Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

VIRUS-ERYTHROCYTE INTERACTIONS[1]

C. Howe[2] and L. T. Lee[2]

Department of Microbiology,
Columbia University, College of Physicians and Surgeons,
New York, New York

I. Introduction 1
II. The Red Cell Membrane 2
III. Methodology 9
IV. Major Virus Groups That React with Erythrocytes 11
A. Myxoviruses 11
B. Paramyxoviruses 19
C. Pseudomyxoviruses 22
D. Adenoviruses 25
E. Arboviruses 29
F. Reoviruses 31
G. Enteroviruses 33
H. Miscellaneous Hemagglutinating Viruses 37
V. Summary 44
References 45

I. Introduction

The observation that fowl erythrocytes were agglutinated by influenza viruses represented a milestone in the development of virology (Hirst, 1941; McClelland and Hare, 1941). The extensive research stimulated by this discovery was the subject of an earlier review in this series (Buzzell and Hanig, 1958). The phenomenon of hemadsorption was also first described in connection with influenza viruses (Shelokov *et al.*, 1958) and reflects the same interaction between erythrocytes and viral envelope components. A number of viruses unrelated to myxoviruses have since been shown to agglutinate erythrocytes of various species. The mechanism in each case appears to differ basically from that of the myxoviruses, and in no instance is there precise knowledge as to the groupings involved in either erythrocyte surface or virion. Regardless of the underlying mechanism, proof that hemagglutination is due to the viral agent in hand depends on whether the visible reaction can be inhibited by antibody directed against the whole viral particle or its active surface structures. Specific inhibition of hemagglutination by antibody has thus

[1] Supported by grants from the National Institutes of Health (AI 03168, 06814; AM 13200).

[2] Present address: Louisiana State University Medical Center, New Orleans, Louisiana 70112.

been a major asset in the recognition of important immunological relationships within and among different groups of animal viruses.

In this review, we have attempted to summarize the experimental evidence bearing on the nature of the virus-erythrocyte reactions characteristic of several taxonomic groups. Such evidence is culled from (1) study of conditions necessary for hemagglutination; (2) examination of specific factors affecting either the cell or the virion to enhance, alter, or abolish the reaction; and (3) direct physicochemical analysis of cells, viruses and "receptor analogs." We have not considered in this discussion (1) passive hemagglutination reactions used for measurement of antibody to viral antigens adsorbed to tanned erythrocytes (e.g., HAA) or (2) hemagglutinins which are formed during viral infection independently of mature virions (e.g., vaccinia).

II. The Red Cell Membrane

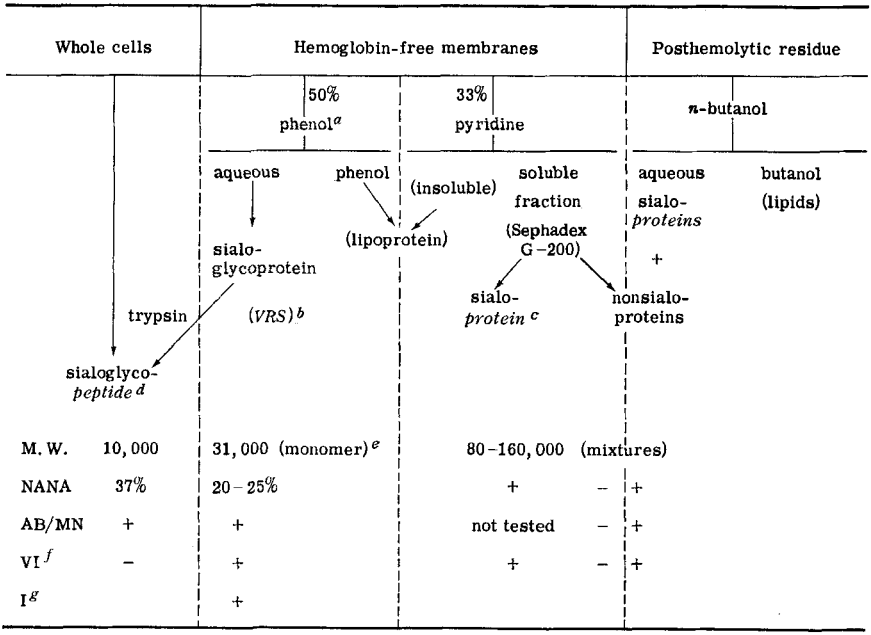
The ultrastructure and composition of erythrocyte membranes constitute a field of study which is expanding so rapidly that no attempt can be made here to encompass the extensive literature on the subject, except for particular aspects which concern this review. It is of more than historical interest that the influenza virus-erythrocyte-glycoprotein inhibitor model has served as an important link in relating biological function to red cell membrane structure. Influenza virus receptors are intimately associated with receptors for isoantibodies, namely blood group antigens (A, B, H, M, N). These serve as additional markers with which to recognize biological activities of purified membrane fractions. In broad analogy with virus receptor analogs, the structure of antigenic determinants of blood group specificity has been elucidated not from primary analysis of erythrocytes but from extensive study of soluble blood group substances derived from sources other than red cells. MN antigens constitute an exception to this statement, since they are found only in erythrocytes, and in fact form a part of the glycoprotein complex which contains virus receptor activity.

A direct approach to the chemical characterization of various receptor activities lies in attempting to render erythrocyte membranes soluble and then to separate components by standard methods of protein and carbohydrate chemistry. Much depends initially on the nature of the starting material which is to be rendered soluble by one means or another. Plasma proteins, leukocytes, and platelets are possible sources of contamination if not separated from the erythrocytes before lysis. The method of lysis used to obtain stroma, or posthemolytic residue, also has an important bearing on the purity of the end product, since hemoglobin and other cytoplasmic constituents are readily occluded and cling tenaciously to membranes of red cells lysed in distilled water.

By hypotonic lysis under carefully controlled conditions of pH and ionic strength, pure erythrocyte ghosts are obtainable (Dodge *et al.*, 1963). Such membranes retain virtually all of the lipid of the intact cell. This fact, along with their antigenic distinction from cytoplasmic constituents, makes such preparations acceptable as intact cell membranes. They appear as biconcave disks when viewed by phase-contrast microscopy and are virtually free of hemoglobin as determined by colorimetric and immunological analysis, and by electron microscopy (Howe and Lee, 1969; Howe *et al.*, 1970b).

Table I presents a simplified summary of several methods which have been used to partition membranes or posthemolytic residue into biologically active subfractions. The term "posthemolytic residue" is here in-

TABLE I
HUMAN ERYTHROCYTE FRACTIONS



[a] Yield of glycoprotein enhanced by prior extraction of membranes with lithium diiodosalicylate. Product antigenically identical to VRS.

[b] Virus receptor substance.

[c] Rich in serine, threonine. Includes some nonsialoproteins.

[d] Rich in serine, threonine, *O*-glycosidically linked to *N*-acetylgalactosamine (alkali labile); asparagine linked *N*-glycosidically to *N*-acetylglucosamine (alkali stable).

[e] M.W. 55,000 proposed on basis of corrected SDS-gel data.

[f] VI = influenza viral hemagglutination inhibition.

[g] I = reactive sites uncovered by removal of *N*-acetylneuraminic acid (NANA) from alkali-stable oligosaccharide side chain.

