1° mouse liver	MIYAZAKI et al., 1957
HCV	229E	2°[c] human embryonic kidney (tc), WI-38 (tc)	HAMRE and PROCKNOW, 1966
		Human embryonic intestine fibroblast (tc)	KAPIKIAN et al., 1969
		1° human embryonic kidney (tc), HeLa (tc)	BRADBURNE and TYRRELL, 1969
		L-132 (tc)	BRADBURNE and TYRRELL, 1969; BRADBURNE, 1969
		human embryonic tracheal organ culture	BRADBURNE et al., 1967
HCV	B814	human embryonic tracheal organ culture	TYRRELL and BYNOE, 1965
		L-132	BRADBURNE and TYRRELL, 1969; BRADBURNE, 1969
HCV	"OC" strains	human embryonic tracheal organ culture	MCINTOSH et al., 1967b
TGEV	all	all strains of TGEV have been isolated primarily in suckling and weanling pigs	
TGEV	Purdue	1° pig kidney (tc)	BOHL and KUMAGAI, 1965; WITTE and EASTERDAY, 1967
		1° fetal pig kidney (tc), 1° fetal pig thyroid (tc)	WITTE and EASTERDAY, 1967
		1° canine kidney (tc)	WELTER, 1965

[a] tc = tissue culture.
[b] 1° = primary.
[c] 2° = secondary.

Table 5.

Corona-virus species	Serotype or strain	Animal or tissue	Reference
TGEV	New York II	1° pig kidney	Bohl and Kumagai, 1965; Lee, 1956
TGEV	SH	1° pig kidney	Bohl and Kumagai, 1965; Harada et al., 1963
TGEV	FS216/64	1° pig kidney	Cartwright et al., 1964; McClurkin, 1965
TGEV	Ukiha	1° pig kidney. 1° pig spleen (tc)	Eto et al., 1962
TGEV	field strains	2° pig thyroid (tc)	Witte, 1971
HEV		most strains of HEV have been isolated primarily in suckling pigs	
HEV	HEV-1	1° pig kidney	Greig and Girard, 1963
HEV	67N	1° fetal pig kidney	Mengeling et al., 1972
RCV	8190	suckling rats. 1° rat kidney (tc)	Parker et al., 1970
SDAV	681	suckling rats. suckling mice	Bhatt et al., 1972
B. Adaptation studies			
IBV	Beaudette	suckling mice	Estola, 1967; Simpson and Groupé, 1959
		1° chick embryo fibroblast (tc)	Lukert, 1966; Fahey and Crawley, 1956
		1° chick embryo kidney (tc)	Lukert, 1966; Churchill, 1965; Chomiak, 1958; Cunningham and Spring, 1965; Mohanty and Chang, 1963
		1° Rhesus monkey kidney (tc), 1° chick embryo heart (tc)	Fahey and Crawley, 1956
		1° chick embryo lung (tc)	Lukert, 1966
		1° chick embryo liver (tc)	Lukert, 1966; Fahey and Crawley, 1956
IBV	Massachusetts	suckling mice	Estola, 1967
		chicken tracheal organ culture	Colwell and Lukert, 1969; Johnson et al., 1969
IBV	F	newborn rabbits, suckling mice, 1° chick embryo kidney, 1° chick embryo fibroblast	Estola, 1966
IBV	16 Japanese field strains	1° chicken kidney (tc)	Kawamura et al., 1961
MHV	A59	Syrian hamsters, WI-38	McIntosh et al., 1969
MHV	MHV (Balb C)	L cells (tc)	Mosley, 1961
HCV	229E	HeLa, 1° human embryonic lung (tc)	Bradburne and Tyrrell, 1969

Table 5.

Corona-virus species	Serotype or strain	Animal or tissue	Reference
HCV	OC 38-43	suckling mice suckling hamsters 1° Rhesus monkey kidney, 1° vervet monkey kidney (tc), BS-C-1 (tc)	McIntosh et al., 1967a McIntosh et al., 1969 Brůčková et al., 1970
		WI-38	Kapikian et al., 1972
TGEV	Ukiha	embryonated hens' eggs	Eto et al., 1962
TGEV	?	dogs, foxes	Haelterman, 1962
TGEV	FS 216/64	pig intestinal and nasal organ culture	Rubinstein, 1970
SDAV	681	1° rat kidney	Bhatt et al., 1972

Several strains of IBV have been successfully grown in chicken tracheal organ cultures (Colwell and Lukert, 1969; Johnson et al., 1969). The viruses produce a rapid ciliary immobilizing effect without the necessity for adaptation by subpassage. This system has not yet been employed either in the primary isolation of field strains or in serologic studies.

It is also clear that some strains of MHV have not shown the fastidiousness in tissue culture of the other coronaviruses. The cell line NCTC-1469 has been particularly useful for the primary isolation and propagation of many MHV strains, both those derived from standard MHV strains and field strains. This cell line is, however, not suitable for the growth of large quantities of MHV for chemical or physical study since it is heavily contaminated with a murine leukemia virus (David-Ferreira and Manaker, 1965). This adventitious virus is of a size and density very close to that of coronaviruses and would be difficult to remove by centrifugation methods.

Of great importance has been the difficulty of growing many HCV strains in laboratory animals or in cell monolayers, and the requirement for human embryonic tracheal organ cultures for primary isolation of most strains. These features of the human coronaviruses doubtless contributed to their not being recognized until very recently. Indeed, if the factors responsible for the extreme fastidiousness of naturally occurring human strains could be discovered and somehow corrected, it seems highly likely that many new and previously unrecognized strains would be found.

Isolation of TGEV directly from animals in primary pig kidney monolayers has been the most frequent mode of cultivation. Recently, however (Witte, 1971), secondary pig thyroid cells have been preferred for the recovery of field strains.

The cytopathic effect (CPE) produced by coronaviruses has been variable. Many of the viruses produce syncytia, including IBV-42 (Akers and Cunning-

ham, 1968; Mohanty and Chang, 1963), HCV strains OC38 and OC43 (Brůčková et al., 1970), TGEV (Cartwright et al., 1965), HEV (Mengeling et al., 1972; Greig and Girard, 1963), MHV (Paradisi, 1968; Mallucci, 1965; Hartley and Rowe, 1963; Laufs, 1967), RCV (Parker et al., 1970) and SDAV (Bhatt et al., 1972). However, not all coronaviruses which do produce CPE form giant cells, so that one cannot assign this capacity as a general feature to the coronavirus group. The CPE produced by the non-syncytium-forming viruses, and in some instances seen in addition to syncytium formation, is of a nonspecific degenerative quality.

The cell monolayer, particularly in early tissue culture passages, merely appears "dirty": scattered cells fall off the glass one by one so that the monolayer is frequently repaired as fast as the infected cells degenerate. This may be an explanation for the observation that some coronaviruses produce a CPE which is more easily recognized when cells are held under agar (Bohl and Kumagai, 1965).

B. Intracellular Development of Coronaviruses

1. Fluorescent Antibody Studies

There is little doubt that all known coronavirus species develop exclusively in the cytoplasm of infected cells. With the exception of a single report (Mohanty et al., 1964a), all fluorescent antibody studies of the intracellular development of coronaviruses have shown only cytoplasmic fluorescence (Hamre et al., 1967; McIntosh, et al., 1969; Konishi and Bankowski, 1967; McClurkin and Norman, 1966; Pensaert, 1970; Stepanek et al., 1971; Berry, 1967; Bito et al., 1971; Colwell and Lukert, 1969; Lukert, 1966; von Bülow and Schneider, 1969; Mallucci, 1965; Mengeling et al., 1972; Lucas and Napthine, 1971). It appears likely that in the one dissenting report the seed virus used (Beaudette strain of IBV) was contaminated with another virus, possibly an adenovirus, as was reported for certain IBV pools by Berry and Stokes (1968). There does not appear to be any characteristic fine morphology of the cytoplasmic fluorescence. Certain studies describe it as beginning in the perinuclear region and spreading to involve the entire cytoplasm (Pensaert, 1970). In cells infected at a multiplicity of about 1, the earliest fluorescence was seen 4 hours after infection (Von Bülow and Schneider, 1969).

2. Electron Microscopic Studies

Although many investigations of the matter have been conducted (Jonas et al., 1969; Mengeling et al., 1972; Svoboda et al., 1962; Watanabe, 1969; Parker et al., 1970; David-Ferreira and Manaker, 1965; Miyai et al., 1963; Starr et al., 1960; Okaniwa et al., 1968b; Witte et al., 1968; Bradfute et al., 1970; Thake, 1968; Vetterlein and Liebermann, 1970; Becker et al., 1967; Hamre et al., 1967; Oshiro et al., 1971; Bucknall et al., 1972a; Nazerian and Cunningham, 1968; Cowen et al., 1971b; Uppal and Chu,

1970; Ruebner et al., 1967) the details of events during morphogenesis of the coronaviruses are not at present finally established. There is agreement in published studies of all members of the group that particles collect during virus development in small and large cytoplasmic vesicles. Likewise there is agreement that virus particles are not observed in the nucleus, and that scattered particles are seen between layers of endoplasmic reticulum (ER) and often in the Golgi apparatus. Some investigators, but not all, have observed budding of virus particles from membranes either of ER or of cytoplasmic vesicles (Hamre et al., 1967; Oshiro et al., 1971; Nazerian and Cunningham, 1968; Bradfute et al., 1970; Vetterlein and Liebermann, 1970; Becker et al., 1967; David-Ferreira and Manaker, 1965; Ruebner et al., 1967; Watanabe, 1969). There appears to be a conspicuous absence of budding from the plasma membranes. In some studies reticular (David-Ferreira and Manaker, 1965; Seamer, 1965) or tubular (Ruebner et al., 1967; Seamer, 1965; David-Ferreira and Manaker, 1965; Becker et al., 1967; Oshiro et al., 1971; Hamre et al., 1967) structures have been seen within the cytoplasm of cells in a stage of active virus formation, but the relation of these structures to development of virus particles is not clear.

Budding into small cytoplasmic vesicles has been observed in studies of all the coronavirus groups. The possible events in the budding process were placed in a hypothetical scheme by Becker et al. (1967). It was postulated that the earliest event in the development of both IBV (strains 41 and 42) and HCV (strain 229E) was the formation of "crescents": areas of membrane which bulged toward the lumen of the vesicle and onto which a layer of dense material was apposed. These events are shown in Fig. 13 for HCV, strain 229E, in WI 38 cells. The crescents appeared to bulge into the lumen, developing more and more of the dense underlying layer, which ultimately formed a complete inner shell, the nucleoid, about 50 nm in diameter. The budding process was completed when the outer membrane was pinched off and the virus particle floated free in the vesicular lumen. These "crescents" were observed by the same authors in their study of IBV in the chorioallantoic membrane and by another group of investigators in the morphogenesis of TGEV (Vetterlein and Liebermann, 1970).

If budding really is the major or only mode of virus morphogenesis, it must occur rapidly, since it is seldom observed, despite the presence of large numbers of virus particles in the cytoplasm of infected cells. Alternative modes of development have been suggested (Uppal and Chu, 1970; Seamer, 1965), but the details of such mechanisms are still vague, even on the morphologic level. Moreover, postulated hypotheses must somehow account for the probable presence in coronavirus particles of host cell antigenic material within the outer envelope (Berry and Almeida, 1968; Hierholzer et al., 1972).

Studies of the morphogenesis of MHV in the livers of infected mice have been in essential agreement with these observations. Certain specific details have been, however, considered to be different. One group of investigators (Ruebner et al., 1967) felt that entire "nucleoids" (or "inner shells") were

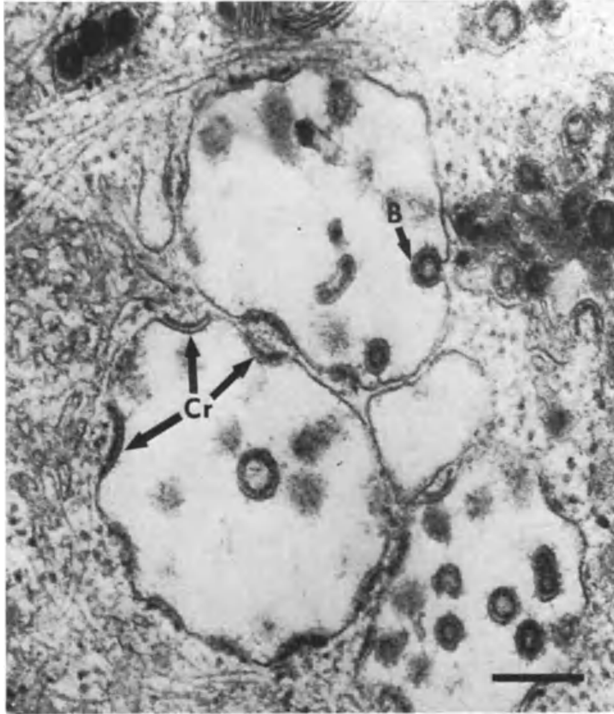

Fig. 13. Cytoplasm of a 229E-infected WI38 cell. Crescents surrounding a large cytoplasmic vacuole, and various stages of budding are seen. Cr: crescents. B: budding particle. Magnification before publication, ×60,000. Bar represents 200 nm

formed in the cytoplasm in close proximity to cytoplasmic vesicles. These nucleoids then approached the membrane of a vesicle and were considered to bud into it, taking their outer coat from the vesicular membrane. Another group pointed to an increase in the number of free ribosome-like particles within the cytoplasm and felt that these particles might represent differentiating viral internal components (Seamer, 1965).

The morphogenesis of different coronavirus species in a single host cell type has not been systematically examined, so that comparative statements cannot be made. It appears likely, however, that different coronaviruses have rather similar modes of intracellular development.

3. Single-cycle Growth Curves

Some insight into the intracellular development of coronaviruses can be gained from observations of single cycle growth curves. Unfortunately, although many experiments have been described, the multiplicity of infection (MOI) in most studies has been either very low (Cartwright et al., 1965; Stepanek et al., 1971; Harada et al., 1968; Hirai and Shimakura, 1971) or undefined (McClurkin and Norman, 1966; Sheffy, 1965; Witte and Easter-

DAY, 1967; VON BÜLOW, 1966; ESTOLA, 1966; AKERS and CUNNINGHAM, 1968; KAWAMURA et al., 1961; BRADBURNE and TYRRELL, 1971). One report (WITTE et al., 1968) described the events following the infection of fetal pig thyroid cell monolayers with the Purdue strain of TGEV, highly tissue culture adapted and at a MOI of about 70. In this study new virus appeared at 4 hours after infection, and the peak of cell-associated infectivity was at 22 hours. These observations agree more or less with those in other well documented reports where other members of the coronavirus group have been studied. At somewhat lower multiplicities and, in certain instances, at undefined multiplicities, new virus appears 4–6 hours after infection and peak titers are reached 12–36 hours after infection (PENSAERT, 1970; LUKERT, 1965; HAMRE et al., 1967; BRADBURNE and TYRRELL, 1971; BECKER et al., 1967; ESTOLA, 1966; AKERS and CUNNINGHAM, 1968; MCCLURKIN and NORMAN, 1966; MALLUCCI, 1965). In general, cell-associated virus titers rise faster than released virus titers (AKERS and CUNNINGHAM, 1968; LUKERT, 1965; HARADA et al., 1968; STEPANEK et al., 1971; HAMRE et al., 1967). Further details, such as the sequential development of various viral antigens and the bio-molecular events during infection, are not available.

V. Immunologic Studies

A. Virus-Specific Antigens from Coronavirus Infected Cells

Precipitating antigens from coronavirus infected cells have been recognized for some time. In 1959 a single antigen in allantoic fluid from IBV-infected eggs was precipitated in agar containing immune chicken serum and 8% NaCl (WOERNLE, 1959). Hypertonic agar has been used since then in systems containing chicken serum. BERRY and STOKES (1968) found a single common band of precipitation from 10 IBV isolates, using the Ouchterlony technique. TEVETHIA and CUNNINGHAM (1968) studied the precipitating antigens from IBV-42 infected eggs and found three soluble antigens. All three were present in crude concentrated allantoic fluid and in sonicates of infected chorioallantoic membrane (CAM). Antigen 1 diffused most rapidly, passed through a filter of 10 nm pore size, was trypsin sensitive but not ribonuclease sensitive, and had a buoyant density of 1.14 in CsCl. Antigen 2 diffused at a moderate rate, passed through 50 nm pores but not those of 10 nm, was ribonuclease and trypsin sensitive, retained its antigenicity at 100°C for 60 minutes and had a density of 1.18 in CsCl. Antigen 3 appeared to be larger and was in low concentrations. These workers found that all three antigens could be released from partially purified virus which had been treated with ether for 2 hours.

CHUBB et al. (1971), using different strains of IBV, were also able to identify 3 soluble antigens released from ether disrupted virus particles. However, they found only a single antigen in crude concentrated allantoic fluid and none in sonicated CAM. HIRONAO et al. (1970) found only a single precipitin band, which was present both in crude allantoic fluid and in homogenized infected CAM. The antigen was trypsin, but not ribonuclease, sensitive and probably,

therefore, represented antigen 1 of Tevethia and Cunningham. Bradburne (1969) reported 2 antigens to be present in IBV-infected mouse brain.

Similarly detailed studies of TGEV have not been performed. However, Phillip et al. (1971) reported that a single soluble antigen was present in lysates from both TGEV and HEV infected cells, and that these antigens were identical by the Ouchterlony technique.

Likewise, there is only a single study of precipitating antigens in suspensions of MHV. Bradburne (1970) described 2 precipitin arcs when MHV 3 was reacted with homologous hyperimmune serum.

Several investigators have described multiple antigens in crude or purified preparations of human coronaviruses. Bradburne (1970) identified two precipitin arcs when strain 229E was reacted with human postinfection sera. These findings have been confirmed by Brůčková and McIntosh (unpublished). Suckling mouse brain grown OC43 contains at least three (Hierholzer, 1972; Brůčková and McIntosh, unpublished) and perhaps four (Bradburne, 1970) separate precipitating antigens. Hierholzer (1972) obtained suggestive evidence that one of these antigens was a host cell antigen, since heterologous serum prepared against highly purified OC43 reacted with normal suckling mouse brain suspensions, and heterologous anti-mouse brain serum reacted with purified virus. This indirect evidence is corroborated by the electron micrographs of Berry and Almeida, which show "holes" in the envelope of IBV created by heterologous anti-IBV serum plus complement. These "holes" may represent material in the membrane which is antigenically host specific since they were not seen when a homologous virus-antibody system was used (Berry and Almeida, 1968).