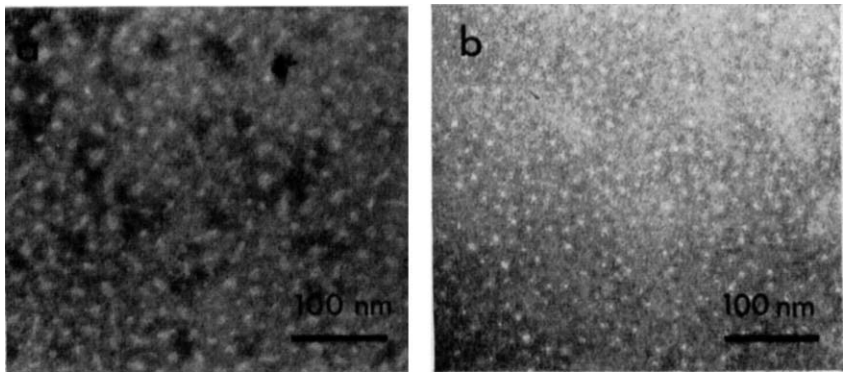


FIG. 1. Purified virus receptor substance (VRS) negatively stained with PTA. (a) VRS from human erythrocytes. (b) VRS from murine (Swiss) erythrocytes. (By Courtesy of Dr. James R. Harris.) × 125,000.

tended to signify relatively crude preparations of stroma as contrasted to membranes that retain both morphological and chemical identity and are uncontaminated with hemoglobin, plasma protein, or formed elements. Hot 50% phenol extraction of membranes separates into the aqueous phase a glycoprotein (Kathan *et al.*, 1961) which can be further purified through fractional ethanol precipitation and various combinations of gel filtration and chromatography (Springer *et al.*, 1966). The final product is free of lipid, contains virtually all the sialic acid and approximately half of the hexoses and amino sugars of the membrane, and is completely soluble in water. By several different criteria, including polyacrylamide gel electrophoresis (PAGE), ultracentrifugal analysis, and immunoelectrophoresis, it represents a single component. Electron microscopic examination of a preparation of erythrocyte glycoprotein considered pure by these criteria was negatively stained with phosphotungstic acid and examined by electron microscopy (Fig. 1a). It is seen that the preparation comprises a homogeneous array of particles, although no conclusions as to size or configuration can be drawn due to hypertonicity of the staining solution. An exactly comparable preparation from murine erythrocytes is shown, in which the particles are seen to be closely similar to the human substance in both size and uniformity (Fig. 1b). The yield of glycoprotein from human erythrocytes was reported to be enhanced if membranes were extracted first with lithium diiodosalicylate (LIS) and then with 25% aqueous phenol (Marchesi and Andrews, 1971). Glycoprotein purified in this manner shows antigenic identity with the VRS and MN substances previously described. Recent electrophoretic studies of various glycoproteins, includ-

ing the LIS-phenol-derived erythrocyte material, have shown that apparent molecular weights vary with the degree of acrylamide cross linking (Segrest *et al.*, 1971). In the light of these findings, some of the molecular weights previously determined for phenol-derived glycoproteins (VRS and MN substances) by PAGE will have to be reevaluated (cf. Table I). The stromal glycoprotein is a potent inhibitor of influenza virus hemagglutination, and contains, in its polysaccharide moiety, the determinants for M and N blood group antigens (Kathan *et al.*, 1961; Howe *et al.*, 1963; Springer *et al.*, 1966). It thus may be considered to encompass the sites on the erythrocyte surface to which myxoviral hemagglutinins attach, i.e., the virus receptor substance (VRS). These surface glycoprotein sites are associated with, and appear to be extensions of intramembranous particles revealed by freeze-etching techniques and electron microscopy (Marchesi *et al.*, 1971). Antibodies evoked in rabbits by injection of VRS in complete Freund's adjuvant may be directed not only to the particular blood group antigens contained in its polysaccharide moiety (Springer *et al.*, 1966), but also to the protein portion of the VRS molecule. The latter antibodies are broadly cross-reactive from one individual VRS preparation to another, independently of blood group antigens. Antibody to VRS, labeled with ferritin, reacts specifically with intact erythrocytes as well as with the external surface of hemoglobin-free membranes. Figures 2 and 3 show electron micrographs of these reactions, both of which can be blocked by prior absorption of the labeled antiserum with pure VRS (Howe *et al.*, 1970b). Trypsin treatment inactivates receptors for myxoviruses both on erythrocytes and in purified VRS. As a result, the electrophoretic motility of both erythrocytes and glycoprotein is reduced. These effects are accounted for by the cleavage of a sialopeptide from the erythrocyte glycoprotein (Cook *et al.*, 1961). A structural model has been proposed for this component and for its insertion into the erythrocyte (Winzler, 1969a). The trypsin-sensitive fragment is considered to be at the amino end of a linear polypeptide which is anchored among the cell lipids at its carboxyl end. Along the polypeptide chain are a series of oligosaccharide residues in which *N*-acetylneuraminic acid (NANA) is terminally bound. This terminally bound NANA accounts for the predominantly anionic nature of the cell surface and the rapid electrophoretic mobility of the intact pure glycoprotein itself (Cook *et al.*, 1961; Howe and Lee, 1969; Danon *et al.*, 1965). The oligosaccharide side chains are attached to the peptide through *O*-glycosidic linkage of *N*-acetylgalactosamine to serine and threonine, and through *N*-glycosidic linkage of *N*-acetylglucosamine to aspartic acid residues. The pure glycoprotein has a characteristically high content of all three of these amino acids (Winzler, 1969a; Kathan