Because of the failure in some studies to find multiple "soluble" antigens in untreated coronavirus preparations (Kaye et al., 1970; Chubb and Cumming, 1971; Berry and Stokes, 1968; Hironao et al., 1970) it appears likely that such antigens are not produced in large quantities, as they are, for example, in adenovirus infected cells. Indeed, those "soluble" antigens which are found in untreated cell culture or allantoic fluids may represent components of spontaneously disrupted whole virion particles.

B. Hemagglutination

Only two of the coronavirus species agglutinate erythrocytes spontaneously. These are human strains OC38 and OC43 (Kaye and Dowdle, 1969), which are serologically identical and probably represent two strains of the same virus serotype, and HEV (Phillip et al., 1971; Mengeling et al., 1972; Greig and Girard, 1963). Strains OC38 and OC43 were shown to agglutinate human type 0 and vervet monkey erythrocytes at 4° C but not at room temperature or 37° C, and chicken, rat and mouse cells at any of the three temperatures. The highest titers were achieved with rat and mouse cells. Rhesus monkey and guinea pig cells failed to agglutinate. HEV (strain 67N) was found to hemagglutinate rat, chicken, turkey, mouse and hamster erythrocytes at 22° C.

Guinea pig, calf, sheep, pig, goose, horse, rabbit and human type O red cells failed to agglutinate under the conditions examined.

Ether destroyed the hemagglutinating activity of both viruses. Trypsin was found to destroy that of the HCV strains. Evidence was obtained by KAYE et al. (1970) that the hemagglutinin of strain OC43 is closely associated with, and probably an integral part of, the infectious virion. Moreover, these workers were unable to free hemagglutinating activity from the virion by various physical and chemical procedures. HIERHOLZER et al. (1972) found that bromelin treatment, which removed the clubshaped projections, removed all hemagglutinating activity as well. Moreover, complement fixing activity and infectivity were destroyed at the same time. These experiments suggest strongly that the projections of the OC43 virion attach to red cells. It is possible that these projections must be part of the intact virus particle to cause hemagglutination.

Both OC38-43 and HEV have been found to cause hemadsorption (PHILLIP et al., 1971; MENGELING et al., 1972; GREIG and GIRARD, 1963; KAPIKIAN et al., 1972). This finding, which was unexpected in view of the absence of budding from the cell surface, was explored further by BUCKNALL et al. (1972a) who found, as others had (HAMRE et al., 1967; OSHIRO et al., 1971), that human coronavirus particles tended to adhere to the surface of the infected cell monolayer. Added erythrocytes, in turn, adhered to the virus particles. These authors preferred to call the phenomenon "pseudo-hemadsorption". It is likely, although unproven, that the mechanism of HEV hemadsorption is similar.

Immune serum has been found to inhibit the hemagglutinating and hemadsorbing activity of both OC38–43 and HEV (KAPIKIAN et al., 1972; KAYE and DOWDLE, 1969; GIRARD et al., 1964). In serologic surveys, rises in hemagglutination inhibiting antibody to OC43 tend to parallel rises in complement fixing antibody, further demonstrating the antigenic specificity of the reaction.

Hemagglutination is also observed in trypsin-treated crude allantoic fluid from IBV-infected eggs (CORBO and CUNNINGHAM, 1959). Further studies have demonstrated that the hemagglutinating activity may be detected in untreated allantoic fluid from infected eggs if it is separated from nonspecific inhibitors by column chromatography (BISWAL et al., 1966). This hemagglutination, however, is not associated with intact infectious virus (BISWAL et al., 1966) and is not specifically inhibited by antiviral antiserum (CORBO and CUNNINGHAM, 1959). IBV hemagglutination is therefore not useful as a serologic tool, and, indeed, may not be a specific viral or virus-coded substance.

C. Complement Fixation

Various strains of human, mouse, rat and avian coronaviruses have been shown to fix complement in the presence of convalescent or hyperimmune serum. There are no published reports of complement fixation (CF) by porcine viruses.

The complement fixing antigen of IBV (Beaudette strain) has been studied using guinea pig serum prepared against chicken kidney grown antigen (Steele and Luginbuhl, 1964). Because concentrated, partially purified virus preparations were used, it appears likely that the antigen measured was attached to the virion. This antigen was found to be heat stable and trypsin resistant.

A "soluble" CF antigen has been reported for MHV (Calisher and Rowe, 1966). Detailed information on the nature of this antigen is, however, not available. The CF antigen from human strain OC43 has been more extensively studied. It appears to be attached to the virion in such a way that, unlike that of myxoviruses, it is not liberated by ether or ether-tween treatment (Kaye et al., 1970). Hierholzer et al. (1972) were able to remove all CF activity from purified OC43 virions by treatment with bromelin. The same treatment removed the projections and destroyed both HA activity and infectivity. The CF antigen, like the HA antigen, was relatively resistant to heat (56°C for 30 minutes).

D. Serologic Interrelationships Among Coronaviruses

Coronaviruses, as a group, display complex serologic variability. This variability has apparently resulted in interrelationships, more or less close, between different strains from the same host species, as well as between strains from different host species. The full picture of these interrelationships is not yet complete, but certain generalizations are warranted. The details of these complex problems are published in recent papers by Dawson et al. (1971), Bradburne (1970),and McIntosh et al. (1969).

The TGEV strains appear to be unique among the coronaviruses in that no antigenic diversity between strains has yet been demonstrated (Bohl and Kumagai, 1965; Sasahara et al., 1958; Cartwright, 1966; Lee et al., 1954; Ristic et al., 1965). Interrelationships between TGEV strains and HEV strains have been briefly explored in a study reported by Phillip et al. (1971). A precipitation arc of identity between the two viruses was described, despite the lack of an antigenic relationship in reciprocal neutralization tests. The possibility of an interrelationship between TGEV and IBV is suggested by the finding that TGEV-coated bentonite particles were flocculated by a chicken antiserum against IBV (strain not specified) at a dilution of 1:80 (Sibinovic et al., 1966). Hyperimmune animal sera against 21 other viruses, including a chicken serum prepared against fowlpox, failed to flocculate the particles at dilutions greater than 1:20, and the homologous swine serum flocculated to a dilution of 1:320. This intriguing finding has not been explored further.

Also of interest is the presence of moderately high titers of neutralizing antibody to TGEV in dogs (Norman et al., 1970). Antibodies were found in dogs with no exposure to pigs, although they were not present in animals raised in a closed colony. This finding suggests the possible existence of a canine coronavirus related to TGEV. Antibodies to TGEV in human sera have not been sought.

On the basis of evidence available to date, IBV appears to be a serologically isolated species: attempts to show relationships between various strains of IBV and other coronaviruses (aside from the brief reference to a possible relation with TGEV mentioned in the previous section) have been uniformly unsuccessful (BRADBURNE, 1970; MCINTOSH et al., 1969). Neutralizing antibodies to IBV strain 41 have been found in human sera (MILLER and YATES, 1968), but exclusively in individuals with close and repeated contacts with chickens. Such antibodies are probably stimulated by exposure to virus aerosols, either from vaccines or from wild virus, and do not necessarily imply that IBV can replicate in man or that a human coronavirus antigenically related to IBV-41 exists. Indeed, the results of more direct studies of known human strains (BRADBURNE, 1970; MCINTOSH et al., 1969) would imply that this was not so. Nevertheless, conclusive evidence of the serologic isolation (or lack thereof) of IBV must await more sophisticated studies of antigenic relationships within the group itself.

In spite of their isolation, however, the various strains of IBV appear to be closely interrelated one with another and form a highly complex and confusing antigenic pattern. All strains of IBV may well be interrelated through the common precipitating antigen described by BERRY and STOKES (1968). However, other serologic techniques, namely neutralization tests in embryonated eggs or tissue culture, cross-protection studies in chickens, and fluorescent antibody studies, show an exceedingly complex picture (COOK, 1968; HITCHNER et al., 1966; HOPKINS, 1969; LUCIO and HITCHNER, 1970; WINTERFIELD et al., 1971; DAWSON and GOUGH, 1971; HOFSTAD, 1958; HOFSTAD, 1961; LUKERT, 1969; COWEN et al., 1971a; RAGGI and LEE, 1957; WINTERFIELD et al., 1964). Interrelationships have been frequently demonstrated by one technique and not by another, so that a satisfactory over-all scheme cannot be constructed. The problem is an important one, since poultry farmers depend on IBV vaccines to protect their flocks. Moreover, although there are between seven and fifteen well recognized different serotypes, most vaccines contain only two, namely the Massachusetts and Connecticut strains (CUNNINGHAM, 1970). This is because, in practice, many strains show interrelationships by cross-protection studies, even though more discriminating tests demonstrate marked differences. It is clear that some systematic effort must be made to obtain plaque-purified strains, to perform tests with standardized sera, and to work, in cross-challenge tests, with virus-free flocks under tightly controlled conditions. It is only through an effort such as this that the picture will be clarified.

It is not unlikely that a complex picture similar to that described above exists for both MHV and the human coronaviruses. Detailed reports of MHV serologic studies are not published. However, those data which are available show that all known strains of MHV are interrelated, either in CF tests or in neutralization tests, although no two strains appear exactly alike (CALISHER and ROWE, 1966; MORRIS, 1959; STARR and POLLARD, 1959a; MANAKER et al., 1961; DICK et al., 1956; GLEDHILL and NIVEN, 1955; MORRIS et al., 1966).

MHV, unlike IBV, is serologically related to coronaviruses from animal species other than the mouse. Several strains are closely related to RCV and SDAV by CF tests and distantly related to RCV by cross-neutralization tests (Parker et al., 1970; Bhatt et al., 1972). Likewise, several strains of MHV are closely related to human coronaviruses OC38 and OC43 (McIntosh et al., 1967a); MHV-3 has also been shown to be related to strain 229E (Bradburne 1970). Antibody to several strains of MHV is commonly found in human sera, probably because of endemic human infection with related coronaviruses (Hartley et al., 1964).

The human coronaviruses are similarly complex. The strains which were primarily isolated in tissue culture monolayers form a fairly homogenous group whose members are similar or identical to strain 229E (Hamre and Procknow, 1966; Kapikian et al., 1969). However, one strain, LP, was originally isolated in HETOC, resembles 229E quite closely, but differs from it by neutralization kinetics (Bradburne, 1970). Other organ culture isolated strains are more heterogenous. The two viruses which were adapted to grow in suckling mouse brain, strains OC38 and OC43, are probably identical (McIntosh et al., 1967a), and another strain OC44, is quite similar (McIntosh et al., 1969). All three are closely related by CF tests to MHV. But other strains, including B814, EVS, OC16, OC37 and OC48 are poorly characterized and variably related to each other, strains 229E and OC38–43, and MHV. It is not unlikely that strain OC38–43 is related, although distantly, to 229E (Bradburne, 1970).

VI. Summary

This review has concentrated primarily on the comparative aspects of coronavirus virology. It is clear that all of the coronavirus species share certain biophysical and chemical characteristics: in negatively stained preparations their size is 80–160 nm, and particles are round, moderately pleomorphic, and covered with widely spaced, 20 nm club-shaped projections; in ultrathin sections they are 75–90 nm in diameter, and possess an outer membrane and an inner shell; their nucleic acid type is RNA; they are ether and chloroform labile; and their buoyant density in sucrose is 1.18–1.19 gms/cu. cm. Some of the species (and, perhaps, types) differ in their sensitivity to acid pH and trypsin. As far as is presently known, the ultrastructural morphogenesis of all species is similar, although perhaps not identical. Two coronavirus species hemagglutinate; the rest apparently do not. The antigenic characteristics of the various species vary widely: TGEV shows little or no variation between strains; MHV and IBV show an apparently limitless antigenic variability between strains, and the HCV types probably resemble MHV and IBV. Some coronavirus species are extremely fastidious in their *in vitro* growth requirements and others are considerably less so.

As a virus genus, the coronaviruses are of great economic importance. They are the cause of several animal diseases of world-wide distribution and high

morbidity and mortality. In man they are responsible for a significant proportion of upper respiratory illness and are major contributors to economic loss due to sick leave. Further investigation into their properties is clearly indicated.

Studies of the biophysical and chemical nature of coronavirus virions have lagged behind other types of research and deserve to be pursued now. These include an examination of purification methods for all the coronavirus species, and chemical and physical characterization of the nucleic acid, core and envelope. The molecular events in coronavirus infected cells have not been examined to any significant extent. The genetics of coronaviruses have been similarly neglected and are particularly important in those species which show marked and complex antigenic variation.

One of the attractive features of coronavirus research is the intriguing possibility that discoveries in one species may have application in another. Thus, examination of the nature of virus-cell interaction of some less fastidious coronavirus species, such as MHV, may elucidate factors leading to more successful cultivation of human strains. Already the necessary use of the electron microscope in the study of HCV strains has led to more rapid and precise virologic diagnosis in IBV infection. Such interplay between veterinary and human virology was the object of a recent coronavirus workshop (*Anonymous*, 1972) and should be encouraged.

Other areas of research, though perhaps more mundane, are not less important. IBV serology, like HCV serology, is of great practical importance and should receive due emphasis. Some of the inter-species antigenic relationships, particularly those involving the porcine coronaviruses, should be explored. Coronaviruses should be sought in other animal species. And, finally, efforts to attenuate coronaviruses should be systematically pursued, since effective vaccines, particularly for IBV and TGEV, are clearly of paramount importance.

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Altered Quantitative Expression of Immunoglobulin Allotypes in Rabbits

ROSE G. MAGE

With 9 Figures

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I. Introduction

In 1962, DRAY showed that the quantitative expression of allelic allotypes in heterozygous rabbits was affected by exposure of the neonatal rabbits to maternal isoantibodies directed against the antigenic type inherited from the father (DRAY, 1962). This phenomenon has become known as allotype suppression. During the decade since the discovery of the phenomenon, a considerable body of information has accumulated about the genetics, structure and localization of the genetically controlled antigenic variants of rabbit immunoglobulins (immunoglobulin allotypes) (reviewed by MAGE et al., 1973a). This review will summarize our knowledge of allotype suppression in the light of current concepts of immunoglobulin structure and cellular immunology.

II. Major Allotypes and their Structural Correlates and Localization

The rabbit has been a particularly valuable experimental animal for study of the genetics and structure of immunoglobulins. The allotypes which will be discussed in the course of this review of allotype suppression are listed in Table 1. There are allotypes which are associated with kappa-type light chains

(b4, b5, b6 and b9), lambda-type light chain (c7 and c21), V_H regions of heavy chains (a1, a2, a3, x32 and y33) as well as genetically linked allotypes on the constant regions of IgG, IgA and probably IgM.

Table 1. Some major rabbit allotypes, their structural correlates, and localization

		Localization and chemical information
Heavy chains		
V	a1, a2, a3	The *a* allotypes are associated with multiple amino acid interchanges within the variable region and are found in association with different constant regions ($C\gamma$, $C\alpha$, $C\mu$, $C\varepsilon$)
	x32 y33	Found in association with different constant regions
$C\gamma$	d11	Methionine (position 225)[a]
	d12	Threonine (position 225)[a]
	e14	Threonine (position 309)[a]
	e15	Alanine (position 309)[a]
Light chains		
Kappa-type		
	b4, b5, b6, b9	Multiple amino acid interchanges, probably within the constant region
Lambda-type		
	c7, c21	No chemical information available

[a] Eu numbering (GALLY and EDELMAN, 1972).

The allotypes of the *a* locus, a1, a2 and a3 as well as genetically linked allotypes on a small proportion of IgG molecules which lack the *a* markers (*a*-negative allotypes x32 and y33) appear to be associated with the variable portions of rabbit heavy chains. They are detectable on IgG, IgA and IgM as would be expected from the fact that different classes of heavy chains share the same set of variable regions (GALLY and EDELMAN, 1972). Data on the compositions of Fd fragments (KOSHLAND, 1967; INMAN and REISFELD, 1968) and amino terminal sequence data (WILKINSON, 1969; MOLE et al., 1971) are also consonant with the localization of these allotypes on the variable regions of heavy chains.

The allotypes of the *b* locus, b4, b5, b6 and b9 appear to reflect multiple amino acid differences in the constant regions of kappa type light chains (APPELLA et al., 1970). Although it is unlikely that the *b* locus allotypes are exclusively due to amino-acid differences in the variable portions of kappa type light chains, there appear to be some differences between normal light chains of different allotypes in the average amino acid sequence at the amino-terminus (CHERSI et al., 1971; HOOD et al., 1971). Thus, it is possible that some antigenic determinants recognized by the usual anti-*b*-locus allotype

antisera are influenced by sequence differences in the V-region of the kappa chains.

The allotypes of rabbit lambda chains, include c7 and c21. They are controlled by the *c* locus which is unlinked to the *a* or *b* loci (GILMAN SACHS et al., 1969). The chemical basis for the antigenic differences between c7 and c21 type lambda chains is not known and they have not been localized to variable or constant portions of the light chains.

Allotypes on the constant regions of IgG, IgA and IgM are closely linked to V_H allotypes of the *a*, *x* and *y* loci. The chemical bases for two sets of variants of rabbit gamma chains have been delineated. The A11 and A12 allotypes (*d* group) in the hinge region, correlate with a replacement of threonine (A12) for methionine (A11), at the residue position immediately N terminal to the heavy-heavy disulfide bond (PRAHL et al., 1969). The A14 and A15 allotypes (*e* group) correlate with the interchange of threonine (A14) and alanine (A15) at position 309 (Eu numbering system) of the $C_{\gamma 2}$ domain (APPELLA et al., 1971). The group *d* and *e* group allotypes are probably controlled by genes for the IgG heavy chain constant region, which can be considered alleles at a *de* locus.

III. Allotype Expression in Normal Rabbits

Newborn rabbits have circulating immunoglobulin derived from their mother (OUDIN, 1960) but secrete little or none of their own. During the first 8–10 weeks of life, the maternal immunoglobulin is catabolized and the young rabbits begin to synthesize and secrete their own immunoglobulin as they develop immunological competence. If the allotypes of the mother and father differ, newly synthesized immunoglobulin can be identified by its content of allotypic markers derived from the father (DRAY, 1962). In normal heterozygous rabbits, paternal allotype becomes detectable in the serum of the developing rabbit between 10 days and three weeks of age. By 12 weeks of age, the percentage of total IgG with paternal allotype reaches a level which is typical for that particular combination of allelic allotypes and remains relatively constant for the remainder of normal life. The serum concentration of IgG reaches typical adult levels between the 12th and 15th week of life although there is much fluctuation in the IgG levels during the lifetime of conventionally raised rabbits, with a trend toward higher concentrations in older animals. Often the fluctuations in IgG concentrations are mirrored by elevations and drops in the concentration of both allelic allotypes in heterozygotes. Hyperimmunization with protein antigens such as BSA (LANDUCCI TOSI et al., 1972a), hapten-protein conjugates (LARK et al., 1965; HOYER and MAGE, 1967; DUBISKI et al., 1970), and bacterial vaccines often elevates both allelic allotypes in parallel fashion although allotype ratios may change slightly. However, marked shifts in allotype ratio can occur after hyperimmunization, especially in instances where high concentrations of antibodies of restricted heterogeneity are stimulated with bacterial vaccines (GELL and KELUS, 1962; CATTY et al., 1969; KINDT et al., 1970).

In heterozygous rabbits, although both alleles at a given allotypic locus are expressed, the products of only one of the alternative alleles are found on any given molecule (DRAY and NISONOFF, 1963; SCHMALE et al., 1969; LAWTON and MAGE, 1969). This phenomenon of allelic exclusion is explained by the fact that cells which secrete serum immunoglobulins have differentiated to produce only one of alternative allelic forms of heavy and light chains. Two allelic types of immunoglobulins are not always found in the serum in equal proportions. Staining of antibody producing cells with fluorescent anti-allotype reagents shows close correspondence between the relative numbers of cells in heterozygous rabbits with allelic allotypes and the relative proportions of the two allotypes in the serum IgG (PERNIS et al., 1965; CEBRA et al., 1966). One is thus led to conclude that relative proportions of molecules with allelic allotypes on IgG are a general reflection of relative proportions of cells differentiated to produce the alternative forms.

There are a number of examples of unequal expression of alternative allelic forms of rabbit immunoglobulins. The *b* locus allotypes are not usually expressed in equal proportions in heterozygotes. The b4 allotype is normally expressed in a greater amount than the other alleles, b5, b6 or b9. The b5 and b6 allotypes are expressed in similar proportions, and all the other *b* allotypes are expressed in greater proportion than b9. In b9 rabbits there is also considerably greater expression of the unlinked lambda type of light chain relative to kappa. Typically b9 rabbits have about 60% kappa, b9 and 40% lambda Ig in their sera while rabbits of other *b*-allotypes have 5–15% lambda (CARBONARA et al., 1969; REJNEK et al., 1969). A most striking imbalance is in the expression of a1 and a2 in heterozygotes. Typical $a^1 a^2$ heterozygous rabbits have four times as much a1 Ig as a2 Ig (MAGE, 1967; LANDUCCI TOSI et al., 1972a). WEIGERT and his colleagues (1970) have suggested that the expression of the lambda type in a given species reflects its relative potential to contribute to immunoglobulin diversity. They also suggested that the ratio of kappa to lambda in the serum might reflect the germ line veriable gene ratio $V_\varkappa$:V_λ. The information we have about expression of light chain allotypes and types in rabbits suggest some modifications of these ideas as well as some further generalizations. It is possible that the normal expression of light chains of a given allotype or type is proportional to the number or variety of V region genes which can associate with that type. For example, if the V region genes which can associate with b9 kappa chains differ from those which associate with b4 either in actual number or in adaptive value for forming antibody sites for environmental antigens, then there would be a lower probability for precursor cells with the b9 type of receptor to be triggered by antigen into differentiation, proliferation and secretion of immunoglobulin. It would also follow that in b9 homozygotes there would be a higher probability for differentiation, proliferation and immunoglobulin production by cells with lambda type light chains utilizing a completely separate and different library of variable region genes. Similar reasoning applied to the *a* locus would suggest that the variable regions available for forming diverse antibody receptors are qualita-

tively or quantitatively deficient if a2 is compared to al as the genetic source of a V region library. The variable regions represented by the *a*-negative group of heavy chains on the other hand, are probably limited quantatively or qualitatively compared to the array of variable regions with allotypic determinants of the *a*-locus. The *a*-negative allotypes of the V_H region such as x32 and y33 are normally expressed on 5–30% of the total IgG. However, several immunized rabbits have produced anti-streptococcal antibodies of restricted heterogeneity with *a*-negative heavy chains (KINDT et al., 1970; WATERFIELD et al., 1972).

There is only limited data on the relative expression of allelic forms of the constant region of IgG. In an al homozygous rabbit, the C_γ markers A14 and A15 were found in equal proportions, however in $a^1 a^2$ heterozygous rabbits, there was expression of the C_γ markers A14 and A15 at a level equal to that of the *a* locus marker (V_H) to which they were genetically linked LANDUCCI TOSI et al. (1970). Thus in a rabbit in which a1 was genetically linked to A14 and a2 to A15, both a1 and A14 were expressed on a large proportion of the total IgG (80%) and a2 and A15 were poorly expressed (20%). In a rabbit with the opposite linkage relationship, where a2 was linked to A14, it was the A14 which was poorly expressed (18%) paralleling the poor expression of the a2 (19%). These quantitative measurements of the expression of allotypes of the variable and constant regions of rabbit γ chains also showed that the great majority (>95%) of differentiated cells producing IgG utilize genetic information for V_H and C_γ regions which resides on the same chromosome. They did not exclude the possibility that a small proportion of molecules might represent recombinant types with the variable region marker present from one parental chromosome and the constant region marker from the other parental chromosome. Such recombinant IgG molecules have indeed been identified in the IgG from rabbits doubly heterozygous for group *a* and group *e* allotypes at a level of about 1% LANDUCCI TOSI and TOSI (1973). Such molecules may represent the products of cells in which somatic crossing over has occurred or in which a gene translocation event has occurred interchromosomally.

IV. Alteration of Normal Expression by Allotype-Suppression

A. Suppression of one Allelic Allotype in Heterozygotes

Persistent and drastic alterations in the normally found proportions of allelic allotypes are observed in heterozygotes which are exposed early in life to anti-allotype antibody derected against a paternal allotype (DRAY, 1962; MAGE and DRAY, 1965; MAGE, YOUNG and DRAY, 1967; MAGE, 1967; DUBISKI, 1967a; DUBISKI and SWIERCZYNSKA, 1971).

Since normal b^4 b^5 heterozygotes generally have more b4 than b5, the effect is especially striking when paternal b4 type is suppressed in expression. Fig. 1 outlines two types of experiments which result in the development of $b^4 b^5$ heterozygous rabbits with abnormally low proportions of paternally derived b4 allotype. In the experiments, dams are homozygous b5 and sires

Fig. 1. Diagrammatic representation of experiments leading to suppression of one allelic *b* locus allotype in heterozygotes (from Mage, 1967)

are b4. The dams may be actively producing anti-b4 (left half of Fig. 1) and the anti-b4 transmitted from mother to young, or the dams may be nonimmunized and anti-b4 can be injected into the newborns (right half of Fig. 1). In the latter type of experiment, the dose and time of administration of antibody can be controlled and normal noninjected littermates can be kept as controls. Fig. 2 shows total IgG, b4 and b5 concentrations in the offspring of normal b5 dams and b4 sires (Mage, 1967). In the untreated control animals, about 20% of their total IgG was of paternal b4 allotype by four weeks of age while littermates which received anti-b4 antiserum on the day of birth (2.5 ml containing 2.2 mg precipitable anti-b4) had no b4 Ig detectable until about $14^1/_2$ weeks of age. Fig. 3 shows data for the same animals as in Fig. 2 (plus an additional control) calculated as relative proportions of b4 and b5. In spite of large variations in total IgG concentrations (Fig. 2), the relative proportions of b4 and b5 were maintained with remarkable constancy in control animals after about 12 weeks of age. The proportion of IgG with b4 allotype rose gradually over the first year of life in the treated animals. Suppression such as that shown in Figs. 2 and 3 has been observed in numerous litters raised in our laboratories (Mage and Dray, 1965; Goldman and Mage, 1972) as well as in the laboratories of Dubiski (Dubiski and Fradette, 1966; Dubiski, 1967a; Dubiski and Swierczynska, 1971), Sell (1968); Dray and coworkers [Reviewed by Dray (1972)], and Lowe (1972). Whereas traces of the suppressed allotype may often be detected between the third and fourth month of life, several injections of antiserum spaced out over the first two weeks generally results in a more prolonged total suppression. Thus, the animal in Fig. 4 (closed triangles) which received anti-b4 on days 1, 2, 3, 7 and 11 had no detectable b4 until it was $6^1/_2$ months old when b4 constituted less than 1% of its total IgG. Dubiski and Swierczynska (1971) have reported that although there is considerable

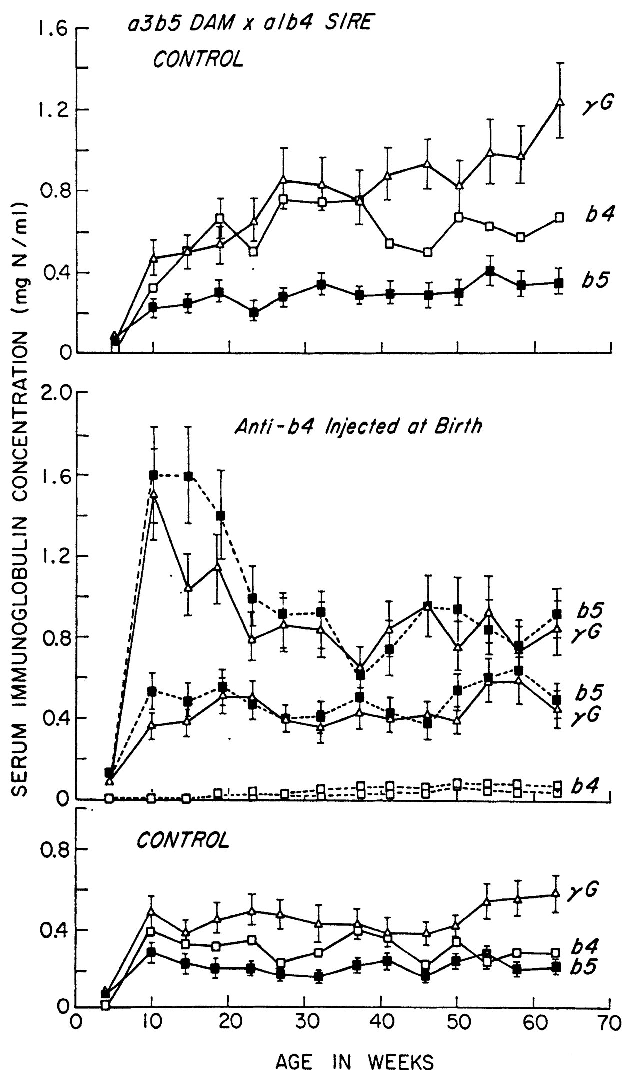

Fig. 2. Serum concentrations (mg N/ml) of IgG (△), b4 (□) and b5 (■) in normal (top and bottom panels) and anti-b4 treated (center panel) b^4b^5 offspring of b5 dams and b4 sires (from MAGE, 1967)

variation, there is a general correlation between the antibody dose given and the duration of total suppression [defined as the period when no paternal allotype is detectable by single diffusion (OUDIN, 1952; CHOU et al., 1967)]. Moreover, several small doses spaced out over 10 days were more effective than fewer injections containing the same total dose of antibody (DUBISKI and SWIERCZYNSKA, 1971).

The altered expression of paternal allotype occurs both for the various combinations of alleles at the *b* locus and for the independently assorting alleles at the *a* locus (MAGE et al., 1967; MAGE, 1967). Fig. 5 shows two types of experiments in which a^1 a^3 heterozygous offspring are obtained which express

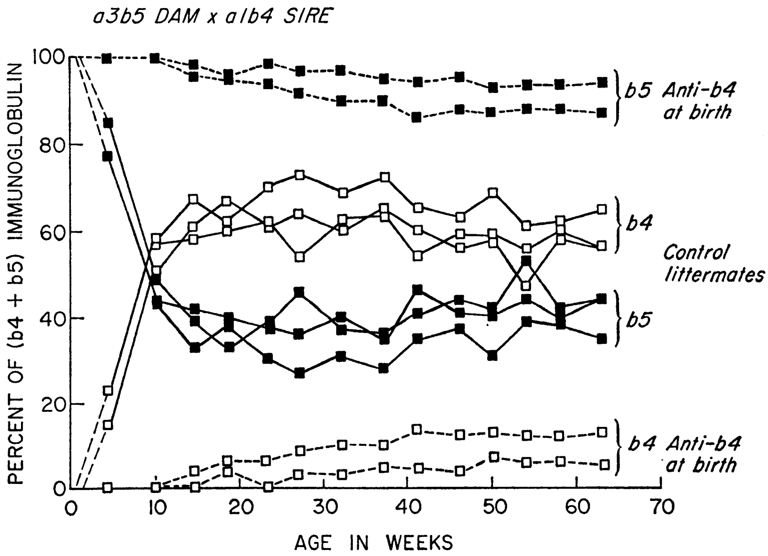

Fig. 3. Relative proportions of b4 and b5 Ig in normal and allotype suppressed b^4b^5 offspring of b5 dams and b4 sires. The percent b4 or b5 is expressed relative to total b4+b5. (From MAGE, 1967)

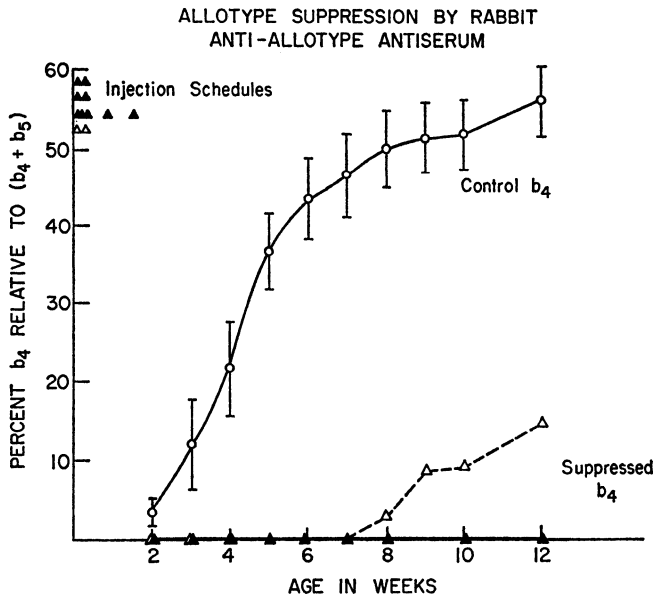

Fig. 4. Development of paternal b4 in normal and in anti-b4 treated b^4b^5 heterozygous rabbits. (▲) Three rabbits which received 2.5 ml of rabbit anti-b4 antiserum on days 1 and 2. One of them received additional injections of 5 ml on days 3, 7 and 11. (△) One rabbit which received 2.5 ml anti-b4 on days 1 and 2 and produced b4 by 8 weeks. (○) Means and standard errors of percent b4 in eight normal b^4b^5 heterozygotes from five different litters. (From GOLDMAN and MAGE, 1972)

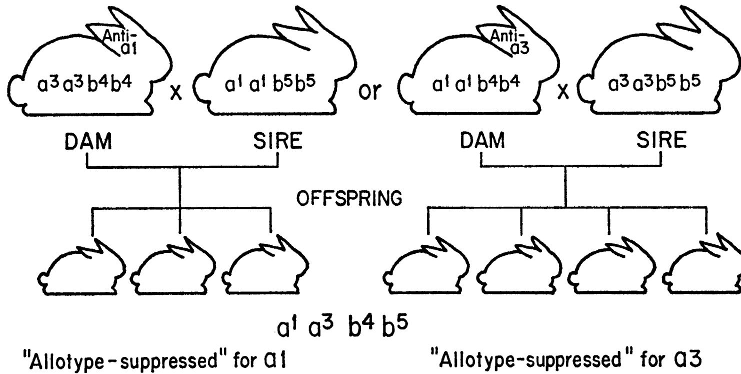

Fig. 5. Two types of experiments which lead to suppression of one allelic allotype in a^1a^3 heterozygous rabbits (From MAGE, 1967)

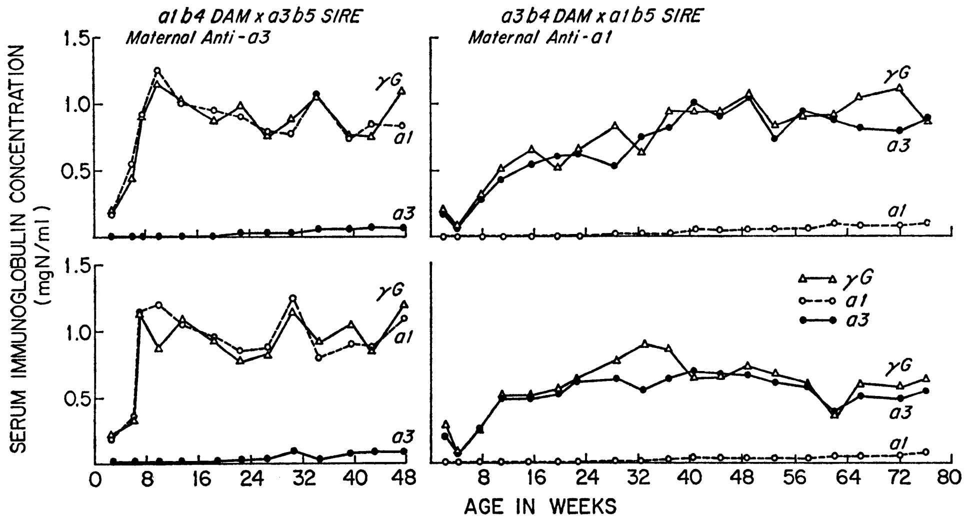

Fig. 6. Serum immunoglobulin concentrations (mg N/ml) in rabbits exposed to maternal anti-a3 (left) or anti-al (right) (△) IgG, (○) al, (●) a3. (From MAGE, 1967)

the a1 and a3 types in very different relative proportions. In the left half of Fig. 5, the $a^3\,a^3$ mother actively immunized against the paternal al allotype has $a^1\,a^3$ offspring which express a low proportion of the paternal a1 allotype (allotype suppressed for a1). In the right half of the figure an $a^1\,a^1$ mother immunized against paternal a3 allotype has $a^1\,a^3$ heterozygous offspring which express the paternal a3 allotype in low proportion (allotype suppressed for a3). The development of immunoglobulin levels in such heterozygotes is illustrated in Fig. 6. In each enimal, the maternal allotype accounts for the majority of the total IgG in the sera measured by a radial diffusion method (MAGE et al., 1967). By this method, paternal allotype was detected in the allotype suppressed animals at 28–30 weeks of age when the concentration became greater than 20 μg N/ml and the suppressed allotype represented 3–7% of the total protein with detectable a-locus determinants. Fig. 7 shows the relative pro-