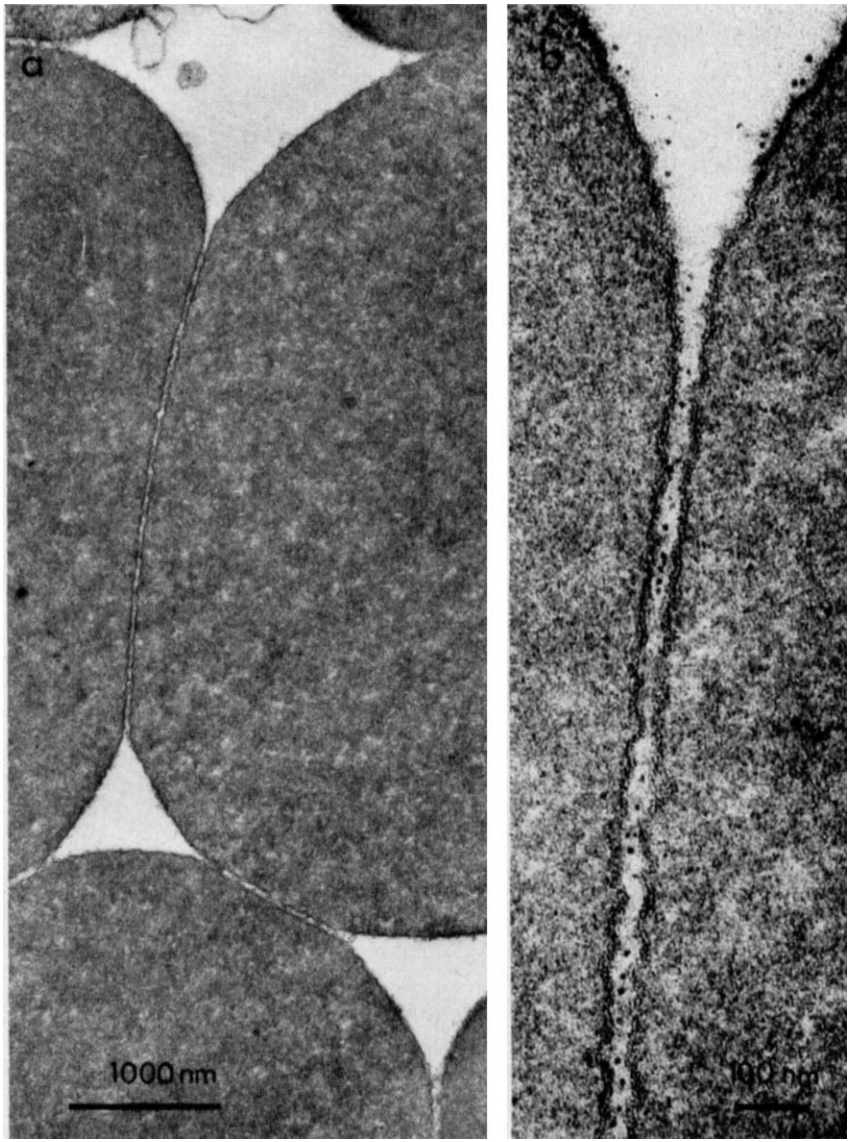


FIG. 2. Specific reaction of human group O erythrocytes with ferritin-labeled antibody to VRS. Glutaraldehyde and osmium tetroxide fixation after reaction with antibody and washing with phosphate-buffered saline, pH 7.2. (By courtesy of Dr. Thomas Bächi.) (a) × 20,000. (b) Detail from (a), × 100,000.

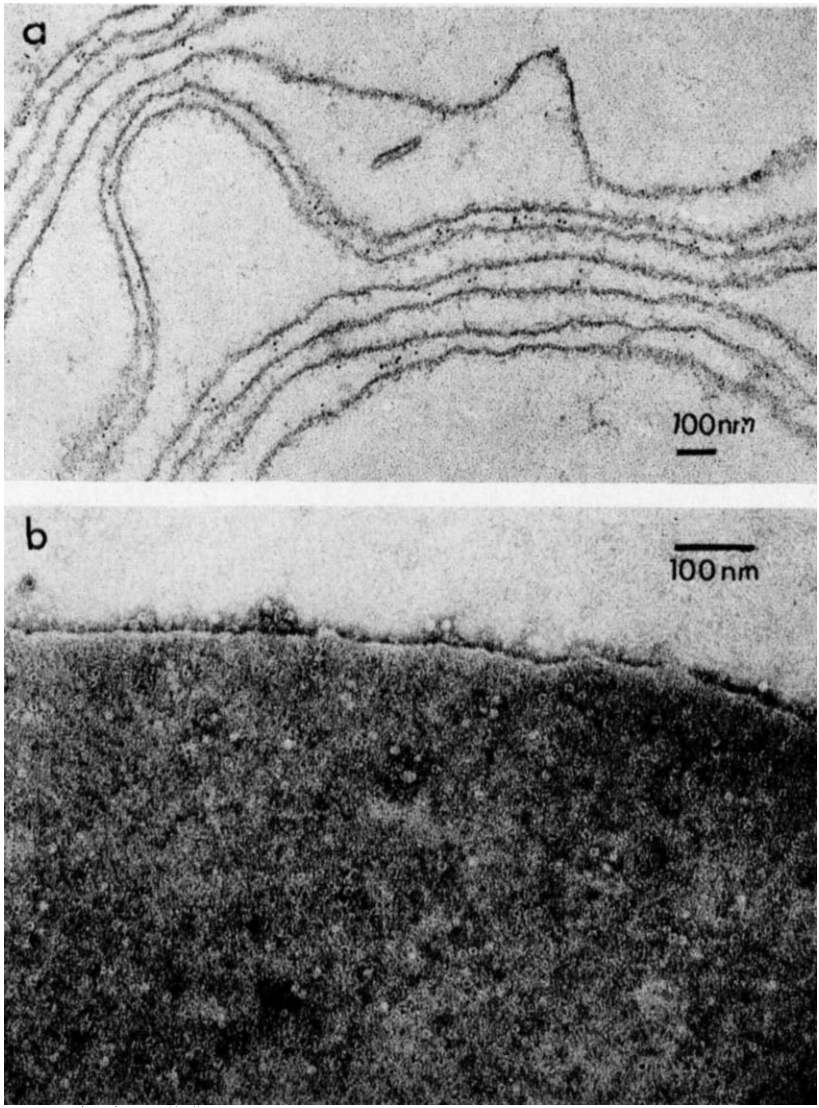


FIG. 3. Specific reaction of human erythrocyte membranes with ferritin-labeled antibody to VRS. (a) Erythrocytes were reacted with the antibody, washed free of nonspecific protein and ferritin, and lysed by the addition of complement. The cell membranes were washed free of hemoglobin, and fixed in glutaraldehyde and osmium tetroxide. × 50,000.

(b) Portion of a hemoglobin-free membrane prepared by hypotonic lysis (Dodge *et al.*, 1963), allowed to react with antibody, washed in phosphate-buffered saline, fixed in glutaraldehyde, and negatively stained with PTA. × 100,000. (By courtesy of Dr. Thomas Bächi.)

et al., 1961; Springer *et al.*, 1966; Howe *et al.*, 1972). The trypsin-released sialopeptide carries the prosthetic groupings necessary for virus attachment to the cell and for inhibition of influenza viral hemagglutinin by the purified glycoprotein. The sialopeptide also carries the determinants for M and N blood group antigens recognized in the intact glycoprotein. Recent evidence (Dzierzkowa-Borodej *et al.*, 1970) shows that determinants reacting with anti-I are contained in an alkali-stable trisaccharide obtained by borohydride degradation of VRS from which NANA has previously been removed by mild acid hydrolysis. Phenol-derived glycoproteins from several species differ from one another significantly with respect to monosaccharide composition and content of the corresponding amino acid residues involved in carbohydrate-protein linkage (Howe *et al.*, 1972).

Treatment of hemoglobin-free membranes with aqueous pyridine resulted in the partition of the protein into a soluble fraction containing most of the membrane sialic acid and insoluble fractions containing membrane lipid (Blumenfeld *et al.*, 1970). On Sephadex G-200, in the presence of pyridine, sialoprotein was separated from components lacking sugar residues. The sialoprotein fraction had at least one component which shared antigenic identity with VRS, and which undoubtedly accounted for the virus inhibitory action of the sialoprotein fraction (Table I). Virus receptor activity of the pyridine-derived sialoprotein was appreciably lower than that of pure VRS. This may have been due to the presence of nonreactive components, or to an arrangement of complementary prosthetic groups less efficient than VRS in binding to the viral surface. In amino acid analysis, the pyridine-derived sialoprotein gave high serine and threonine values comparable to those for the purified VRS, strongly supporting the conclusion that this large molecular weight glycoprotein was an entity distinct from the smaller proteins in the soluble fraction, which contained little or no sialic acid (Howe *et al.*, 1971).

n-Butanol has been used by a number of investigators (Howe *et al.*, 1963; Maddy, 1964) to solubilize membrane components. A portion of the membrane protein was thus rendered water soluble, presumably by separation from lipids and lipoproteins which entered the butanol phase. Several extractions were required to remove all lipid from the water-soluble fraction, which comprised several components and contained sialic acid, virus receptor, and blood group activities (Whittemore *et al.*, 1969). One component cross-reacted antigenically with pure VRS (Howe and Lee, 1969). As will be noted subsequently, erythrocyte receptors for several groups other than myxoviruses have been described on the basis of the inhibitory activity demonstrable in mixtures of mem-

brane components, such as the water-soluble fraction referred to above. These "receptors," however, cannot be recognized in a state of purity comparable to that of VRS.

Combinations of various methods have been used to determine the total number of individual proteins present in erythrocyte membranes prepared by hypotonic lysis and free of hemoglobin and cytoplasmic constituents. Rosenberg and Guidotti (1969) subjected ghosts to sequential extraction with EDTA, 0.8 *M* NaCl and ethanol–ether. The resulting lipid-free residue contained most of the sialic acid of the membrane and was resolved by gel electrophoresis into five additional fractions, two of which accounted for 90% of the sialic acid in the starting material. These two sialoprotein fractions had molecular weights in the same range (25,000–30,000) as that of the monomeric form of VRS obtained by phenol extraction of membranes. The other components separated on gel electrophoresis were in the size range from 10,000 to 170,000. NH_2-terminal end group analyses indicated consistent differences among the proteins of the various fractions. A minimum of twelve different polypeptide chains were judged to be present in the membrane. These results are to be compared with those of another study in which hemoglobin-free membranes were dissolved in SDS and heated at 100°C for 3 minutes in order to disaggregate the proteins completely. Sephadex G-200 chromatography revealed the presence of at least 14 components of molecular weight ranging from 32,000 to 255,000, two of which contained carbohydrate—one with a molecular weight of 108,000; the second, a smaller, more rapid component thought to be glycolipid (Lenard, 1970). In this latter study, the multiplicity of components in SDS gel was not critically excluded as being due to random aggregation.

III. Methodology

The visible result of viral hemagglutination is the "pattern" formed at the bottom of a test tube or well plate by lattices of red cells lightly conjoined by viral hemagglutinin (Salk, 1944). The stability of this pattern varies inversely with the ability of the virus to elute. Hemagglutination serves as a useful direct means of titering intact viral particles or hemagglutinating subunits. Since hemagglutination is a simple two-component system, the titer of a given preparation will depend on the concentration of viral particles and their state of aggregation. The concentration of erythrocytes, the pH, the temperature, and in some instances the presence of divalent cations or other supplementary factors may affect the titer. Details of special significance will be mentioned hereinafter with the discussion of individual groups of hemagglutinating viruses. The techniques generally applicable to the

quantitation of viral hemagglutination are well described in standard compendia of virological diagnostic procedures (Lennette and Schmidt, 1969). With a few exceptions, titrations of viral hemagglutinin and corresponding antibodies are readily adaptable to microtechniques (Sever, 1962) which are highly reproducible and effect substantial economies of valuable diagnostic and experimental reagents.

While the pattern technique is the one in widest use, other approaches to quantitation of viral hemagglutination have been developed, particularly with influenza viruses. Densitometric procedures, first devised by Hirst and Pickels (1942), depend on the rate of settling of cells during their interaction with viral hemagglutinating particles resulting in the formation of aggregates. Readings of optical density taken at given intervals of time, and related to a normal cell suspension without virus, bear a direct relationship to the concentration of viral particles. Levine *et al.* (1953) were able to extend this principle to establish methods for determining the absolute number of hemagglutinating particles present in a virus suspension. Their method was based on spectrophotometric measurement of the number of erythrocytes dimerized by viral hemagglutinin under conditions precluding the formation of larger cell aggregates. The presence of dimers under the standard conditions used was established by direct microscopy. These workers found that the conversion factor for transforming tube dilution hemagglutination titers into absolute numbers of hemagglutinating particles was the same for influenza and Newcastle disease viruses. More recently, a photometric method has been described (Drescher *et al.*, 1962) which depends on determining the highest dilution of virus causing maximal agglutination by extrapolation from data obtained with constant numbers of cells and varying dilutions of virus. At this point, variability in reactivity of virus with different samples of erythrocytes is minimal. Actual titers are then calculated mathematically from this point of reference and are expressed as "hemagglutinin concentration units." A high degree of reproducibility was claimed for this method, which has advantages primarily for detecting minor antigenic variations among related strains of influenza virus.

In studies of the antigenic composition of the influenza virus envelope, constituents of the hemagglutinin have been obtained which, on separation from the parent molecule, do not hemagglutinate directly. Such components are considered to be monovalent with respect to hemagglutinating activity, but are antigenically related to the hemagglutinin complex. Such for example is the "host antigen" found in influenza viruses which have been propagated in chicken embryos and which will be discussed subsequently in greater detail. The method for detecting these

monovalent fragments of hemagglutinin depends on their capacity to combine with antihemagglutinin and thereby to block its inhibition of hemagglutination by complete virus (hemagglutination-inhibition blocking or HIB test) (Harboe, 1963). The specificity of the antibody is critical and purified virus must be used, since nonviral host tissue fragments may combine with antibody to block hemagglutination.

The hemadsorption phenomenon is based on the attachment of erythrocytes to infected cells in culture which have hemagglutinin at their surfaces (Shelokov *et al.*, 1958). Monolayers suspected of containing infected cells are drained of culture fluids; a suspension (usually 0.5%) of washed guinea pig erythrocytes is added in a quantity sufficient to immerse the cells. The reaction takes place at 4°C almost immediately. After 15–20 minutes the monolayers are drained and washed with cold balanced salt solution to remove unattached cells. The preparations are then examined immediately under the microscope, before any elution of erythrocytes occurs. Done in this manner, hemadsorption may substitute for cytopathic effect in estimating titers or units of infectivity. Hemadsorption plaques represent a variation of this technique. Monolayers are infected in the same manner as for plaque titrations with a cytolytic virus. Instead of the usual solid overlay, a semisolid overlay (e.g., methyl cellulose or 0.2% agar) is used which can be removed at the end of the total incubation period without disturbing attached cells. Residual traces of medium are removed by washing with prewarmed balanced salt solution, and hemadsorption is carried out. Plaques thus revealed display a dose response quite comparable to infectivity measured by $TCID_{50}$ or hemadsorption end points (Howe *et al.*, 1970a) and offer a reliable means for quantitation of myxoviruses. Hemadsorption can be directly quantitated by lysing attached erythrocytes with distilled water and measuring the concentration of free hemoglobin (Finter, 1964). With either fluid or semisolid overlays, urea–cyanide (Aculute, Ortho Pharmaceutical Corporation, Raritan, New Jersey) may be used as the lysing agent. This renders both erythrocytes and freed tissue cells soluble. There is thus no interference with the spectrophotometric determination of hemoglobin converted to cyanomethemoglobin. Optical density is read at 410 nm, and the results are expressed as percentage of a standard solution of cyanomethemoglobin.

IV. Major Virus Groups That React with Erythrocytes

A. Myxoviruses

Since the discovery of myxoviral hemagglutination (Hirst, 1941; McClelland and Hare, 1941), much has been learned about the nature

of the viral subunits involved in the reaction as well as about the chemistry of the "receptors" on erythrocytes. In addition, this virus-erythrocyte interaction has constituted a model for the study of the first stages of viral attachment to host cells capable of synthesizing new virus. The extensive literature on influenza viruses has been well reviewed in recent years (Buzzell and Hanig, 1958; Hoyle, 1968).

The reaction of myxoviruses with red cells involves two relatively independent phenomena, namely adsorption and elution. The viral components primarily responsible are, respectively, hemagglutinin and neuraminidase. Both are components of the viral envelope, and both are coded for independently by the influenza viral genome. Recombinants of A_2 strains of influenza virus with an A strain (NWS) served to demonstrate that the hemagglutinin of recent A_2 isolates (Hong Kong) differed antigenically from that of A_2 strains isolated prior to 1968, but that the neuraminidases of all A_2 strains examined were antigenically identical with one another (Schulman and Kilbourne, 1969). These observations showed that hemagglutinin and neuraminidase varied independently as antigens; and that antibodies to the enzyme were of primary importance in protection against Hong Kong virus. It is also evident from such studies that the antigenic classification of influenza viruses must take into account the specificity of both of these major envelope components.