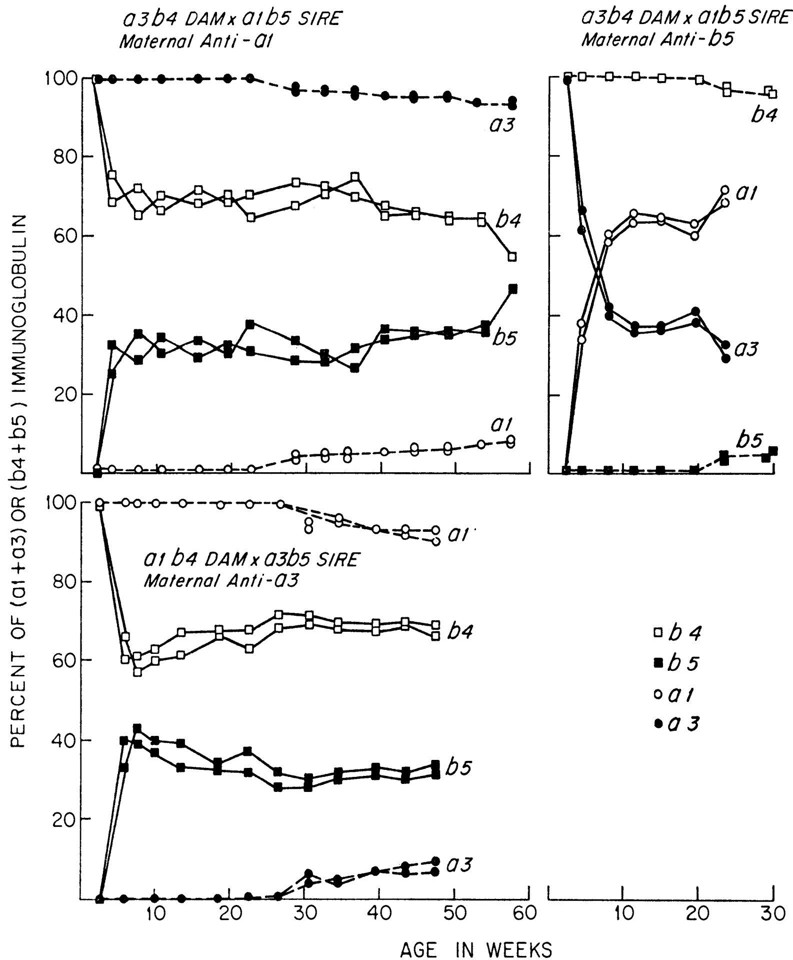

Fig. 7. Relative proportions of a1 and a3 in the suppressed a^1a^3 heterozygotes whose serum Ig concentrations are shown in Fig. 6. Also shown are the normal proportions of paternal b5 and maternal b4 allotypes. The upper right panel shows normal expression of a1 and a3 in a heterozygote exposed to anti-b5. Proportions of *b* allotype are expressed relative to total b4+b5 and *a* allotype relative to a1+a3. (From MAGE, 1967)

portions of a1 and a3 in the sera of the same animals whose IgG and allotype concentrations are shown in Fig. 6. The relative proportions of the a1 and a3 allotypes are strikingly different in the two groups of heterozygotes. Also shown in Fig. 7 are the relative proportions of b4 and b5 in these same animals. The paternal b5 allotype in each group was expressed in a normal proportion (compare with proportions in Fig. 3). In the upper right panel of Fig. 7, the proportions of a1 and a3 in double heterozygous offspring of an a^3 a^3 dam which was making anti-b5 but not anti-a1, are shown for comparison.

When one allelic allotype at the *a* locus is suppressed, the linked marker on the constant region of the IgG γ chain is also suppressed (LANDUCCI TOSI et al., 1970). An animal of genotype 1,14/2,15 was born from a mother with circulating anti-a1 antibody. The neonatal exposure to anti-a1 resulted in suppressed

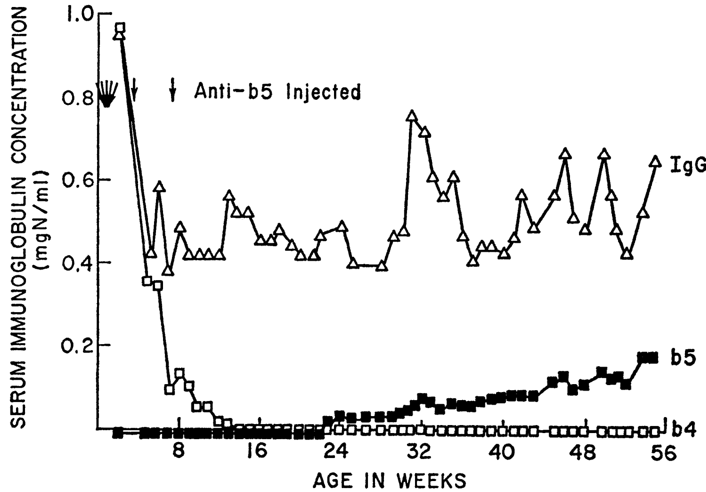

Fig. 8. Serum Ig concentrations (mg N/ml) in a rabbit which received five injections of anti-b5 antiserum (16 mg antibody protein) during its first week of life, 8.5 mg antibody protein on day 24 and 4.2 on day 52. (From MAGE et al., 1970)

homozygous at a given locus. DUBISKI (1967a, 1967b) first reported total suppression of *b* locus allotype in homozygous b^5b^5 rabbits. This report was confirmed by VICE and coworkers (1969a), DAVID and TODD (1969) and by MAGE and coworkers (1970). In the initial experiments of DUBISKI (1967a, 1967b) and in our first experiments, a heterozygous b^4b^5 dam already allotype-suppressed for b5 was mated with a b^5b^5 sire so that maternal b5 immunoglobulin was not transmitted to the offspring. Approximately half of the newborn rabbits received the b5 gene from their dams and thus were b5 homozygotes. The newborns were injected with anti-b5 repeatedly during the first week of life, and the animal shown in Fig. 8 received additional anti-b5 at intervals until 7 weeks of age. After catabolism of maternal and injected globulin with b4 allotype (open squares), the animal shown in Fig. 8 had concentrations of serum IgG (open triangles) ranging from 0.4 to 0.8 mg N/ml of serum during the first year of life. In numerous suppressed b^4b^4 and b^5b^5 animals which we have now examined, total IgG levels are within the normal range for age matched or littermate control rabbits [see e.g. HARRISON et al. (1973a)]. It can be seen in Fig. 8, that IgG with b5 allotype (filled squares) was not detectable in this animal's serum until it was 5 months old when it constituted about 3 % (15 μg N/ml) of the total serum IgG. All of the suppressed homozygous rabbits which have been produced in my laboratory have "escaped" from total suppression and begun to produce small amounts of circulating Ig with kappa-type light chains within the first year of life (usually between 5 and 7 months of age). VICE et al. (1969b) reported a rabbit which was still totally suppressed for b5 at 14 months of age. An even more efficient way to obtain suppressed homozygous rabbits was independently developed by VICE et al. (1969a) and by DAVID and TODD (1969). Although the two

expression of the a1 allotype. Even at 11 months of age the IgG with a1 was only 25 % of the total whereas it was 73 % a2, essentially the reverse of the normal relative expression of these allotypes. The constant region allotype A14 which was genetically linked to the a1 was also expressed in low proportion (20 %) while the A15 allotype linked to a2 was found on 70 % of the IgG. This animal was immunized with bovine serum albumin (BSA) and the non-antibody and anti BSA IgG were separately analyzed for the V and C heavy chain markers LANDUCCI TOSI et al. (1972a). Only about 12 % of the anti-BSA had the suppressed a1 allotype and this was paralleled by 15 % detectable A14. The linked a2 and A15 markers were found on 77 and 80 % of the anti-BSA IgG molecules, respectively. The linked suppression of V_H and C_γ markers suggests that the *a* locus differentiation step preceded or coincided with the γ chain differentiation and that generally markers on the same chromosome were expressed together in an individual cell. It would be of great interest to know whether the reverse experiment of suppressing the constant region marker could suppress expression of the linked V_H marker, but to date we have not succeeded in obtaining chronic suppression with anti-A15 or anti-A14 reagents (MAGE, 1971). Since the differentiation of cells producing γ chains may occur relatively late in the developing newborn rabbit, in one experiment, we injected four of six littermates from a mother which was actively making anti-A15 with additional anti-A15 at birth and at weekly intervals through eight or nine weeks of age. At five weeks of age, two of the four injected animals had no circulating IgG with A15 and anti-A15 was demonstrable in serum collected seven days after their last injection. There was also no detectable a2 to which the A15 was genetically linked. These two animals received anti-A15 until nine weeks of age. However, by eight weeks, we found that all the animals had detectable A15. The levels of a2 were close to normal (12–25 %), and by 12 weeks all the rabbits had 18–25 % a2. Thus although there may have been some delay in the appearance of a2 and A15 in the serum, chronic suppression was not established by administration of anti-A15 to the newborn and developing rabbits. We cannot exclude the possibility that a more pronounced effect would have been obtained if we had given more antibody. Since anti-A15 is a hemagglutinating antibody directed against a discrete region of IgG associated with a single amino acid interchange (see part II) it may not be of sufficient potency to react with receptors on target cells and lead to allotype suppression. It is also possible that the cells which are the targets for allotype suppression in rabbits do not have receptors with γ chains at all. All of the other allotypes of rabbits which have been successfully suppressed are represented on all classes, as light chains or V-region markers of the *a* locus.

B. Suppression in Homozygotes

Since one allelic allotype can be suppressed by exposure of heterozygous rabbits to antibody to the paternal type, it was considered possible that total suppression of the expression of an allotype could be produced in rabbits

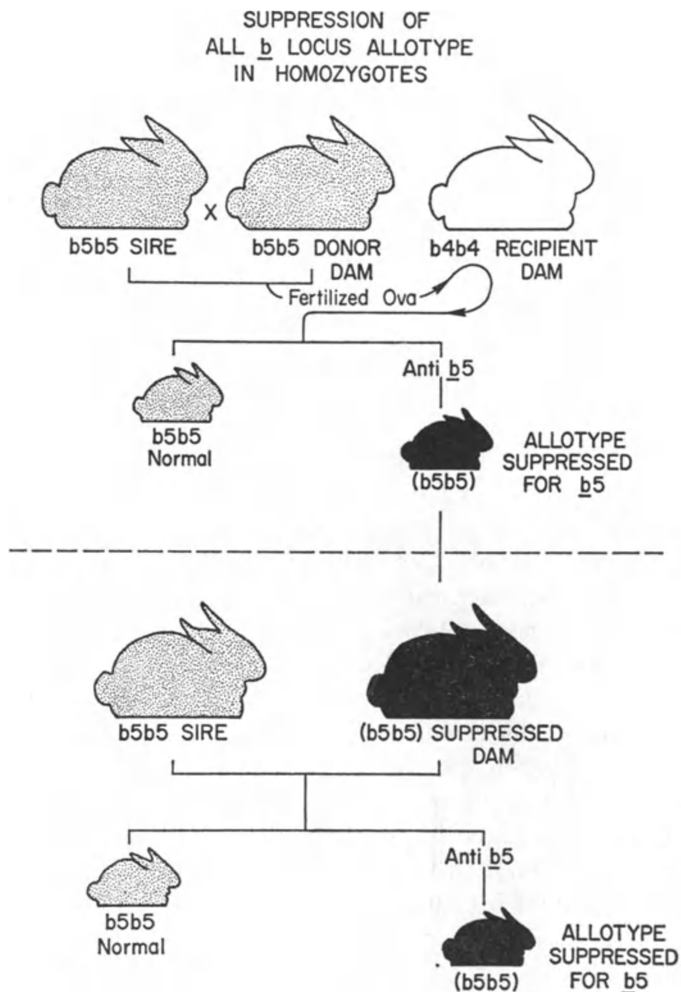

Fig. 9. Diagrammatic representation of the production of suppressed homozygotes at the *b* locus

techniques differ in technical detail, the principle is the same. Transfer of maternal serum containing the allotype one wishes to suppress is avoided by transplanting fertilized ova (2–16 cell stage) to the uteri of recipient does of another allotype. These does may be actively immunized against the allotype of the transferred zygotes, or may be non-immunized and the newborns injected with the appropriate anti-allotype antiserum. In my laboratory, we have followed the procedure of VICE et al. (1969a) and produced suppressed homozygous b^4b^4 and b^5b^5 rabbits. Several of the suppressed b^5b^5 females were also mated with b^5b^5 sires, and their offspring treated with anti-b5. In this way, we produced a total of ten suppressed b^5b^5 homozygous rabbits as well as two control littermates (in one of the litters) with out doing any additional surgical procedures. Fig. 9 outlines diagrammatically, the production of suppressed

homozygotes at the *b* locus by zygote transfer and by subsequent utilization of totally suppressed homozygous does.

When one allelic *b*-alocus allotype is suppressed in heterozygotes, there is compensatory increased production of the alternative allelic form. When the *b*-locus allotypes are suppressed in homozygotes, the rabbits produce no kappa-type IgG but compensate by production of IgG with lambda-type light chains (APPELLA et al., 1968; MAGE et al., 1970). Thus, upon suppression of all kappa-chain expression, the products of an unlinked locus (*c* locus) are used to assemble and secrete normal levels of IgG. In normal rabbits, the proportion of IgG with lambda-type light chains ranges from 5–40% (depending upon the strain and *b*-locus allotype) (MAGE et al., 1970). In suppressed *b*-locus homozygotes, all of the IgG has lambda type light chains until "escape" from suppression commences, and in many animals the proportion of IgG with lambda type light chains remains abnormally high for the entire life of the animal.

The IgG and light chains from suppressed homozygous rabbits provided the materials which permitted chemical studies of the lambda-type light chains of rabbits (APPELLA et al., 1968; APPELLA et al., 1970). It was only when the IgG of such rabbits became available that it could be proven that lambda type light chains carried the c7 allotype (MAGE et al., 1968). It had been known for some time that the c7 allotype [originally designated "P" (DRAY et al., 1963)] was associated with a minor component of normal rabbit IgG (DRAY, 1964). IgG and light chains from suppressed homozygotes reacted strongly with anti-c7 antisera. When quantitative determinations of the total IgG bearing the c7 allotype were made using ^{125}I labeled antigens, no more than 50% of the labeled antigens could be shown to carry the c7 marker (MAGE et al., 1968; VICE et al., 1969b; 1970a). When the c21 allotype was discovered, (GILMAN-SACHS et al., 1969) it could be shown that this allotype accounted for the remainder of the lambda-type IgG in some of the rabbits. However in other rabbits which lacked c21 only about half of the IgG lacking *b*-allotypes had detectable c7 allotype. Similarly, in a rabbit totally suppressed for b4 which lacked c7, less than half of the IgG had detectable c21 (MAGE, unpublished observations). It is probable that the IgG not precipitable by the anti-c7 or anti-c21 reagents, has lambda-type light chains with determinants for which we do not have specific ragents. These data as well as genetic data [discussed in GILMAN-SACHS et al. (1969); MAGE et al. (1970); MAGE et al. (1973a)] have suggested that there are additional alleles (or pseudoalleles) at the *c* locus.

Similar increased understanding of the small proportion (5–30%) of IgG molecules which lack *a*-locus allotypes (*a*-negative) has been gained through production of homozygous rabbits totally suppressed for expression of *a*-locus allotypes (DAVID and TODD, 1969; VICE et al., 1970b). The suppressed rabbits were obtained by zygote transfers in experiments exactly analogous to those described for production of suppressed *b*-locus homozygotes. KNIGHT et al. (1971) obtained *a*-negative IgG from an a^2a^2 rabbit suppressed for a2 and used it as immunizing antigen to obtain isoantibodies specific for *a*-negative IgG.

The allotypic specificities were found to be present on the Fab-fragments. Moreover, Fc-fragments from *a*-negative IgG were indistinguishable from Fc-fragments prepared from normal IgG (KNIGHT et al., 1971; PRAHL and TODD, 1971) suggesting that the suppressed *a*-locus homozygotes produced the major class of rabbit IgG with variable regions contributed by V_H genes controlling structures lacking *a*-locus determinants. Further support for the peptide mapping studies came from the demonstration (LANDUCCI TOSI et al., 1972b) that the C_γ allotypic determinant A15 was present on nearly all the *a*-negative molecules from an $a^2a^2e^{15}e^{15}$ rabbit suppressed for a2.

In order to further define *a*-negative allotypes, KIM and DRAY (1972) took the multispecific anti-*a*-negative antiserum [originally designated anti-A31 by KNIGHT et al. (1971)] and absorbed it with individual rabbit sera to yield three reagents; anti-A31; anti-A31, x32 and anti-A31, y33. The anti-A31, after removal of antibodies specific for x32 and y33 allotypes reacted only with the donor's serum whereas the anti-x32 and anti-y33 sera appeared to recognize allotypes present in the sera of other rabbits. Progeny data of different mating combinations indicated that x32 and y33 were not controlled by allelic genes but by closely linked genes. The markers were present on IgA and IgM as well as IgG, suggesting that there are at least three sets of closely linked V_H genes: V_Ha; V_Hx and V_Hy. Thus, in contrast to the rabbits totally suppressed for expression of kappa-type light chains which compensate by production of the unlinked lambda-type light chains, the suppressed *a*-locus homozygotes compensate by production of increased amounts of a closely linked form of variable region which is usually poorly expressed. The fact that the A31 specificity is so far found present only in the one suppressed rabbit raises the possibility that the A31 represents determinants on clonal products uniquely produced by the suppressed animal. Alternatively, the failure to find the A31 determinants in the sera of other normal rabbits and in one other suppressed homozygote may reflect the fact that although present in the genotype of other rabbits this specificity is rarely present in the "clonotype" GALLY and EDELMAN (1972)[1] of normal animals.

V. Cellular Studies of Allotype Suppression and Some Approches to the Determination of the Mechanism

A number of new approaches to the investigation of the mechanism of chronic allotype suppression have been suggested by the delineation of the B and T groups of lymphoid cells in mice and the concomitant developments of criteria for identification of cell types based on their surface antigens and specific membrane receptors (RAFF, 1973). In addition, provocative implications can be found in the increasing body of evidence that there are stimulatory and

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1 These authors define the "clonotype" as the "different Ig molecules an animal produces which can be detected and classified according to antigen-binding specificity, class, antigenic determinants, primary structure, allotype, or any other experimentally measurable property". Usually a number of molecules greater that obtained from a single cell must be present for detection and classification.

suppressive interactions between lymphoid cells with different surface properties during immune responses.