Hemagglutinin and neuraminidase have been recognized as distinct entities by electron microscopic examination of negatively stained preparations of virus after disintegration with ether or detergents, and separation by electrophoresis, column chromatography or density gradient centrifugation. Laver and Valentine (1969) disrupted strains of influenza A virus with sodium dodecyl sulfate (SDS) and subjected them to electrophoresis on cellulose acetate. Electron microscopic examination of the eluted, biologically active subunits, in the presence of SDS, revealed hemagglutinin to comprise rods 40×140 Å; the neuraminidase appeared as oblong structures 85×50 Å with a centrally attached fiber 100 Å in length. Removal of SDS by acetone precipitation resulted in the formation of polymers of either subunit and, in mixtures of the two, in the formation of copolymers. The results suggested that the spikes on the intact virion are of two kinds corresponding, respectively, to the two independently active subunits described. Rott *et al.* (1970) subjected SDS-treated influenza virus to sucrose density gradient centrifugation. They isolated hemagglutinin which was reactive with chicken erythrocytes and which appeared on negative staining as a rodlike structure 50 Å in diameter and 200 Å in length; a second morphological form, composed of spherical particles about 40 Å in diameter, was reactive

with guinea pig cells. Biologically active neuraminidase and hemagglutinin have been isolated from one and the same preparation of virus by treatment with Tween-20 and separation on Sephadex G-200. Residual hemagglutinin could be removed from neuraminidase by absorption with chicken erythrocytes. The sedimentation coefficient for hemagglutinin subunits was 8.1 S; and that for neuraminidase, 10.8 S. Structures seen by electron microscopy in negatively stained preparations of neuraminidase consisted of aggregates in rosette formation with a central knob (Webster, 1970). In other studies, purified influenza A_2 virus was treated with ether, and the envelope components separated from nucleocapsid by electrophoretic techniques and by analytical ultracentrifugation. The isolated neuraminidase–hemagglutinin complexes tended to aggregate into rosettelike clusters and appeared to comprise either tubelike structures, rings attached to stalks, or variations between these two forms. In this biologically active form, the neuraminidase–hemagglutinin complex was comparable in size to the tubular structures forming the characteristic spikes of the influenza virus envelope (Hjerten *et al.*, 1970).

The chemical nature of the reactive groups of the viral hemagglutinin responsible for attachment to soluble or cell-surface glycoproteins is not at all clear, except that peptides are involved. The attachment is pH dependent, and therefore may be mediated by ionizable amino acid groupings of the hemagglutinin. The work of Hoyle and Hana (1966) pointed to disulfide linkages as "active centers" for two major biological activities of the influenza virus particle, namely hemagglutinin and neuraminidase. Chemical cleavage of S—S bonds resulted in the inactivation of one or the other of these activities, depending on the strain of virus used. Similar observations were made by Eckert (1967) on the hemagglutinin of the reassociated envelope protein of influenza virus (PR8). Hemagglutination, and its inhibition by mucoids, is generally independent of neuraminidase activity. That this dichotomy may not be absolute is suggested from recent studies (Hoyle, 1969) which indicate a close resemblance between the active sites of both neuraminidase and hemagglutinin in several strains of influenza virus. Through the use of reagents reacting directly with amino acids in proteins of A and A_2 strains, centers containing histidine were found which appeared to possess both hemagglutinin and neuraminidase activity. It was concluded that histidine was essential for neuraminidase activity and that tyrosine could substitute for histidine in the hemagglutinin-active centers.

Included in the hemagglutinin of influenza virus is a sulfated glycoprotein, originally referred to as "host antigen" (Harboe, 1963). Antibody to normal chicken tissue inhibited strains of influenza virus hemagglutinin due to the reaction of a portion of the antihemagglutinin with this

host factor on the viral particle. It was subsequently shown that this same antigen was present in large quantities and in soluble form in chorioallantoic fluid from both infected and normal chicken embryos, and that it could be quantitated by the hemagglutination inhibition blocking (HIB) test (Section III) (Haukenes *et al.*, 1966; Lee *et al.*, 1969). The host antigen showed structural similarities to blood group substance and to certain keratosulfates from cartilage (Haukenes *et al.*, 1966; Meyer *et al.*, 1967). Rabbit antisera to the pure substance contained antihemagglutinin reactive with purified intact influenza virus particles.

Besides fowl erythrocytes, cells of many mammalian species are agglutinated by influenza viruses. Adsorption appears to be relatively independent of temperature, and takes place with most strains equally well at 4°C and 37°C. This is largely an electrostatic interaction, and in general there are upper (pH 9.0) and lower (pH 5.0) limits to the pH range in which the reaction is maximal (Tischer, 1963). With respect to the molecular basis for viral attachment (hemagglutinin), much has been learned from the study of receptor analogs, the prototype of which was originally described as a nonspecific inhibitor of viral hemagglutination distinct from antibody in human serum (Francis, 1947). Receptor analogs have since been obtained from many sources. They are all glycoprotein and bind preferentially to reactive sites on the virus so as to prevent attachment to erythrocytes. Inhibitory action in all cases is abolished following treatment with reagents (e.g., periodate oxidation, trypsin, neuraminidase or receptor-destroying enzyme of *Vibrio cholerae* or *Clostridium perfringens*) which modify the polysaccharide moiety by alteration of NANA residues, or their removal from the macromolecule. A partial list of inhibitory glycoproteins includes, besides human and animal sera: egg components, urinary mucoprotein, ovarian cyst fluid, meconium, Collocalia mucoid (Howe *et al.*, 1961), and extracts of tissues, e.g., lung and erythrocytes, of several species. Extensive study of purified glycoproteins has shown that the residue essential for interaction with influenza virus is *N*-acetylneuraminic acid (Gottschalk, 1966a,b). Recent studies have shown further that carbons 7, 8, and 9 in the polyhydroxyl side chain of NANA are involved in the binding of sialoproteins to influenza virus (Suttajit and Winzler, 1971). Although all inhibitors of influenza virus hemagglutination contain NANA, not all sialoproteins are active inhibitors. The inhibitory potency of a glycoprotein is determined by the number of negatively charged terminal sialic acid residues which can attach simultaneously to complementary groupings of the viral surface. The attachment in turn is a function of the number of sialic acid residues available per molecule of glycoprotein and the degree

of complementarity of the viral surface (Fazekas de St. Groth and Gottschalk, 1963). The basis for the latter is still unknown. A relationship between molecular size, sialic acid content, and inhibitory activity has been recognized among a number of naturally occurring inhibitors of influenza virus hemagglutination (Springer *et al.*, 1969). Such a relationship was also demonstrated artificially with serum orosomucoid, which in the native state is a weak inhibitor. Polymers, produced by heating under controlled conditions, had molecular weights greater than 200,000 and on a weight basis were found to be much more potent inhibitors than the monomeric form (Whitehead and Winzler, 1968). Inhibitory activity was lost after treatment with *V. cholerae* neuraminidase. Concordant results were obtained with orosomucoid polymerized by cross-linking with acetaldehyde (Morawiecki and Lisowska, 1965). A polymer of molecular weight in excess of 800,000 showed activity approximately 600-fold greater than that of the monomer. A filamentous form of heat-polymerized orosomucoid was found to be more active against certain strains of influenza virus than a globular form of the same macromolecule (Barclay *et al.*, 1969). This observation suggested that multiple reactive sites on the viral particle might be more reactive with sialic residues in a linear disposition than on a convoluted molecule, thus accounting for the greater inhibitory efficiency of the "chain" polymer over that of the "ball." However, hemagglutination by viral subunits was equally sensitive to inhibition by the two morphological forms of orosomucoid. Therefore, it seems that factors other than simply the spatial configuration of NANA residues must be essential for interaction with viral hemagglutinin.

A heat-labile inhibitor, unrelated to antibody or complement, is found in many animal sera (C. M. Chu, 1951). This factor (β inhibitor) is not inactivated by periodate or neuraminidase, either of bacterial or viral origin; it requires calcium ions for activity and is inactivated by trypsin. These properties suggest that the antiviral activity involves peptide rather than glycosidic linkages, and by analogy, that erythrocyte receptors for influenza virus may exist which do not involve terminal NANA residues. The β inhibitor seems to have greater activity against A_1 than against B strains of influenza virus (Hoyle, 1968).

A third type of nonspecific inhibitor (gamma inhibitor) is present in some normal animal sera, particularly those of horses and guinea pigs. The horse serum inhibitor is a glycoprotein distinct from antibody and is highly active against A_2 strains of influenza virus, with little activity against A_1 or B strains. It is stable to heat and neutralizes hemagglutinin as well as infectivity *in ovo*. This neutralizing effect was ascribed to the unusual avidity of some strains of virus to complex

with receptor groupings on the glycoprotein. The firmness of the inhibitor-virus union may also have been due to incomplete receptor destruction by viral enzyme and consequent failure of virus and inhibitors to dissociate. Fractionation and structural studies (Levinson *et al.*, 1969) of horse serum inhibitor showed that it comprised two active substituents in the polysaccharide moiety, namely 4-*O*-acetyl-NANA as well as NANA. The former represented the grouping active against A_2 influenza virus, the latter the residue reactive with a recent B isolate. In accord with this interpretation was the fact that the A_2 inhibitor was sensitive to viral neuraminidase only after treatment with mild alkali. In addition, metaperiodate treatment enhanced the inhibitory activity against the B virus. Since this type of enhancement had not been encountered in strains of influenza virus isolated prior to 1965, it was postulated that this biological change was related to natural antigenic modifications emerging at the same time. These findings suggested that the structure of NANA itself might play a determining role in the inhibition of A_2 viral hemagglutinin.

Strains of influenza were obtained from isolates of A_2 virus in the 1957 pandemic which were distinguished by either sensitivity ("+") or insensitivity ("—") to horse serum (gamma) inhibitor. Reactivity with antibody paralleled receptor destruction in the serum inhibitor and on erythrocytes (Choppin and Tamm, 1960a). The avidity of the "+" strains for glycoprotein substrates was explained by the failure of substrate hydrolysis and hence failure of virus to be liberated from the complex (Choppin and Tamm, 1960b). A parallel observation was reported which indicated that avid strains were still able to agglutinate fowl cells after removal of receptors for other influenza viruses by treatment with RDE, either bacterial or viral. Moreover, the avid A_2 strain eluted incompletely from neuraminidase-treated erythrocytes. These findings were taken to mean that avid strain receptors, uncovered by neuraminidase or periodate treatment, differed qualitatively from those for nonavid strains of influenza virus (Takatsy *et al.*, 1959).

It has been assumed that the combination of influenza viruses with glycoproteins from various sources depends on the same forces and molecular requirements that are responsible for viral attachment to glycoprotein receptors on the surface of erythrocytes. As described in an earlier section, VRS, or soluble virus receptor substance from human erythrocytes has the oligosaccharide residues required for viral attachment. Moreover, all the NANA on the erythrocyte is terminally bound and can be cleaved by neuraminidase, both bacterial and viral. The findings cited above with respect to avid strains of A_2 virus suggest the presence on erythrocytes of determinant inhibitory groupings which

do not include NANA; but this has not been substantiated. On the other hand, the presence of differently substituted neuraminic acids has not been entirely excluded. It is of some importance that the sialopeptide split from erythrocytes by the action of trypsin is itself a poor inhibitor of hemagglutination. The parent material, however, is recognized as a macromolecule of 31,000 monomeric molecular weight. It is itself a potent inhibitor and increases in potency with polymerization (Springer *et al.*, 1969).

There is some evidence showing that after removal of sialopeptide by trypsin, resulting in complete removal of NANA, erythrocytes are still capable of hemagglutination by influenza virus (Winzler, 1969b). Hence, other portions of the peptide backbone may be involved in this interaction, including the proximal portions of oligosaccharide side chains. Other soluble sialic acid-containing fractions of erythrocyte membranes, whether derived by butanol or by pyridine extraction (Table I) are several orders of magnitude less potent than VRS as viral inhibitors. This may be explainable by the fact that these fractions are mixtures of active less aggregated glycoprotein with inactive proteins. There is no certainty that the frequency or distribution of the necessary receptor configurations are the same in these sialoprotein fractions as in the glycoprotein purified by the phenol method.

Adsorption of virus to erythrocytes in suspension causes their aggregation into a fragile lattice which, on settling, forms the characteristic agglutination pattern at the bottom of the tube or titration well plate. The visible reaction is followed by elution of intact viral particles when the reaction mixture is brought from 4°C to 37°C at pH 7.2. Elution of influenza virus was initially recognized as being enzymatic in nature (Hirst, 1942) and is now known to be due to the action of neuraminidase in the viral envelope. While the completeness with which influenza viral particles elute from the erythrocyte surface may vary from strain to strain, the basic mechanism can be considered the same in all cases. Elution of the viral particle is accompanied by release of NANA from the receptor site. Similarly, in the interaction with soluble analogs, viral neuraminidase effects a degree of dissociation which varies with the strain, as intimated above. Structural studies with orosomucoid have indicated that the prosthetic groupings necessary for interaction with influenza virus include oligosaccharide side chains, linked *O*-glycosidically to serine and threonine (Gottschalk, 1965). NANA occupies a terminal nonreducing position in α-ketosidic linkage to *N*-acetylgalactosamine; it is at this latter bond that viral neuraminidase acts to free NANA from glycoprotein inhibitors (Graham and Gottschalk, 1960; Carubelli *et al.*, 1965; Gottschalk, 1966a). The polyhydroxy side chain

of NANA must be intact for maximum activity of the enzyme to take place (Suttajit and Winzler, 1971). In general, the completeness of elution from the erythrocytes appears to be inversely proportional to the avidity for glycoprotein inhibitors, as exemplified by the reaction of "+" strains of A_2 influenza virus with the gamma inhibitor of horse serum, cited earlier. In studies with low molecular weight oligosaccharides as substrates for neuraminidase, the enzyme from *V. cholerae* (the classical RDE) was shown to hydrolyze both 2-6′ and 2-3′ ketosidic linkages, whereas the viral enzyme attached only to the 2-3′ bond. Although the 2-6′ compounds were able to bind the viral enzyme, the latter acted only slightly, if at all, in releasing NANA (Drzeniek, 1967). This suggested that enzyme itself may have some role, albeit limited, in binding the viral particle to cell receptor material. Probably both 2-3′ and 2-6′ linkages exist in the erythrocyte, as deduced from studies of an alkali labile tetrasaccharide obtained by borohydride reduction of stromal sialopeptide. The latter was derived by trypsin treatment of either virus receptor substance (VRS) or erythrocytes (Table I) (Winzler, 1969b).