It is reasonable to hypothesize that an early event in the establishment of allotype suppression is the interaction of the injected antibodies with allotypic determinants expressed on the surface of target cells which are presumably lymphoid cells or their precursors. Potential target cells were identified in newborn heterozygous rabbits which stained with fluorescein conjugated anti-allotype reagents specific for paternal allotype (HARRISON, 1973; HARRISON and MAGE, 1973). In normal b^4b^5 rabbits, cells bearing endogenously synthesized Ig of paternal allotype were present in peripheral blood (6–12% fluorescent positive), spleen (11–13% positive) and appendix (~5% positive) shortly after birth while in comparable heterozygotes injected with antiserum to the paternal allelic type no cells with detectable membrane Ig of the paternal allotype were detected. Within 24 hours of injection of the suppressing anti-*b*-allotype antiserum, membrane Ig of the paternal *b* allotype was no longer demonstrable by fluorescent antibody staining of cells in spleen, peripheral blood or bone marrow. Moreover 24 hours after injecting b9 anti-b5 into b^4b^5 heterozygotes, the suppressing antibody was not detectable on the surface of cells in these organs by sensitive fluorescent staining with b4b5 anti-b9 antibody. The absence of the b9 marker on the cells demonstrated that the injected antibody was not merely masking Ig of b5 type on the membranes (HARRISON and MAGE, 1973). The cells were either eliminated from the organs or their membrane Ig was modulated from the surface (BOYSE and OLD, 1969) and not replaced with the original light chain form.

It has been reported that the interaction of heterologous anti-mouse Ig reagents with membrane Ig on mouse B lymphocytes is followed by rapid migration of the complexes within the membrane to form aggregates (patches or caps). This is followed by endocytosis, pinocytosis or in some instances sloughing of the aggregated complexes (TAYLOR et al., 1971; UNANUE et al., 1972). Similarly, recent *in vitro* studies of the consequences of interaction of anti-*b*-allotype antibody with membrane Ig showed endocytosis of surface Ig by lymphocytes labeled at 0°C, warmed for 1–2 minutes at 37°C, and immediately fixed. In less than 10 minutes the membrane was found cleared of label (LINTHICUM et al., 1973). Unfortunately, the subsequent fate of such cells *in vivo* is not known. Since it is necessary to maintain an excess of circulating anti-allotype antibody in the milieu during establishment of chronic allotype suppression, it is likely that any membrane Ig of the same allotype which reappeared on the cell surface would immediately encounter additional anti-allotype antibody. It is conceivable that such cells are eventually killed by the continued assault upon the membrane Ig, although direct cytotoxicity of anti-allotype for allotype-bearing lymphocytes in the presence of complement is generally not demonstrable *in vitro* (MOND et al., 1972); (and unpublished observations of M. HARRISON and others). It is possible that the target cells are cleared by reticuloendothelial cells, killed by non-specific cytotoxic cells (e.g. lymphocytes or polymorphonuclear leukocytes), or that they die out after

transformation into blast cells of the type seen in cultures of cells exposed to anti-allotype antibodies *in vitro* (SELL and GELL, 1965). Recently however, LOWE (1972) showed that sheep antibody specific for b6 light chains which was able to stimulate blast transformation *in vitro* was not able to establish chronic allotype suppression when administered to newborn heterozygous b^4b^6 rabbits. Her studies confirmed and extended those of GOLDMAN and MAGE (1972) who found that administration of a variety of goat anti-allotype reagents did not lead to chronic allotype suppression in b^4b^5 heterozygotes. In addition, DUBISKI and SWIERCZYNSKA (1971) showed that phytohemagglutinin which induces the majority of rabbit peripheral blood lymphocytes to transform into blast cells (KNIGHT et al., 1965), when administered together with anti-allotype antiserum, did not affect immunoglobulin synthesis or the duration of antibody induced suppression. Simply causing blast transformation or even the killing and elimination of membrane Ig-bearing lymphocytes from the developing lymphoid system for several weeks is not necessarily sufficient for the establishment and chronic maintenance of a suppressed state. The allotype which has been suppressed remains totally absent from the circulation and from the surface of B lymphocytes for a period longer than that during which circulating antibody could be expected to exert direct effects by eliminating the Ig or cells with membrane Ig of that type. HARRISON et al. (1973a) found that a group of 5 totally suppressed homozygous b5 littermates had no circulating b5 Ig and no peripheral blood lymphocytes which stained with ^{125}I anti-b5 before 7 months of age. This observation was extended in studies of b^4b^5 heterozygotes totally suppressed for b5 where in addition to the peripheral blood, spleen, appendix, lymph nodes and bone marrow were shown to be devoid of cells bearing membrane immunoglobulin of b5 type detectable with fluorescent anti-b5 (HARRISON, 1973; HARRISON and MAGE, 1973). During spontaneous escape from suppression, b5 bearing cells appeared in bone marrow and other organs and in the peripheral blood, before circulating b5 Ig was detectable. However, the proportion of peripheral blood lymphocytes in b5 homozygous suppressed rabbits which stained for b5 approached that of normal littermates while the total proportion of b5 Ig in serum was markedly depressed. It appeared that was a real discrepancy between the proportions of b5 bearing lymphocytes and of circulating b5 Ig produced in the rabbit recovering from total kappa (b5) suppression. Experiments in which lymphocytes were stripped of membrane b5 and allowed to regenerate membrane Ig *in vitro* (HARRISON et al., 1973b) by a method described by JONES et al. (1973), showed that the majority of b5-bearing cells from the peripheral blood of the suppressed rabbits could actively synthesize the b5 Ig detected on their membranes, and were not simply passively coated with some of the small amounts of b5 Ig present in the circulation. Thus, even when small lymphocytes with membrane Ig of the suppressed kappa (b5) type did appear, normal levels of kappa-type serum Ig were not produced. A number of possible explanations can be advanced. The b5 bearing lymphocytes may be deficient functionally and not differentiate and develop into Ig producing cells. It is

conceivable that although precursors of Ig producing cells (B cells) with detectable membrane b5 are present, the animals are still deficient in T cells which must specifically function in activating kappa-type B cells. Clonal proliferation and antibody production by B cells often requires a specific antigenic stimulus and a T lymphocyte stimulus (KRETH and WILLIAMSON, 1971). Carrier specific T cells may have an enhancing or an inhibiting influence on antibody production (KATZ et al., 1973). To date however, there is no evidence that cooperating T cells have specificity for the type of immunoglobulin on the B cell (e.g. b4 or b5, kappa or lambda).

By the time b5-bearing cells appear in the recovering suppressed animals, an array of lambda-bearing memory cells or other Ig producing cell precursors may have been established in the animals which compete effectively for most antigenic challenges and thus perpetuate a serum Ig composed largely of lambda type. Some experiments relevant to this point have been performed. DUBISKI and SWIERCZYNSKA (1971) showed that hyperimmunization of suppressed heterozygotes with pneumococcal vaccine did not lead to an increased proportion of suppressed allotype compared to control heterozygotes, confirming and extending earlier work of MAGE and DRAY (1966) who reported that antibenzenearsonate antibody purified from the serum of a b^4b^5 heterozygote suppressed for b4 had the same b4:b5 ratio as the whole serum and non-antibody IgG. In addition, as noted earlier (part IVA) anti-BSA produced by an al suppressed rabbit had less total al (12—13 %)than the non anti-BSA IgG (27%) (LANDUCCI TOSI et al., 1972a).

However, in several instances in my laboratory, hyperimmunization of suppressed rabbits with benzene arsonic-acid (ars) coupled to edestin (a plant protein) has resulted in a large antibody response including a nearly normal proportion of the suppressed allotype. One b5 homozygous rabbit which was recovering from total suppression of b5 eventually produced b5 antibody of restricted heterogeneity after a second course of immunization with the antigen. Even after one course of immunization, the anti-ars was predominantly of b5 type whereas only half of the non-antibody IgG was of kappa (b5) type (MAGE et al., 1973b).

An exciting possibility is that extrinsic regulatory factors such as suppressor T cells prevent the cells with membrane b5 Ig in recovering suppressed homozygotes, from differentiating normally into b5 producing cells. In one particular combination of mouse strains, BALB/c females immune to an Ig allotype of SJL sires produce SJL $\times$ BALB/c F_1 offspring which exhibit chronic suppressed expression of the paternal allotype (HERZENBERG et al., 1971; JACOBSON and HERZENBERG, 1972). The maintenance of this chronic state of suppression appears to be an active process. Cell transfer studies have provided strong evidence that T cells from the suppressed mice exert a suppressing influence on Ig producing cells or their precursors (JACOBSON et al., 1972). Although in such chronically suppressed mice, the precursors of cells which would ordinarily differentiate and secrete the paternal Ig allotype are prevented from doing so by regulatory T cells, this mechanism may be excep-

tional, since chronic suppression has only been produced using this particular combination of mouse strains. It is not at all clear that the mechanism is a general one with relevance to allotype suppression in rabbits. Experiments to explore the possibility that such regulatory T cells are functioning in allotype-suppressed rabbits are difficult to do without inbred strains. It is hoped however, that future work will follow this promising lead, since delineation of the mechanism of allotype suppression in rabbits will contribute much to our overall understanding of the control of B cell differentiation by humoral and cellular regulatory mechanisms.

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The SV40 "S" Antigen and other Papovavirus-Induced Surface Antigens

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I. Introduction

Although a variety of properties which characterize virus-transformed animal cells (e.g., morphological changes, loss of growth control, agglutination by plant lectins) have been linked to surface membrane alterations (BLACK et al., 1971), perhaps the most important surface alteration which occurs in the transformed cell is that involving antigenic changes, since the definitive feature of the neoplastic cell is the ability to produce a tumor in an appropriate host. Whether a transformed cell will produce a tumor in the autochthonous or syngeneic host involves many factors, but of great significance is the presence of "new" surface membrane antigens associated with the transformation process and the immunological response they evoke.

Although the direct evidence is still relatively meager, several lines of experiments suggest that such so-called transplantation antigens must be ex-

pressed on the cell surface (KLEIN, 1967). First, the reactivity of specific antisera raised in genetically identical animals can be completely removed by absorption with intact viable transformed cells in suspension. Second, such antisera can be used to specifically stain the appropriate intact transformed cells in suspension by the indirect membrane immunofluorescence technique (MIF; MÖLLER, 1961). Under these conditions, penetration of antibody into the cell interior is prevented and the reaction is limited to the cell surface. In addition, the specific cytotoxicity of sensitized lymphoid cells can be blocked by prior reaction of the target tumor cells with immune serum. Thus, the presence of exposed antigenic sites on the target cell surface is essential for a reaction simulating graft and tumor rejection (KLEIN, 1967), which is primarily a function of host cell-mediated immunity (KLEIN, 1968).

Other evidence has demonstrated the surface membrane location of transplantation antigens much more directly in that such antigens have been solubilized from plasma membrane fractions (HOLLINSHEAD and ALFORD, 1969; SMITH et al., 1970). In addition, membrane fractions have been shown to be capable of inducing specific transplantation immunity *in vivo* (TEVETHIA and RAPP, 1966; PANTELEAKIS et al., 1968; COGGIN et al., 1969; HOLLINSHEAD and ALFORD, 1969; SMITH et al., 1970). Furthermore, the development of *in vitro* assays for virus-induced "tumor-specific transplantation antigens" (TSTA), in which the reaction target is intact transformed cells (PEKÁREK et al., 1968; SJÖGREN and MOTET, 1969; TING and HERBERMAN, 1971; WRIGHT, 1971; WRIGHT and LAW, 1971; SMITH and MORA, 1971), provides additional evidence for the expression of transplantation antigens at the cell surface. It should be noted, however, that the detection of transplantation antigens on the cell surface does not preclude their presence at other cellular sites (e.g., internal membranes).

In short, information pertaining to the surface membrane antigenic composition of transformed cells is extremely relevant to the tumorigenic potential of these cells, as well as to other membrane-associated manifestations of their oncogenicity. In fact, it has been proposed that the definitive feature of neoplasia is a heritable alteration of the cell surface which interferes with normal regulation and which, as a by-product, results in new surface antigens (PREHN, 1968).

Given the importance of transplantation antigens in determining cell oncogenicity, it is not surprising that extensive investigation of the specificity of such markers has been carried out. Although somewhat cumbersome, the *in vivo* transplantation resistance assay has proved the most reliable in examining this question. In general, it appears that the transplantation antigens present in viral-induced tumor cells are specific for the virus involved, while those in chemically-transformed cells are unique for each tumor (KLEIN, 1966; KLEIN, 1968; PREHN, 1968). Thus, pre-inoculation of appropriate hosts with virus or transformed cells can protect against a later challenge of a tumor-producing dose of cells transformed by the same virus, but will not protect against cells transformed by other viruses (HABEL and EDDY, 1963; KOCH and SABIN,

1963; DEFENDI, 1963). Furthermore, cells of different species transformed by the same virus contain the same transplantation antigen (GIRARDI, 1965). In contrast, studies with tumors induced by a wide variety of chemical carcinogens have demonstrated the presence of transplantation antigens unique to each individual tumor induced by the same carcinogen (PREHN and MAIN, 1957; RÉVÉSZ, 1960; KLEIN et al., 1960; OETTGEN et al., 1968), even when primary tumors were induced with the same chemical at different sites on the same host animal (GLOBERSON and FELDMAN, 1964; ROSEMAN and MORTON, 1966). It should be noted, however, that more recent evidence indicates that this general distinction between virally- and chemically-induced tumors is not absolute. Thus, unique transplantation antigens have been found in murine mammary carcinomas induced by the mammary tumor virus (MTV; VAAGE, 1968; VAAGE et al., 1969; MORTON et al., 1969; HEPPNER and PIERCE, 1969) and cells transformed by different strains of polyoma virus (PV) contain different transplantation antigens (JARRET, 1966). Furthermore, some indications of common antigenicity of different tumors induced by the same chemical carcinogen have been reported (TAKEDA et al., 1966; REINER and SOUTHAM, 1967, 1969; ZBAR et al., 1969; COLNAGHI et al., 1971).

Despite the suggestion that the numerous surface alterations associated with neoplastic transformation, including the appearance of TSTA, are essential for initiation and maintenance of the transformed state (PREHN, 1968; TENNANT, 1970), it is clear that this is not universally true. One strain of PV has been found which does not induce its TSTA yet induces tumors perfectly well (HARE, 1967a). Cells transformed *in vitro* by this variant demonstrate expected properties for growth in liquid medium or semi-solid agar and for transplantation to animals (HARE, 1967b). Other polyoma- and SV40-transformed cells have been found to lose their transplantation antigen during growth of the primary tumor (DEFENDI and LEHMAN, 1965; DEICHMAN and KLUCHAREVA, 1966), a process which may act to hasten tumor metastasis (DEICHMAN and KLUCHAREVA, 1966). In addition, the SV40-specific TSTA has been reported to be synthesized in productively infected, nontransformed cells (GIRARDI and DEFENDI, 1970), further weakening the unique association between its presence and the transformation event, although the synthesis of TSTA during productive SV40 infection has recently been questioned (SMITH and MORA, 1972). In any event, the role of the TSTA in the molecular mechanism of viral transformation remains unclear at this time.

II. The SV40 S Antigen

Although the classic *in vivo* assay for virus-induced TSTA has provided the functional definition of these antigens, the technique is cumbersome, time-consuming, requires the use of many animals, and perhaps most important, does not have a level of precision or quantitation necessary for detailed biochemical and/or molecular characterization of these antigenic moieties. Thus, considerable effort has been expended to develop a rapid and specific *in vitro*

assay for virus-induced TSTA. Toward this end, TEVETHIA, RAPP and coworkers attempted to detect the SV40-specific TSTA through the utilization of the MIF technique and published the results of a series of investigations dealing with the "SV40-specific surface (S) antigen". Numerous other workers, employing a variety of *in vitro* techniques, have subsequently also reported the detection of "virus-specific" surface antigens on papovavirus-transformed cells. Unfortunately, in many cases these have also been denoted as S antigen(s), although there is now good evidence that they are neither identical with each other nor with S, which has led to considerable confusion in the literature. For our purposes in this review, we will reserve the term S antigen solely for the specificity detected by TEVETHIA and coworkers on SV40-transformed cells. We should like to consider the nature of this antigenic specificity and will attempt to clarify the numerous contradictory reports pertaining to other papovavirus-induced surface antigens, both in regard to their viral specificity and their relation to S, TSTA and each other. Last, a hypothetical model is proposed which attempts to reconcile the fact that several of these surface neoantigens appear to be virus-associated yet are clearly not coded for by the viral genome (i.e., represent virus-induced, host-coded antigens).

The initial report of TEVETHIA et al. (1965) indicated that antisera raised in hamsters in response to repeated subcutaneous inoculations with SV40 virus and transformed hamster cells contained antibodies directed against an SV40-specific surface antigen, as detected by the MIF technique. This "virus-specific" membrane neoantigen (subsequently denoted the SV40 S antigen) was demonstrated to be present on SV40-transformed hamster cells while it was absent from normal hamster cells and hamster cells transformed either spontaneously or by adenovirus 12 (Ad. 12). A similar specificity was reported utilizing a cytotoxicity assay with heterologous sera from rabbits immunized with SV40-transformed hamster cells and absorbed with normal hamster cells (TEVETHIA and RAPP, 1965). Subsequent papers (TEVETHIA and RAPP, 1966; TEVETHIA et al., 1968a) attempted to link the identity of the S antigen with that of the SV40-specific TSTA, primarily on the basis of the established surface localization of the former and the (then) presumed surface localization of the latter, as well as the somewhat similar kinetics of anti-S antibody production and the development of SV40-specific transplantation immunity in immunized hamsters. This linkage, however, is obviously dependent upon the assumption that the S antigen itself is truly SV40-specific.

The first indication that the nature of the S antigen was more complex than the initial results implied was provided by the examination of certain SV40-transformed hamster embryo cell lines. These cells were shown by MIF to contain the S antigen yet were incapable of responding to established SV40-specific transplantation immunity (TEVETHIA et al., 1968b). The existence of such S^+TSTA^-cells clearly demonstrated a lack of identity between these two antigens, although not eliminating a partial relationship between them (e.g., S representing a component of a more complex TSTA). Nevertheless, studies of a number of these SV40-transformed hamster embryo cell lines in-

dicated a definite association of the S antigen with SV40-transformation (DIAMANDOPOLOUS et al., 1968). That this association, however, is not one of virus-specificity (which implies a virus-coded product) has been demonstrated by further investigations of the S+TSTA⁻hamster cells. Utilizing nucleic acid hybridization techniques, these cells were found to lack detectable amounts of either SV40 DNA (LEVINE et al., 1970) or SV40 complementary RNA (LEVIN et al., 1969), indicating that S is not directly coded for by the virus genome. However, one cannot completely exclude the possibility that a non-detectable amount of SV40 DNA, including the gene for the S antigen, is present and transcribed (at low levels) in these cells, although this is considered unlikely. In any event, additional evidence against the S antigen being SV40-coded has been the detection of an anti-S-like antibody activity in the sera of multiparous female hamsters collected late in the gestation period (DUFF and RAPP, 1970). These animals had never been inoculated with SV40 virus or transformed cells, suggesting that S may represent a virus-induced host-coded antigen which is normally expressed during embryogenesis.

Recent results from other laboratories have raised additional doubts as to the nature of the SV40 S antigen. Utilizing hamster anti-S antiserum prepared and characterized by RAPP, BERMAN (1972) has reported positive MIF staining against a series of hamster cells transformed spontaneously, or by Ad. 12 or murine sarcoma virus, as well as those transformed by SV40. SV40-transformed mouse cells were non-reactive, as were cells derived from an Ad. 12-induced mouse tumor. Furthermore, while cells from hamster embryos taken early in gestation were positive, the antigen(s) in question seemed to have disappeared on cells obtained from tissues of term embryos, newborns or adults. Thus, the S antigen has again been linked with a host-coded specificity normally expressed during fetal development. Attempts to prepare anti-S antiserum by the protocol of TEVETHIA et al. (1965) were unsuccessful (L. BERMAN, personal communication).

We also have investigated the identity and specificity of the SV40 S antigen. Using a highly controlled system, however, we were unable to detect an antigen with the characteristics of the S antigen, both with respect to its virus-association and its relationship to normal hamster fetal antigens (COLLINS and BLACK, 1973a). An experimental approach was devised in order to eliminate the possibility that the immunized hamsters of earlier studies (TEVETHIA et al., 1965; TEVETHIA and RAPP, 1966; DUFF and RAPP, 1970) had reacted against contaminants in the non-purified virus preparations used and/or isoantigens on the outbred hamster cells. Thus, these experiments were repeated using purified SV40 and autochthonous transformed hamster kidney cells (see Fig. 1). Under these conditions, no serum MIF activity against any surface antigen, SV40-associated or otherwise, could be detected. In an endeavor to show directly that the previously reported anti-S serum activity was directed against either a hamster isoantigen or a contaminant in the virus preparation these studies were repeated by immunizing outbred hamsters with non-purified, clarified SV40 and homologous transformed cells by the identical immunization

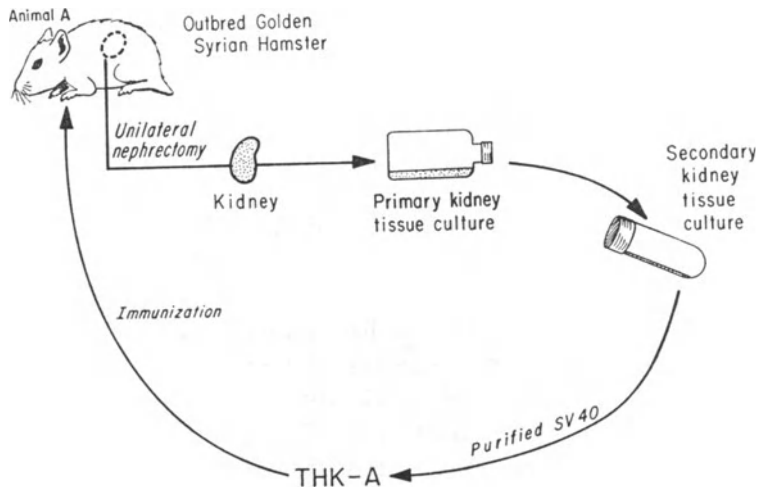


Fig. 1. Protocol for the isolation of autochthonous SV40-transformed hamster kidney cell lines for immunization of the donor animal. Immunizations were carried out by a variety of procedures, including that used by TEVETHIA et al. (1965) for production of anti-SV40 S antisera, as well as by the growth and excision of a subcutaneous tumor induced by the autochthonous transformed cells. (Figure taken from COLLINS and BLACK, 1973a)

schedule (TEVETHIA et al., 1965). Again, the sera obtained lacked detectable MIF activity. Since the majority of the studies of TEVETHIA and coworkers have utilized sera prepared against the H-50 line of SV40 hamster tumor cells it is possible that the SV40-transformed hamster cell lines utilized in the present study do not contain the S antigen (which would be further evidence of its non-viral specificity). However, anti-S activity has also been induced with SV40-transformed human and marmoset cells (TEVETHIA et al., 1970), although the immunization schedule used differed from that of the earlier studies.

It is difficult to understand the wide species range of the SV40 S antigen on transformed cells when it is clearly not coded for by the viral genome. However, if it should represent a normal fetal antigen which is expressed after SV40-transformation of adult cells (DUFF and RAPP, 1970), then it may be a determinant present in several different species. In accord with this suggestion, embryonic hamster, mouse and human cells have all been reported to be capable of interfering specifically with the transplantability of SV40-transformed cells in hamster hosts (COGGIN et al., 1970; AMBROSE et al., 1971). Furthermore, using the same approach, these investigators have found that immunization of BALB/c mice with syngeneic fetal cells can prevent the growth of mouse cells infected with Rauscher leukemia virus (HANNA et al., 1971). Whether the fetal antigen(s) involved in the SV40 and Rauscher virus system are related remains to be determined. It should be noted, however, that our attempts to repeat the studies of DUFF and RAPP (1970) which indicated a relationship between the SV40 S antigen and a hamster fetal antigen have been unsuccessful (COLLINS and BLACK, 1973a).

III. Other "SV40-Associated" Surface Antigens

In addition to the SV40 S antigen of TEVETHTA, RAPP and coworkers, several other groups have reported the existence of "virus-specific" surface neoantigens on cells transformed by SV40. As already mentioned, the unfortunate tendency has been to also refer to these as S antigens; however, it now appears that in most cases these represent additional examples of virus-induced, host-coded surface antigens. The early studies of KLUCHAREVA et al. (1967) reported the detection by MIF of an SV40-specific surface antigen which was suggested to be the TSTA. The sera used in these experiments, however, were prepared in response to inoculations with non-purified SV40 and could well have been directed against soluble monkey antigens in the clarified virus preparation. Only SV40-hamster and human cells were examined, a rather limited spectrum to conclude virus-specificity. In addition, the experiments examining the relationship between this virus-induced antigen and the TSTA utilized the immunization of outbred hamsters with homologous SV40-infected cells. Tumor rejection could therefore have been due to the expression of host-coded antigens subsequent to virus-infection and/or transformation. The immunized hamsters were not tested for tumor immunity to the same hamster cells transformed by other viruses (e.g., PV and Ad.).

Using the mixed hemagglutination (MHA) technique, METZGAR and OLEINICK (1968) reported, in a preliminary study, the detection of an "SV40-associated" antigen on transformed hamster and mouse cells and implied that it might represent the virus-specific TSTA. However, unabsorbed heterologous hamster serum prepared against SV40-transformed human fibroblasts was used, raising the obvious objection that they may have been detecting antigens on the transformed cells cross-reacting with human isoantigens. No qualitative or quantitative differences in the isoantigenic pattern of the transformed human cells used for immunization were found when compared to the corresponding untransformed cells. In addition, several important questions were not examined, including the testing of the hamster antiserum against untransformed human cells of the same cell line and against hamster cells transformed by other viruses.

A more extensive investigation of SV40-induced surface antigens has been carried out by KLEITMANN and SEEMAYER (1971), who also utilized the MHA technique. Employing immune hamster sera made in a variety of ways, they reported the existence of an SV40-specific surface antigen on transformed mouse, hamster and monkey cells. Unfortunately, all immunizations utilized unpurified virus and/or homologous or heterologous transformed cells. Nevertheless, specificity controls did not detect the antigen on untransformed hamster, mouse and monkey cells or on PV-transformed hamster cells. Preliminary data supported a relationship between the antigen and the SV40-specific TSTA. Furthermore, the expression of this antigen was decreased in cells transformed by UV-irradiated SV40, suggesting the involvement of some viral function. However, whether this viral function is direct coding of the

antigen involved has been made questionable by the work of HÄYRY and DEFENDI (1968, 1970).

Initial studies by these investigators strongly suggested that they could detect an SV40-specific surface antigen by the MHA procedure (HÄYRY and DEFENDI, 1968). Although some experiments utilized immune hamster sera raised against transformed homologous or heterologous cells, similar results were achieved with serum produced in response to immunization with purified SV40. The antigen was present on SV40-transformed hamster and mouse cells, but not on the corresponding untransformed and PV- or Rous sarcoma virus (RSV)-transformed cells. The MHA procedure was reported to be considerably more sensitive than MIF, and the antigen detected correlated well with the SV40-specific TSTA. In agreement with the work previously cited (KLEITMANN and SEEMAYER, 1971), cells transformed by UV-irradiated SV40 demonstrated diminished amounts of antigen.

However, subsequent analysis of the nature of this determinant has yielded somewhat paradoxical results: although the antigen continued to appear strongly "SV40-associated" it was shown clearly not to be coded for by the SV40 genome (HÄYRY and DEFENDI, 1970). This was indicated by the demonstration that mild proteolytic treatment of normal and PV-transformed mouse or hamster cells converted these previously negative cells to a positive reaction, both by MIF and the MHA test. Thus, it appears that the MHA-detected "SV40-specific" surface antigen is also present on normal cells, but in a concealed form. Exposure of the antigen by proteolysis presumably operated in much the same way that cell agglutination sites, previously thought to be specifically associated with the transformed cells, can be revealed on normal cells by similar enzyme treatments (BURGER, 1969; INBAR and SACHS, 1969). Since this surface antigen is present in a "cryptic" arrangement on normal cells it is obviously not coded for by the SV40 genome. Nevertheless, its strong association with SV40-transformation implies that some virus-specific function may be involved in uncovering of the antigenic site; this could be the UV-sensitive function indicated earlier (HÄYRY and DEFENDI, 1968; KLEITMANN and SEEMAYER, 1971).

The results of HÄYRY and DEFENDI (1970) may also account for the rash of claims as to the SV40-specificity of various surface antigens. Thus, cells removed from monolayer culture with trypsin and then used as target cells in MIF reactions may give spurious results and yield misleading conclusions as to the distribution of an antigenic determinant (TEVETHIA et al., 1965; KLUCHAREVA et al., 1967). Although the MHA procedure has the advantage that the tissue culture target cells can be reacted with directly on the glass surface, frequently the immune sera have been prepared against tissue culture cells obtained by trypsinization (METZGER and OLEINICK, 1968; KLEITMANN and SEEMAYER, 1971). This, too, may lead to incorrect conclusions. For these reasons, it is considered desirable to use chelating agents (EDTA and EGTA) for the collection of immunizing and/or target cells with "*in vitro*" assays of

surface antigens (COLLINS and BLACK, 1973a, b). While these agents are by no means totally benign in regard to membrane damage (COLLINS, 1972; CULP and BLACK, 1972a; RUGGIERI et al., in preparation) they are considerably less severe than trypsin treatment and have not yet been reported to reveal cryptic membrane sites.

Studies which indicate the exposure on virus-transformed cells of normal cryptic membrane sites suggest that if a specific virus function is involved, it may be responsible for a failure to complete the synthesis of membrane components. Such a model has been proposed (ROBERTSON and BLACK, 1969) to explain the appearance of Forssman antigen on SV40-, PV- and RSV-transformed BHK-21 cells (O'NEILL, 1968; ROBERTSON and BLACK, 1969). That Forssman antigen may indeed be a cryptic antigen is indicated by the ability to detect it on the plasma membrane of normal BHK-21 cells after mild proteolysis (BURGER, 1971). It should be noted that the Forssman antigen, which is present in the cells of a wide variety of animal species, is an excellent example of a virus-induced host-coded antigen in the virus-transformed cells mentioned above (O'NEILL, 1968; ROBERTSON and BLACK, 1969).

Another class of virus-induced host-coded antigens is indicated by several studies which demonstrated cross-reactions between SV40-induced surface antigens and normal fetal antigens. Such a relationship with the SV40 S antigen has already been mentioned (DUFF and RAPP, 1970; BERMAN, 1972), but other cases have been reported as well. COGGIN et al. (1970) have found that immunization of hamsters with hamster or mouse fetal cells induces SV40-specific "cytostatic antibody" which prevents the growth of SV40-transformed cells in lymphocyte-impermeable Millipore diffusion chambers placed in the hamsters' peritoneal cavity. Human fetal cells were subsequently shown to function equally well in this system (AMBROSE et al., 1971), raising questions as to whether or not all transplantation rejection antigens are truly virus-coded. These results also emphasize the need to examine a wide range of transformed cells of different species when attempting to establish the viral-specificity of a surface antigen; this is a shortcoming in several of the previously mentioned studies (KLUCHAREVA et al., 1967; METZGER and OLEINICK, 1968; HÄYRY and DEFENDI, 1968, 1970; DUFF and RAPP, 1970). KOPROWSKI and coworkers have also demonstrated an antigenic relationship between an SV40-induced surface antigen and a normal mouse fetal antigen (BARANSKA et al., 1970; KOPROWSKI et al., 1971). Using a cytotoxicity assay, sera from guinea pigs immunized with unfertilized mouse eggs of an inbred strain were highly reactive against SV40-transformed mouse cells of several strains, but not against SV40-transformed cells of other species. The antisera were not cytotoxic for rat eggs, mouse lymph node cells, chemically-transformed or normal mouse cells. Mouse cells transformed by PV or Ad. 12 or infected with Moloney leukemia virus were only slightly reactive. The specificity of this antigen clearly differentiates it from the fetal determinant detected by COGGIN and coworkers on SV40-transformed cells (COGGIN et al., 1970; AMBROSE et al., 1971) and further underlines the complexity of the antigenic make-up of transformed cell surfaces.

Work in our laboratory, already mentioned with respect to the S antigen, has resulted in the detection of a series of surface antigens induced by SV40 yet unique to individual lines of transformed hamster kidney cells (COLLINS and BLACK, 1973a). Using the autochthonous system demonstrated in Fig. 1, outbred hamsters were immunized by the progressive growth and subsequent surgical excision of a subcutaneous tumor induced by the inoculation of their own kidney cells transformed by SV40. The resulting humoral activity was assayed *in vitro* by the MIF test and from the spectra of positive target cells it was apparent that the autochthonous hosts had responded to non-identical virus-induced surface neoantigens. In addition, as demonstrated by the tumor excision studies, the level of serum MIF activity was influenced by the presence (and size) of a tumor mass.

Similar studies with SV40-transformed rabbit kidney cell lines and clones of SV40-transformed kidney cells from the inbred LSH hamster strain have indicated once again the multiple types of new surface antigens which can appear after virus-transformation (COLLINS and BLACK, 1973b). Using the cloned LSH cells we have shown that unique virus-induced host-coded surface antigens are associated with each transformation event (and not just each transformed cell line). Furthermore, in both the rabbit and LSH hamster systems the *in vitro* detection of these antigens was dependent upon prior treatment of the target cells with phospholipase C. The expression of these antigens was also influenced by the density at which the target cells were grown in tissue culture and the method by which the cells were harvested for MIF staining.

Despite our skepticism as to the earlier claims of the *in vitro* detection of SV40-specific surface antigens, recent work from several independent groups has provided the strongest evidence to date that an SV40-specific surface antigen, perhaps the TSTA, is being assayed *in vitro*. Using a cytotoxicity assay with ^{51}Cr-labeled target cells and mouse sera raised against SV40-transformed syngeneic mouse cells, an SV40-associated surface antigen on transformed mouse and hamster cells has been demonstrated (WRIGHT, 1971; WRIGHT and LAW, 1971). The antigen correlated well with the SV40-specific transplantation antigen and could be clearly differentiated from the S antigen in that the previously mentioned S+TSTA$^-$; cells (TEVETHIA et al., 1968b) did not react with the test serum. Before it can be concluded that the antigen in question is truly SV40-specific, however, a wider range of transformed cells must be examined. In addition, reactivity with various fetal cells and with trypsin-treated normal cells should be examined.

Utilizing a nearly identical system, SMITH and coworkers have reported analogous results (SMITH et al., 1970; SMITH and MORA, 1972). Comparable results were achieved in a cytotoxicity assay using syngeneic mouse sera prepared in the same strain (AL/N) utilized by WRIGHT and LAW, whether measured by trypan blue exclusion or release of ^{51}Cr label. Again, excellent correlation with the SV40 TSTA was demonstrated both *in vitro* and *in vivo*, and a lack of identity with S antigen was apparent. In addition, preliminary

successful attempts at solubilization of the TSTA were reported, which could be monitored with the *in vitro* cytotoxicity assay. Unfortunately, these workers also restricted the scope of their target cells to hamster and mouse, a point we shall consider below.

Last, employing the sensitive isotopic antiglobulin technique with AL/N mouse serum raised against syngeneic SV40-transformed cells, TING and HERBERMAN (1971) have also detected an SV40-associated surface antigen on transformed mouse and hamster cells. As in the previous studies, normal and PV-transformed AL/N cells and S+TSTA⁻hamster cells did not react with their sera. In addition, this antigen has been shown to be distinct from normally expressed mouse fetal antigens, although other antigens on the virus-transformed cells examined did cross-react with embryonic tissue (TING et al., 1972a). Again, we must express the same reservations about these studies as with the above investigations, particularly in regard to the failure to test SV40-transformed cells of species other than mouse and hamster, although TING and HERBERMAN (1971) did present preliminary evidence that their antigen could not be exposed on PV-transformed hamster cells by mild proteolysis. We cannot emphasize too strongly that before an antigen can be accepted as truly virus-specific a wide range of transformed cells must be examined. This is particularly true in the case of SV40 and hamster and mouse cells, since this species cross-reactivity has been found with a wide range of "SV40-associated antigens" which are clearly not virus-specific (HÄYRY and DEFENDI, 1968, 1970; COGGIN et al., 1970; COLLINS and BLACK, 1973a). In addition, since the recent studies cited above (SMITH et al., 1970; SMITH and MORA, 1972; TING and HERBERMAN, 1971; WRIGHT, 1971; WRIGHT and LAW, 1971) all used the AL/N mouse system, albeit different immunization procedures, it would be desirable to carry out similar experiments in other syngeneic systems so as to eliminate the possibility that the results reflect an unusual immune response of AL/N mice. Nevertheless, despite these reservations, it seems probable that the above workers are detecting the same antigen, which is quite likely SV 40-specific and identical to the TSTA. If this is the case, these *in vitro* assays should prove to be of great benefit in examining the structure, biochemistry, cellular distribution, and mechanism of appearance of SV40-induced TSTA.