It was demonstrated that after adsorption and elution of influenza virus, the electrophoretic mobility of erythrocytes was greatly reduced, in concert with their resistance to reagglutination by freshly added virus (Hanig, 1948; Stone and Ada, 1952; Cook *et al.*, 1961). Treatment with RDE of *V. cholerae*, with trypsin or with periodate achieved the same results. These early observations, subsequently confirmed and extended, are readily explained in each instance by the removal of sialic acid residues, whether as NANA alone or as part of a sialopeptide fragment. The predominantly negative charge of the erythrocyte at physiological pH can be ascribed entirely to ionized free COO^- groups of sialic acid. Similarly, the electrophoretic mobility of purified VRS from human RBC was greatly reduced following reaction with influenza virus and removal of sialic acid (Howe *et al.*, 1963). Besides changes in electrophoretic mobility, removal of NANA residues from human erythrocytes by either viral or bacterial neuraminidase effects striking changes in the immunological reactivities of the cell. These concern several specificities: (1) the M and N blood group antigens which are inactivated by myxoviruses acting either on whole cells or on purified erythrocyte glycoproteins bearing these determinants; (2) Forssman heterophile antigens, including those associated with infectious mononucleosis; and (3) the specificities related to broadly reactive serum "panagglutinin" and phytohemagglutinins. The subject of antigenic alteration of erythrocytes by myxoviruses and soluble enzymes has been reviewed elsewhere (Springer, 1963; Drzeniek, 1970.

B. Paramyxoviruses

The paramyxovirus group, now set apart from the orthomyxoviruses (influenza viruses), includes the viruses of mumps and Newcastle disease and the parainfluenza subgroup which comprises 5 members. In contrast to the influenza viruses, paramyxoviruses are capable of lysing erythrocytes of several species, and of fusing cells in culture to form polykaryons. Neither hemolysis nor cell fusion caused by exogenously applied virus bore any relation to the virulence of several strains of NDV (Kohn and Fuchs, 1969). In contrast, polykaryocytosis induced during active infection in chick embryo fibroblasts (endogenous fusion) was correlated with the virulence of several strains of NDV for chicken embryos (Reeve and Alexander, 1970). Moreover, the fusing capacity of avirulent strains was enhanced by blockade of cellular RNA synthesis. Inhibition of protein synthesis with *p*-fluorophenylalanine or cyclohexamide resulted in parallel inhibition of fusion by virulent strains. These findings suggested that protein synthesis was required for fusion to occur and that the cytopathology induced by avirulent strains was related to failure of viral maturation and release and to the intracellular accumulation of viral products (Reeve and Poste, 1971). The molecular basis for cell fusion, whether exogenous or endogenous is still largely unknown, however. The same is true for viral hemolysis.

The paramyxoviruses hemagglutinate the erythrocytes of avian as well as a number of mammalian species. As with the influenza group, the viral hemagglutinin is situated on the spikes of the viral envelope in close association with viral neuraminidase. Hemagglutination occurs with strains of paramyxoviruses over a wide range of pH (5–9), optimally at about 7 (Tischer, 1963), and presumably by much the same mechanism as with influenza virus. The same receptors on the erythrocyte are involved, as proved by the capacity of influenza viruses to inactivate receptors for paramyxoviruses, and vice versa. Trypsin and *V. cholerae* enzyme are equally effective in this regard, receptor destruction in all instances being associated with the release of diffusible NANA. While most strains of paramyxoviruses, particularly those adapted to laboratory passage, are relatively insensitive to the alpha (Francis type) hemagglutination inhibitor, they are nonetheless capable of splitting NANA from receptor analogs as well as from purified VRS (Howe and Morgan, 1969). One exception appears to be purified ovine α-1-glycoprotein which has been reported to inhibit NDV hemagglutination to high titer independently of viral enzyme activity (Campbell *et al.*, 1967). Paramyxoviruses elute from the erythrocyte surface with lower efficiency than influenza viruses. Those viruses which are not actively hemolytic

can be shown to elute at neutrality. Those which hemolyze at temperatures above 4°C (NDV, mumps, Sendai) can be adsorbed to formalinized fowl erythrocytes at 4°C and eluted at 37°C (Kis.l'ov *et al.*, 1969). Eluted virus may show a relative increase in hemagglutinin titer but a decrease in infectivity, both effects being due to disruption and fragmentation of viral particles (John and Fulginiti, 1968).

Viral hemolysis was first described for mumps virus (Morgan *et al.*, 1948; L. Chu and Morgan, 1950) and then for NDV (Kilham, 1949); and somewhat more recently for Sendai virus (Sato, 1958) and type 3 parainfluenza virus (Hermodsson *et al.*, 1961). The exact mechanism which mediates viral hemolysis remains to be elucidated, however. Several facts have been established which throw some light on this phenomenon. For hemolysis to occur, intact viral particles or large subunits were required as shown with both Sendai and NDV (Sagik and Levine, 1957; Sokol *et al.*, 1961). Heterogeneity of NDV particles with respect to hemolytic activity was demonstrated (Sokol, 1963). Not only was "incomplete" Sendai virus less hemolytic than complete virus (Sokol *et* al., 1964), but it was shown that sonication of infective virus progressively inactivated hemolysis which was ultimately eliminated when hemagglutinating particles were reduced below a certain size range (Hosaka, 1970). This was in accord with the earlier finding that subunits of Sendai virus obtained by ether disruption of the viral envelope were nonhemolytic (Sokol *et al.*, 1964). With respect to the cell surface, it is clear that the same receptors are required for hemolysis as for hemagglutination, since the virus must attach in order to hemolyze. This was originally shown for mumps virus (L. Chu and Morgan, 1950) and has since been demonstrated for Sendai virus (Sokol and Neurath, 1962). Accordingly, antibody to purified virus receptor substance prevents the agglutination as well as the lysis of human group O erythrocytes by Sendai virus. The hemolytic property is inversely proportional to the capacity of a given strain to elute from the red cell surface, as has been demonstrated with NDV (Sagik and Levine, 1957). These observations suggested that the degree of contact, i.e., the resultant of particle size and its "avidity" for cell receptors, might be of critical importance. Electron microscopic examination of erythrocyte ghosts lysed by Sendai virus gave a clue as to the nature of this contact. Concentrated Sendai virus, when adsorbed to erythrocytes at 4°C, caused invaginations in the plasma membrane. After elevation of the temperature to 37°C, the plasma membrane became fused with viral envelope. Cell lysis followed and was accompanied by rapid and total loss of hemoglobin to the extracellular space and depletion of membrane NANA. Concomitantly, rupture of the virions occurred, with release of nucleocapsid (Howe and Morgan, 1969). It

appeared that the viral particles joining erythrocytes in the initial agglutination reaction were torn apart as a result of the sheering forces engendered by rapid cell lysis at 37°C. More recently, it was shown that fusion of human erythrocytes occurred if cells agglutinated by virus at 4°C were packed by light centrifugation and the undisturbed pellet then brought to 37°C. Large, stable polymembranes, retaining appreciable amounts of hemoglobin, were thus formed in which no viral envelope components were recognized on morphological grounds. However, free nucleocapsid filaments were seen in the cytoplasm of fused erythrocytes. By freeze-etching techniques, it was demonstrated that smooth, plaque-like areas had formed, through obliteration or redistribution of normally well-ordered and numerous intramembranous particles (Bächi and Howe, unpublished). This rearrangement of intramembranous particles as a result of viral action was reminiscent of the regrouping which was effected by proteolytic enzymes, and which constituted the basis for proposing that sialoglycoprotein receptors exposed at the cell surface are associated with the intramembranous particles (Marchesi *et al.*, 1971).