IV. Surface Antigens Induced by Other Papovaviruses

Although surface antigens on SV40-transformed cells have been studied in the greatest detail, the antigens on cells transformed by other papovaviruses have also been analyzed to some degree with similar results. As in the SV40 system, TING and HERBERMAN (1970) appear to have the strongest evidence for the presence of a PV-specific surface antigen. Using the same approach as outlined with SV40 for the production of antisera and assaying of antigen (TING and HERBERMAN, 1971), a PV-specific surface antigen was detected on transformed mouse cells. SV40-transformed and untransformed cells of the same strain were negative, but the cells of other species transformed by PV

must be examined to confirm the specificity of the antigen. In a subsequent study (TING et al., 1972b) the expression of this antigen in several PV-transformed mouse cell lines was shown to be closely correlated quantitatively with the PV-specific TSTA, as determined by the immunogenicity and immunosensitivity of the various transformed cell lines. This is analogous to the situation with their SV40-induced surface antigen, which correlated well with the SV40-specific TSTA, but not with the S antigen (TING and HERBERMAN, 1971). A further similarity was the finding that the PV-induced surface antigen did not crossreact with mouse fetal antigens (TING et al., 1972a).

Results of an early study by IRLIN (1967) were interpreted as indicating the detection by MIF of a PV-specific surface antigen identical to the virus-specific transplantation rejection antigen. However, the sera utilized were prepared in mice of one strain against PV-transformed cells of another and isoantigenic differences could well account for the serum activity. No data on the specific absorption of the homologous sera were presented. Similar objections may be raised against the later study of MALMGREN et al. (1968). Antisera were prepared in mice against either impure virus or transformed homologous cells and used unabsorbed. In addition, the serum MIF activity was highly variable with a very low value considered positive ($\sim$5 %) and the cross-reaction between different PV-transformed mouse cell lines was extremely erratic.

Results have also been obtained which suggest a cross-reaction between a PV-induced surface antigen and hamster fetal antigens (PEARSON and FREEMAN, 1968). It was concluded that the PV-induced antigen being detected was the virus-specific TSTA, but the experimental approach makes this unlikely. Heterologous rabbit or homologous hamster sera were prepared against impure PV or spontaneously-transformed hamster embryo cells. In some cases, hamster immunizations were carried out by tumor induction and subsequent tumor excision. The antigens were assayed by cytotoxicity, using target cells obtained by trypsinization. Although both the heterologous and homologous sera raised against the virus-transformed cells were absorbed with the spontaneously-transformed control cells it is quite possible that some anti-isoantigen activity remained. In addition, the use of trypsinized target cells could have exposed the virus-induced antigen on normal embryonic cells. Nevertheless, the *in vivo* tumor rejection experiments support the possibility of an antigenic cross-reactivity between a PV-induced surface antigen and normal hamster embryonic tissue, which would be in accord with the results presented in the SV40 system (DUFF and RAPP, 1970; COGGIN et al., 1970).

The induction of another "PV-specific" surface antigen in both stably- and abortively-transformed hamster cells has been reported by MEYER and coworkers (MEYER and BIRG, 1970; STOKER et al., 1972). This antigen was detected by MIF, and while claimed to be specific for PV, could be differentiated from the PV-specific TSTA on the basis of different inactivation rates for the induction of these antigens by PV irradiated with ultraviolet light, as well as the ability of a PV mutant defective in TSTA induction (HARE, 1967a) to

induce the surface neoantigen. Several objections, similar to those already discussed for SV40-induced surface antigens, may be raised to their assertion that the antigen in question is virus-specific. Although the antisera used were obtained from hamsters rejecting tumors induced by PV, it is not stated whether purified virus preparations were used. Second, trypsinized cells were used as target cells for the MIF reaction and the 2 hours provided after trypsinization may not be sufficient for regeneration of membrane components. Last, only PV-transformed hamster and mouse cells have been examined (data being presented only for PV-transformed BHK-21). Thus, as stated before, a wider range of cells transformed by PV must be shown to have this specificity before it can truly be considered virus-specific.

Shope rabbit papilloma virus (SPV) has also been reported to induce the appearance of a "virus-specific" surface antigen on transformed rabbit cells (Ishimoto and Ito, 1969, 1971). Using antisera obtained from papilloma-bearing rabbits and the MIF procedure, the antigen was shown to be susceptible to trypsin inactivation in that approximately 50% of EDTA-harvested transformed target cells had detectable antigen as compared with ~5% of trypsin-harvested cells. However, since the distribution of the antigen on other types of infected or transformed cells has not been examined further, little can be said as to its virus specificity at this time. The trypsin-sensitivity of the SPV-induced surface antigen is similar to that reported, in a preliminary study, for an Ad. 12-induced surface antigen on transformed hamster cells (Vasconcelos-Costa, 1970). Freshly trypsinized Ad. 12-transformed hamster cells were non-reactive by the MIF procedure, but became positive after being held overnight at 4°C. Normal and PV-transformed hamster cells did not react, but cells of other species transformed by Ad. 12 were not tested. In addition, since test sera were produced in hamsters against clarified virus and homologous transformed cells, the specificity of the reaction remains unclear.

The above discussion leads to the inescapable conclusion that DNA oncogenic viruses in general, and SV40 in particular, are capable of inducing the appearance of a wide range of "new" antigens on the surface of transformed cells, most of which are not directly coded for by the viral genome. Of critical importance in the detection of these various virus-induced antigens is the mode of immunization and the serological technique utilized. Differences in the immunizing cells, schedule, host animal, detection assay and many other associated parameters may well account for the variable results from different laboratories. This could include the unsuccessful attempts (Berman, 1972; Collins and Black, 1973a) to confirm previous reports (Tevethia et al., 1965; Tevethia and Rapp, 1966; Duff and Rapp, 1970) on the distribution and nature of the SV40 S antigen. It has already been shown that the level of expression of cell surface antigens is dependent on a number of variables (Cikes, 1970, 1971; Cikes and Friberg, 1971; Collins and Black, 1973b), thus the growth and preparation of immunizing and/or target cells could primarily account for discrepancies and confusion in the literature.

V. General Consideration of Transplantation Antigens

A. Tumor-Specific Transplantation Antigens (TSTA) of Virally- and Chemically-Transformed Cells

At this time, the TSTA is undoubtedly the best candidate for a virus-coded surface antigen which is present on transformed cells. However, as mentioned earlier, even the general distinction between the absolute specificity of virus-induced transplantation antigens and the uniqueness of the transplantation antigens of chemically-transformed cells (KLEIN, 1966, 1968; PREHN, 1968) is undergoing modifications. Several groups have reported results which indicate that MTV-containing murine mammary carcinomas bear non-cross-reacting transplantation antigens which are unique for individual tumors. Utilizing syngeneic hosts tolerant to MTV (due to neonatal infection) VAAGE and co-workers (1968, 1969) demonstrated that 5 of 23 mammary carcinomas contained immunologically active transplantation antigens which were unique for each tumor and unrelated to the MTV-specific transplantation antigen. All 23 tumor lines, when tested in non-tolerant mice, demonstrated the presence of the MTV-specific transplantation antigen. The possibility that minor histocompatibility differences within the inbred strain were involved was excluded by the finding that tumors arising at separate sites on the same animal could have either the MTV-specific or a unique transplantation antigen (VAAGE et al., 1969). Although these authors refer to the non-cross-reacting transplantation antigens as "non-virus-associated", it seems probable that they are in fact virus-induced host-coded antigens analogous to those present on cells transformed by the DNA oncogenic viruses.

Using a similar approach, MORTON et al. (1969) demonstrated unique transplantation antigens on 5 of 11 mammary carcinomas. These studies were further extended by HEPPNER and PIERCE (1969), who examined the antigenicity of various mammary carcinomas using the *in vitro* colony inhibition test with immune lymphocytes. Using lymph node cells obtained from immunized MTV-tolerant syngeneic mice, 7 of 8 tumor lines examined were characterized by unique non-cross-reacting antigenicity. Furthermore, in contrast to the previous studies (VAAGE, 1968; VAAGE et al., 1969; MORTON et al., 1969), unique antigens were detected on 7 of 12 tumor lines even with lymphocytes obtained from immunized MTV-free syngeneic hosts. Thus, the unique transplantation antigens can represent the major immunogenic stimulus in mice capable of reacting against the MTV-specific TSTA. That such virus-induced host-coded antigens can serve as transplantation antigens tends to cloud the significance and reliability of the so-called "virus-specific" transplantation rejection antigens.

Consistent with this interpretation is the finding that different strains of PV can induce non-cross-reacting transplantation antigens in Py-BHK cells (JARRET, 1966). Although each PV strain may code directly for its specific transplantation antigen, in the absence of further information, it seems equally likely that different host-coded antigens are being expressed on these cells

after virus-transformation. It is clear, however, that the expression of virus-specific TSTA is not a requirement for successful transformation, since transformed lines have been established subsequent to infection with PV or SV40 which lack the associated transplantation antigens (HARE, 1967a; TEVETHIA et al., 1968b). A point previously mentioned which further casts doubts on the universal specificity of virus-induced transplantation antigens is the ability of normal fetal tissues to immunize appropriate hosts against the transplantation of virus-transformed cells (COGGIN et al., 1970; AMBROSE et al., 1971; HANNA et al., 1971). That such antigens can cross-species bariers (COGGIN et al., 1970; AMBROSE et al., 1971) indicates their wide distribution and suggests that they may be of great importance in the immunological rejection of virus-transformed cells. Thus, virus-transformed cells may contain several transplantation antigens, including the virus-specific TSTA and non-specific antigens shared with normal fetal tissues.

On the other hand, the absolute uniqueness of the transplantation antigens on chemically-transformed cells has also been questioned. REINER and SOUTHAM (1967, 1969), working with a series of 10 methylcholanthrene (MCA)-induced murine sarcomas in inbred mice, confirmed that each tumor contained unique transplantation antigens. Utilizing immunization by tumor induction and excision, they found, however, that if mice were immunized by the simultaneous inoculation of several (from 2–4) of the tumor cell lines they were then capable of rejecting one of the other previously non-cross-reacting tumor lines. It was suggested that although the major transplantation antigen of these MCA-induced tumors was unique to each individual tumor line, the transformed cells were characterized by the presence of multiple minor antigenic components on their surface. These would be shared by some tumor lines, but not necessarily all. For this reason, and also because of their proposed "weak" antigenicity, immunization with several tumors would be needed to yield effective tumor immunity.

ZBAR et al. (1969), studying 6 hepatomas induced in inbred guinea pigs by diethylnitrosamine, found that two of these tumor lines initially shared cross-reacting transplantation antigens, while three of them contained unique antigens and one was non-antigenic. Of great interest was the observation that after several transplant generations two of the three lines which originally contained unique antigens had acquired a transplantation antigen which cross-reacted with one of the first two lines (containing a shared antigen). A similar development of cross-reacting transplantation antigens subsequent to continuous *in vivo* transplantation has also been reported with MCA-induced rat sarcomas which were originally antigenically unique (TAKEDA et al., 1966). In a recent study using an *in vitro* microassay for cell-mediated immunity, a majority of urethran-induced lung adenomas in inbred mice were found to contain common antigens (COLNAGHI et al., 1971). In summary, the distinction between the specificity of transplantation antigens on virus- and chemically-transformed cells is somewhat less clear than initially thought. Likewise, the supposition that all the transplantation antigens present on virus-transformed

cells represent materials directly coded for by the viral genome is no longer acceptable. If virus-induced surface antigens, including at least some transplantation antigens, do represent host cell products, what might be the mechanism of their appearance in virus-transformed cells?

B. Mechanisms for the Expression of Virus-Induced Surface Antigens

The results of numerous investigations reported to date lead one to consider two general models to explain the "new" expression of host-coded surface antigens on virus-transformed cells: 1) the direct derepression of host cell genetic information and 2) the exposure of previously concealed membrane determinants as a result of alterations in membrane biosynthesis and/or organization, or cleavage of membrane components. The strongest evidence in support of the derepression model is provided by the large number of reports demonstrating cross-reactions between the surface antigens of virus-transformed cells and antigens of normal embryonic tissues (PEARSON and FREEMAN, 1968; DUFF and RAPP, 1970; BARANSKA et al., 1970; COGGIN et al., 1970; AMBROSE et al., 1971; HANNA et al., 1971; KOPROWSKI et al., 1971; BERMAN, 1972). In several cases, the available evidence suggests that the cross-reacting fetal antigen is present only at certain periods during gestation (DUFF and RAPP, 1970; COGGIN et al., 1970; BERMAN, 1972), implying that the corresponding host gene is subject to genetic control during normal embryogenesis. Studies of the carcinoembryonic antigen (CEA) of the human digestive system (GOLD and FREEDMAN, 1965a; COLLINS and BLACK, 1974c) have also demonstrated its presence in fetal tissue only during the first two trimesters of gestation (GOLD and FREEDMAN, 1965b). Such temporal expression of fetal antigens strongly supports a mechanism based on the genetic control of *de novo* synthesis, and by analogy, implies that a similar mechanism is involved in their appearance subsequent to virus transformation. One could envisage the integration of the viral genome into a host regulator gene controlling the expression of the repressed fetal antigen in adult tissue; the inactivation of this regulator gene would lead to the subsequent synthesis and appearance of the fetal antigen. A similar situation has been reported with lysogenic bacteriophage Mul (TAYLOR, 1963), in which integration of the prophage into a host structural gene resulted in the cessation of synthesis of the protein coded for by that gene.

Alternatively, investigations of a wide variety of surface membrane alterations in virus-transformed cells have yielded results completely in accord with the second model (BLACK et al., 1971). Detailed analysis of surface sites (both antigenic and agglutinin receptors) initially thought to be specific for transformed cells have revealed them to be present on normal cells as well, but in a different orientation. These determinants could be detected on normal cells after mild proteolysis (BURGER, 1969; INBAR and SACHS, 1969; HÄYRY and DEFENDI, 1970), which led to the postulate that the protein-containing membrane components which covered these sites on normal cells were missing from the membrane of virus-transformed cells. However, recent investigations of

such "cryptic" agglutination sites have yielded a somewhat different interpretation of this phenomenon. Thus, radioactively-labeled concanavalin A (Con A) and wheat germ agglutinin have both been found to bind equally well to a variety of normal and transformed cells (CLINE and LIVINGSTON, 1971; OZANNE and SAMBROOK, 1971), even when calculated on the basis of surface area. While mild trypsin treatment greatly enhanced cell agglutinability, it did not increase binding of the lectin (OZANNE and SAMBROOK, 1971). However, in contrast to these results, more recent experiments demonstrated approximately 5-times greater binding of ^{3}H-Con A by SV-3T3 cells than by normal 3T3 cells (NOONAN et al., 1973).

Nicolson (1971), using ferritin-conjugated Con A and electron microscopy, demonstrated that 3T3 and SV-3T3 cells were capable of binding approximately equal amounts of the plant-derived lectin. However, while the distribution of ferritin-Con A on 3T3 cells was essentially random, that seen on the surface of SV-3T3 was characterized by large, randomly dispersed clusters. It was suggested that the more clustered distribution of the Con A binding sites after SV40 transformation of 3T3 cells is responsible for the increased agglutinability of these cells by Con A and that it is unnecessary to invoke a mechanism involving the exposure of cryptic sites. Subsequently, mild proteolytic treatment of normal 3T3 cells was shown to result in surface alterations including (or leading to) a similar redistribution of the agglutinin sites (NICOLSON, 1972). It should be noted that the rearrangement of receptor sites on transformed cell surfaces may occur in response to the actual binding of the lectin (DE PETRIS et al., 1973; INBAR and SACHS, 1973; ROSENBLITH et al., 1973; NICOLSON, 1973). This would imply that the difference between the surface properties of normal and transformed cells is not so much one of pre-existing site distribution but of the ability of the sites to be redistributed (possibly a measure of membrane fluidity), although not all the available data (DE PETRIS et al., 1973) is consistent with this interpretation.

Utilizing radioactively-labeled Con A, BEN-BASSAT et al. (1971) have independently confirmed these results. Analyzing hamster and mouse cells transformed by PV, SV40, and a chemical carcinogen, they also reported a redistribution of Con A binding sites. In addition, their results supported the idea that in some cases the increased agglutinability of transformed cells could indeed be due to the exposure of cryptic sites, or even to an increased concentration of the binding sites resulting from the decreased size of the transformed cell. Subsequent studies by these investigators (INBAR et al., 1971) have indicated that, in addition to the binding site, agglutination by Con A also requires the presence of a temperature-sensitive metabolic site. The active form of the metabolic site was present only in transformed cells, and its temperature-sensitivity was interpreted as indicating that its activation required some enzyme-mediated membrane alteration. This dual site hypothesis could account for the equal binding of Con A by normal and transformed cells with the subsequent agglutination of only the latter cells. More information is needed, however, on the precise mode of activation of the metabolic site. In short, whether

the detection of lectin receptor sites on normal cells after mild proteolysis reflects the uncovering of "cryptic" sites or a redistribution of membrane components is still not completely clear.

The appearance of an SV40-associated surface antigen on normal cells after mild proteolysis (HÄYRY and DEFENDI, 1970) has already been mentioned. Although this is generally taken to support the cryptic site hypothesis, it too could reflect the redistribution of membrane components after both transformation and enzyme treatment. The unmasking of surface antigens on tumor (GASIC and GASIC, 1962; BAGSHAWE and CURRIE, 1968; CURRIE and BAGSHAWE, 1968; VASUDEVAN et al., 1970) and normal (CURRIE et al., 1968) cells by enzymatic treatment has also been demonstrated in several other systems. Providing strong support for the hypothesis that virus-induced surface antigens appear primarily as a result of alterations in the plasma membrane are the results of comparative biochemical studies carried out in a variety of cell systems. Examination of the membrane glycoproteins (WU et al., 1969; MEEZAN et al., 1969; GRIMES, 1970; CULP et al., 1971) and glycolipids (HAKOMORI and MURAKAMI, 1968; HAKOMORI et al., 1968; MORA et al., 1969, 1971; CUMAR et al., 1970; ROBBINS and MACPHERSON, 1971a, b; SAKIYAMA et al., 1972; FISHMAN et al., 1972; CRITCHLEY, 1973; CRITCHLEY and MACPHERSON, 1973) of virus-transformed cells and comparison with the same components from the corresponding normal cells strongly suggest that tumor cells may be characterized by a general failure to complete the synthesis of membrane constituents. This, in turn, could account for the exposure of previously concealed membrane sites or the reorganization of the membrane architecture (BLACK et al., 1971). Incomplete synthesis of membrane components, particularly glycoproteins, has also been postulated (ROBERTSON and BLACK, 1969) to explain the appearance of Forssman antigen on the surface of certain virus-transformed cells (O'NEILL, 1968; ROBERTSON and BLACK, 1969). It should be noted that this model could even explain the appearance of virus-induced antigens which cross-react with fetal tissues; in this regard, it has recently been shown that embryonic cells can be agglutinated by Con A (MOSCONA, 1971). Since this agglutination site can be expressed on normal adult cells only after proteolysis, its ready detection in malignant and embryonic cells is much more in accord with a mechanism based on alterations in membrane architecture than direct genetic controls. Likewise, CEA has been reported to be present in normal adult tissues, albeit at much lower levels than found in tumor cells (COLLINS and BLACK, 1974).

VI. Hypothetical Model to Account for the Virus-Associated Nature of Some Virus-Induced Host-Coded Surface Antigens

A. Role of Virus-Coded Product

Given that virus-induced host-coded surface antigens in transformed cells may be a reflection of membrane alterations rather than direct changes in genetic controls, one must reconcile the apparent randomness of these neoantigens

with the paradoxical association of some of them with a particular virus (e.g., SV40 S antigen). This dilemma can be overcome by postulating that a specific virus-coded function can result in widely divergent effects in different host cells, particularly when these effects are assayed as surface antigenic alterations. How such a mechanism could operate to result in the expression of membrane neoantigens in virus-transformed cells is discussed in the following hypothetical model using SV40 as the prototype oncogenic DNA virus.

Because of the small amount of genetic material contained in both SV40 and PV ($\sim 3 \times 10^6$ daltons), the number of proteins for which these viruses can code is extremely limited (BLACK, 1968). Any model based on the effect of a specific virus function must be compatible with this fact. At this point, the material found in transformed cells which most likely represents a virus-coded product is the tumor (T) antigen (BLACK, 1966, 1968). For this reason, as well as for the sake of simplicity, the model will be discussed with the SV40-specific T antigen assumed to be the virus-coded product involved. It cannot be emphasized too strongly, however, that some other, as yet unidentified, virus-coded material could serve just as well.

Despite their use as a convenient marker of virus-transformation, very little is actually known as to the chemistry and function of T antigens. Partial purification of the SV40 T antigen has yielded widely divergent results as to its composition and size (GILDEN et al., 1965; KIT et al., 1967; DEL VILLANO et al., 1968; DEL VILLANO and DEFENDI, 1973; POTTER et al., 1969) and preliminary analysis of its function suggests that it is not related to the DNA synthetic enzymes whose levels rise markedly after SV40 infection (KIT et al., 1967). Even the site of synthesis of the SV40 T antigen remains unresolved; although it is detected only in the nucleus by immunofluorescence (POPE and ROWE, 1964) and electron microscopy (LEVINTHAL et al., 1967; LEDUC et al., 1969), this may only reflect a higher concentration of the antigen. Some evidence that SV40 T antigen is synthesized in the cytoplasm has been provided by studies with an Ad.-SV40 hybrid mutant virus. Cells infected and/or transformed by this virus contain T antigen only in the cytoplasm, which may reflect the absence of a virus-coded function responsible for transport of the antigen into the nucleus (BUTEL et al., 1969). In the case of Ad. 2, evidence that the T antigen is synthesized in the cytoplasm and subsequently transported into the nucleus has been presented (THOMAS and GREEN, 1966).

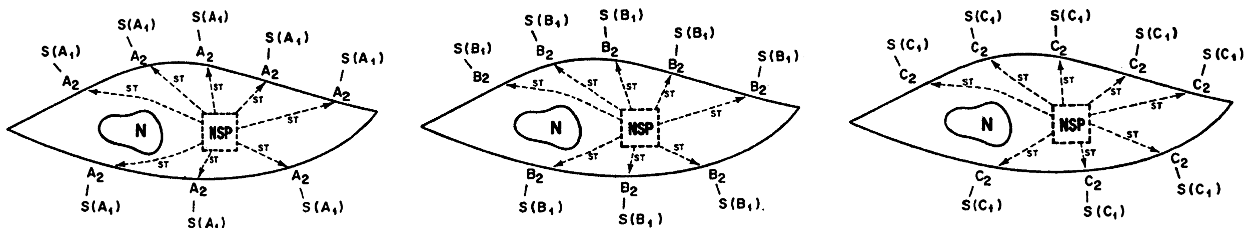
The following model will assume the presence of some T antigen in the cytoplasm of SV40-transformed cells, in addition to its well-demonstrated nuclear location. It is proposed that the T antigen may function by binding to, or in some other manner (enzymatic?) inactivating, one or more host enzymes involved in membrane biosynthesis. Prime candidates for the host enzyme(s) involved would be those instrumental in the addition of terminal membrane components. Interference with the addition of such membrane constituents could then result in either an uncovering of cryptic (antigenic or other) sites previously concealed by these materials or in a reorganization of the membrane architecture with the same consequences. It is apparent that the membrane

properties of the host cell will be of paramount importance in determining the precise nature of the new sites exposed as a result of this virus-specified function; i.e., interference with the same enzymatic activity in different transformed cells could result in the appearance of distinct membrane antigens. In addition, the different virus T antigens, which are immunologically distinct, could act on different biosynthetic enzymes, thus accounting for the virus-associated nature of some of the surface neoantigens.

This model is presented in schematic form in Fig. 2. In a simplified manner, it is illustrated with SV40 T antigen serving as the active virus-coded intermediate and the membrane biosynthetic enzyme involved being a sugar transferase (see below). The normal cells (of the same species) are indicated to possess at least some non-cross-reacting isoantigens (denoted as A_1, B_1, and C_1) whose immunogenicity is dependent upon the presence of the terminal sugar. These isoantigens are depicted as covering, or in some other way preventing the expression of subterminal non-cross-reacting antigens (denoted as A_2, B_2, and C_2), whose immunogenicity is dictated by a glycolipid or glycoprotein component that acts as the normal membrane acceptor of the terminal sugar. In the untransformed cells, the sugar is transported from the nucleotide-sugar pool to the membrane acceptor by a specific sugar transferase (ST). Subsequent to virus-transformation, some of the ST molecules are inactivated by the binding of T antigen (ST-T), thus allowing the exposure of some subterminal sites. Note that not all of the enzyme is inactivated, but enough new sites are uncovered to allow the host animal to respond immunologically to them. Since the different normal cells were characterized by unique subterminal sites, the virus-induced inactivation of the same transferase enzyme subsequent to the transformation of these cells (of the same species) would result in the appearance of non-cross-reacting surface neoantigens.

B. Involvement of Host-Coded Sugar Transferase Enzymes

Currently available data already suggests several good candidates for the membrane biosynthetic enzymes which may be interfered with in virus-transformed cells. Diminished levels of sialyl transferase activity have been found to correlate quantitatively with the decreased membrane sialic acid concentration of SV-3T3 cells (WU et al., 1969; MEEZAN et al., 1969; GRIMES, 1970). In addition, the equivalent levels of the intracellular nucleotidesugar precursor pool in the 3T3 and SV-3T3 cells, despite the lower membrane sialic acid content of the latter, also supports interference with the enzymatic transfer of membrane constituent sugars at the site of membrane synthesis (whether at the plasma membrane or in the endoplasmic reticulum) as the mechanism responsible (WU et al., 1969; MEEZAN et al., 1969). Py-BHK cells are also characterized by decreased levels of membrane sialic acid (OHTA et al., 1968); although the levels of L-glutamine: D-fructose-6 phosphate amino-transferase, the first enzyme of sialic acid biosynthesis, were about the same in these normal and transformed cells, the activity of sialyl transferase was not examined.



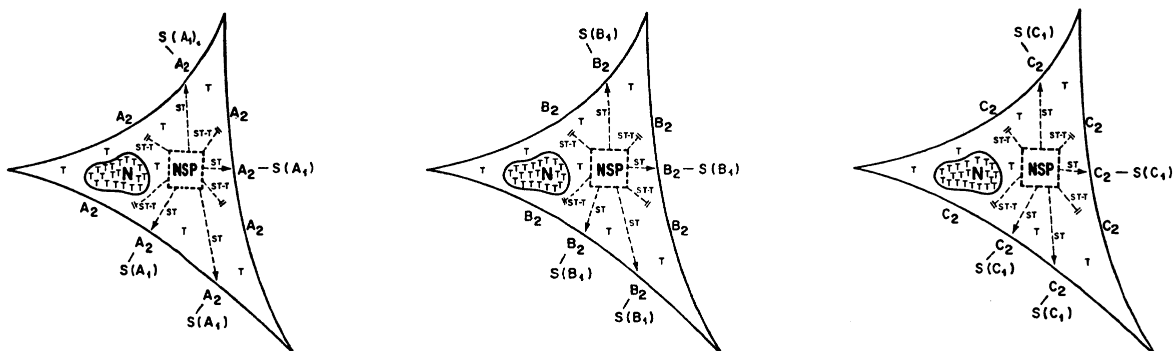


Fig. 2. Diagrammatic presentation of the proposed model for the appearance of virus-induced host-coded surface antigens on virus-transformed cells. It is suggested that the virus-coded T antigen can inactivate certain host-coded sugar transferases (ST) by binding to them (ST-T) and thereby interfering with normal membrane biosynthesis and/or organization. The inability to transfer the sugar (S) from the nucleotide-sugar pool (NSP) to the membrane acceptor could then lead to the appearance of previously concealed membrane antigens ($subscript_2$). In addition, the specificity of the normal "covering" isoantigens ($subscript_1$) is suggested to be at least partially dependent on the presence of the particular sugar. The model is presented here in terms of the transformation by SV40 of three different cells of the same species (e.g., hamster kidney, see COLLINS and BLACK, 1973a) containing at least some isoantigens which are distinct. A considerably more detailed discussion of this hypothesis is presented in the text. (Figure taken from COLLINS, 1972)

Subsequent examination of this latter enzyme activity in another laboratory (Den et al., 1971) has, in fact, demonstrated an 85 % reduction in its level in Py-BHK cells compared to the untransformed cells, as well as an altered response to activation by cardiolipin, which is consistent with an enzyme modification after transformation.

Another surface-associated alteration of transformed cells, namely the synthesis and excretion of collagen (Green et al., 1966a, b), has also been shown to involve a sugar transferase enzyme. Collagen-glucosyl transferase, which transfers glucose from a nucleotide-sugar precursor to the collagen molecule, was found to decrease in activity in parallel to the diminished synthesis of collagen by PV- and SV40-transformed 3T3 cells (Bosmann and Eylar, 1968). It would be of interest to measure the activity of this enzyme in SV40-transformed BALB/c 3T3 cells, which have been reported to produce greater amounts of collagen than the corresponding untransformed cells (Culp and Black, 1972b).

Furthermore, it has recently been suggested that at least some of the changes in membrane glycolipids which occur subsequent to virus transformation (Hakomori and Murakami, 1968; Hakomori et al., 1968; Mora et al., 1969, 1971; Cumar et al., 1970; Robbins and Macpherson, 1971a, b; Sakiyama et al., 1972; Fishman et al., 1972; Critchley, 1973; Critchley and Macpherson, 1973) may also reflect interference with a sugar transferase enzyme. Thus, the decreased content of higher giangliosides in membranes of Py-3T3 and SV-3T3 cells (Mora et al., 1969) was found to correlate with the decreased activity of the enzyme catalyzing the transfer of N-acetylgalactosamine from the nucleotide-sugar precursor to glycolipid acceptors (Cumar et al., 1970; Mora et al., 1971; Fishman et al., 1972). Although the normal level of enzyme activity in spontaneously transformed cells was reduced somewhat when these cells were co-cultivated with the corresponding SV40-transformed cells (Mora et al., 1971), attempts to demonstrate the presence of a soluble enzyme inhibitor in homogenates of the latter cells have been unsuccessful (Cumar et al., 1970; Mora et al., 1971). However, it is possible that the preparation of the cell homogenates resulted in the destruction of a labile inhibitor; it would be of interest to determine the T antigen titer of these cell homogenates. A similar enzyme defect (Kijimoto and Hakomori, 1971) appears to be the basis of the glycolipid changes in PV-transformed hamster cells as well (Hakomori and Murakami, 1968; Hakomori et al., 1968).

In any event, it is clear that there is sufficient precedent available to support the notion that virus-induced cell surface alterations may be mediated via interference with enzymes involved in membrane biosynthesis. The abnormally high levels of some sugar transferases in virus-transformed cells (Bosmann et al., 1968; Bosmann, 1972a; Warren et al., 1972) reinforces the idea that modifications of these enzymes could account for the surface-associated alterations characteristic of tumor cells. It should be noted that glycosyl transferases have also been implicated (Kijimoto and Hakomori, 1971) in the control of density-dependent glycolipid changes in normal hamster cells

(HAKOMORI, 1970; ROBBINS and MACPHERSON, 1971a, b; SAKIYAMA et al., 1972; CRITCHLEY, 1973; CRITCHLEY and MACPHERSON, 1973) and as primary mediators of cell-to-cell communication and adhesion (BOSMANN, 1971; ROTH et al., 1971; ROTH and WHITE, 1972). The antigenic changes which characterize transformed cells may, in fact, represent secondary effects of the primary biochemical lesion. Whether an effect on the membrane synthetic enzymes is indeed mediated by a virus-coded product, such as T antigen, remains to be proven. The model does, however, account for both the random and quasi-specific aspects of the virus-induced surface antigenic alterations. It is interesting that polyoma T antigen has also recently been proposed to have a genetic control function; it was suggested that T acts to trigger the virus-induced replication of host cell chromosomes which accompanies the production of PV in permissive mouse cells (WEIL and KÁRA, 1970).

C. Alternative Mecchanisms

A less appealing alternative to the involvement of a virus-coded intermediate (e.g., T antigen) is the hypothesis that the synthesis of the appropriate transferase enzyme(s) is turned off by the virus, possibly by integration into the corresponding host structural gene (see earlier discussion of the direct derepression hypothesis and TAYLOR, 1963). This mechanistic modification is more in line with the inability to find an inhibitor of glycosyl transferases in transformed cells (CUMAR et al., 1970; MORA et al., 1971; DEN et al., 1971), but does not explain the fact that the sugar transferases studied thus far are not completely absent in the transformed cell (BOSMANN, 1968; GRIMES, 1970; CUMAR et al., 1970; MORA et al., 1971; DEN et al., 1971; FISHMAN et al., 1972); this could, however, be a reflection of gene dosage effects. In addition, until more is known about the sites where SV40 integrates into the host DNA, it seems excessively speculative to try to account for the wide range of membrane effects by integration into specific genes. It is also possible that the increased level of proteolytic activity in transformed cells (SCHNEBLI, 1972; BOSMANN, 1972b) may lead to the removal of protein-containing membrane components and the subsequent exposure of "new" sites (SCHNEBLI and BURGER, 1972). This would parallel the findings of several *in vitro* studies (BURGER, 1969; INBAR and SACHS, 1969; HÄYRY and DEFENDI, 1970). In any event, extensive investigation is required to verify the validity of any of these models.

D. Uncovering of "Cryptic" Sites vs. Membrane Reorganization

One may question the model presented in Fig. 2 as to whether the basic membrane characteristics of the cells of different species, much less the cells from the same species, differ sufficiently to be able to account for the observed diversity of virus-induced surface neoantigens on the basis of an interference with the addition of terminal membrane components. In this regard, it may be

noted that even clones of the same cell line differed markedly in their membrane glycolipid composition (SAKIYAMA et al., 1972). Thus, it would not be surprising that the elimination of a particular membrane component (e.g., sialic acid) could lead to the expression of unique antigens on cells of the same and/or different species.

The results obtained in our earlier studies are completely in accord with the model discussed. The experiments with SV40-transformed outbred hamster cells demonstrate that at least some of the surface antigens which appear subsequent to virus-transformation are unique for an individual transformed cell line and need not be present on other SV40-transformed hamster kidney cells (COLLINS and BLACK, 1973 a). Thus, the same type of cells transformed by the same virus were characterized by different surface antigens. The antigens expressed on the transformed outbred hamster cells were in a non-cryptic form while those detected on SV40-transformed inbred hamster cells and rabbit cells had first to be exposed by enzymatic treatment (COLLINS and BLACK, 1973 b). This further underscores the significant differences evident when comparing both intraspecies and interspecies cell mambrane architecture and reemphasizes the critical role that the host cell plays in determining what antigenic moieties are finally expressed.

The random nature of the virus effect on the cell surface was even manifested when comparing the antigenicity of SV40-transformed inbred hamster kidney cell clones (COLLINS and BLACK, 1973 b). In addition, the cryptic antigens of the transformed inbred hamster cells and the transformed rabbit kidney cells did not cross-react either with each other or with the exposed antigens on the transformed outbred hamster cells. Since the cloned inbred hamster cells all presumably contain the same genetic information it would be difficult to explain this antigenic uniqueness solely on the basis of the uncovering of cryptic sites; however, the involvement of a more severe and, perhaps random membrane reorganization subsequent to virus transformation could account for such unique effects. These results suggest that interference with the addition of membrane constituents (e.g., various sugars), as depicted in Fig. 2, need not result directly in the exposure of host-coded antigenic determinants, but may, in fact, lead to a considerable reorganization of the membrane architecture.

The enhanced expression of surface antigens, both virus-induced (HÄYRY and DEFENDI, 1970; COLLINS and BLACK, 1973 b) and otherwise (GASIC and GASIC, 1962; BAGSHAWE and CURRIE, 1968; CURRIE and BAGSHAWE, 1968; CURRIE et al., 1968; VASUDEVAN et al., 1970; HERSCHMAN et al., 1972) on animal cells subsequent to enzymatic treatment is now a well know phenomenon. However, in the case of virus-induced antigens, the action of the enzyme may reflect either the direct exposure of a cryptic antigenic site by removal of covering material or a further alteration of the membrane structure beyond that previously induced by the virus. The distinction between uncovering of cryptic sites and membrane organization which is discussed here is analogous to the question of whether the agglutination of virus-transformed cells by plant lectins reflects the exposure of preexisting, but covered, cryptic sites (BURGER,

1969; INBAR and SACHS, 1969), or the redistribution of normally exposed sites into a clustered arrangement (NICOLSON, 1971).

VII. Conclusion

In summary, virus transformation of animal cells can lead to the appearance of both virus-specific and virus-associated surface antigens, which the autochthonous or syngeneic host can perceive and respond to immunologically. Some virus-associated antigens appear to be unique for individual transformation events and such surface neoantigens may be organized in markedly different ways (i.e., exposed or cryptic), which is primarily dependent on the host cell. Whether these antigenic moiete is have any functional role in the virus-transformed cell other than serving as immunological markers is not clear at present, but future investigations will hopefully address themselves to this question, as well as further delineating the mechanism of their appearance and their relationship to the numerous other surface-associated characteristics of neoplastic cells.

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