Hemagglutinin as well as hemolysin are trypsin sensitive, suggesting that peptide residues on the viral envelope are involved (Neurath, 1963, 1964c; Fontanges *et al.*, 1964). In addition, the hemolytic property of Sendai virus may be host dependent for its expression, since virus grown in mouse lung cell cultures was nonhemolytic whereas the same virus from chick embryos was strongly hemolytic (Ishida and Homma, 1960; Matsumoto and Maeno, 1962).

In sum, it appears that with Sendai virus, as probably also with the other paramyxoviruses, red cell lysis is a complex interaction between intact virion, or its larger subunits, and the cell surface. Those factors which are known to be operative are the requirement for firm adsorption of hemagglutinin to sialoprotein cell receptors, readily effected at 4°C, and a sufficient area of contact between intact virion or larger envelope fragments and the cell receptor sites. Temperature and pH dependence suggest that some form of enzymatic activity is involved in effecting the initial breach in the integrity of the cell membrane. This results either in lysis of individual cells or, if virus-coated cells remain in close apposition to one another, in fusion and relatively restricted loss of hemoglobin. In the latter instance, it must be assumed that the increased permeability of the membrane is transient, its duration and extent limiting the egress of hemoglobin. Except for porcine kidney cells (Hosaka, 1970) the chicken embryo is the only susceptible host which yields hemolytic virus. The possibility has been considered that host-derived lysosomal enzymes or other factors (Neurath, 1963, 1964b), such as lysoleci-

thin (Rebel *et al.*, 1962), adsorbed or otherwise attached to the virus *in ovo*, may somehow contribute to its hemolytic activity. Definitive confirmation of this possibility is lacking. Likewise, the relationship of a "hemolysin" reported to be present in normal chorioallantoic membrane to the hemolytic action of Sendai virus is not clear (Neurath, 1964a).

The hemadsorption phenomenon is accounted for by viral antigen(s), particularly hemagglutinin, present on the surface of infected cells before and during the release of virions by budding from the membrane. As shown by electron microscopic studies using ferritin-labeled antibody, maturation and release of paramyxovirus particles was accompanied by the accumulation of ribonucleoprotein strands under segments of the membrane which had been altered to display antigenic specificity identical to that of the viral envelope (Howe *et al.*, 1967). Similarly, viral antigens replaced normal cell membrane antigens in the course of influenza virus infection (Duc-Nguyen *et al.*, 1966). The presence of viral hemagglutinin at altered membrane sites was demonstrated by electron microscopic examination of mumps virus hemadsorption which took place at the surface of infected cells even before the maturation and release of virions (Duc-Nguyen, 1968). The presence of neuraminidase has also been demonstrated in monolayers as early as 6 hours after infection with influenza virus and before release of progeny virus. Viral enzyme activity was associated with membranes as well as with cell sap released by freezing and thawing (Kendal and Apostolov, 1970).

C. Pseudomyxoviruses

The pseudomyxovirus group comprises the agents of measles (rubeola), canine distemper, and rinderpest (the medipest group) (Imagawa, 1968), and, with some reservations, respiratory syncytial (RS) virus. This group resembles the large paramyxoviruses morphologically and in certain biological properties. The complete virion, approximately the same size as the paramyxoviruses, contains RNA in helical symmetry. It is enveloped and has the projections characteristic of myxoviruses. The envelope, being ether sensitive, is thereby assumed to have essential lipid. The differences from the paramyxoviruses, however, are as striking and consistent as are the resemblances and concern important properties of the envelope. None of the pseudomyxoviruses possess neuraminidase, and measles is the only one which hemagglutinates. The extensive literature on the biology of measles virus has been well reviewed (Waterson, 1965).

The hemagglutinin of measles is a property of the viral envelope. "Soluble," noninfective hemagglutinating particles in cultures of measles virus are now recognized as being subunits, or fragments of envelope

which accumulate in cultures as cytopathology progresses. These have been referred to as "large hemagglutinin," infective and noninfective hemagglutinin, and small hemagglutinin, respectively (E. C. J. Norrby *et al.*, 1964). The large hemagglutinin has been characterized as either intact infective virus or virions which have undergone partial disorganization and hence are no longer infective, but retain the physical properties of the particle. The small hemagglutinin, being noninfective, has no RNP core and varies in density over a relatively wide range. A certain proportion of the small hemagglutinins are seen as high density particles with a characteristic rosette appearance. Treatment of virus with Tween 80 and ether (E. Norrby, 1962; Funahashi and Kitawaki, 1963) results in the conversion of most of the hemagglutinin to this latter form, at the same time substantially increasing the hemagglutinin titer of the preparation. Ether by itself disrupts the viral envelope with consequent reduction in hemagglutinin titer. The function of the Tween is to protect the smaller protein subunits from denaturation and hence to preserve their activity. Tween 80–ether-treated hemagglutinin is more sensitive to inhibition by antibody and therefore constitutes an improvement over crude culture fluids as a reagent for use in hemagglutination-inhibition tests. Treatment of virus with sodium deoxycholate (SDS) results in the disintegration of viral particles and allows better separation of nucleocapsid from the small rosette hemagglutinin than is achieved with Tween 80–ether treatment. For this reason, SDS would appear to be a satisfactory reagent for the separation of nonhemagglutinating, complement-fixing antigens (nucleocapsid) from envelope constituents (Norrby, 1966). A small hemagglutinin has also been described which was found to have a lower buoyant density than the others. This low density particle, lacking RNA, was capable of hemolysis and of causing an early CPE. The latter was in all probability the same phenomenon as the early, rapid fusion produced by the application of high titers of measles virus to cell monolayers (Cascardo and Karzon, 1965). The character of the cytopathology and of the viral progeny resulting from the infection of monolayers with measles virus has been found to vary with the multiplicity of infection. Virus passaged as undiluted culture fluid caused the formation of incomplete virus consisting of antigen with high hemagglutinating and hemolytic titers and relatively low infectivity; whereas virus passaged at high dilution resulted in production of virus with almost no hemagglutinin or hemolytic activity, but with high infectivity. (E. Norrby *et al.*, 1970).

The hemagglutinins discussed above, all referable to the virion or subunits directly derived from it, adsorb readily to erythrocytes in the presence of minimal concentrations of monovalent or polyvalent cations,

and none can be made to elute. It was found that the addition of salts such as ammonium sulfate to measles virus pools enhanced the hemagglutinating titer (Schluederberg, 1968). The degree of enhancement was dependent on the relative concentrations of particles which showed increased hemagglutination titers in high salt concentration and those which did not. Moreover, sonication of such preparations altered this ratio toward higher titers. Evidence suggested that the change was referable to the physical disintegration of viral envelope. Sonicated measles virus, in the presence of ammonium sulfate, was also more sensitive to inhibition by antibody than untreated virus. A class of particles with an average sedimentation coefficient of 22 S, and smaller than any of the hemagglutinins previously described, was demonstrated in extracts of cells infected with measles virus (Schluederberg and Nakamura, 1967). These particles in contrast to the "normal" hemagglutinin, including those enhanceable by hypertonic salt, were strictly dependent on the presence of polyvalent anions for both adsorption to and agglutination of erythrocytes. Their agglutinating activity was greater at pH 7.8 than at pH 5.6, and increased with elevation of temperature from 4°C to 37°C. Moreover, this salt-dependent agglutinin (SDA), after adsorption to erythrocytes in hypertonic solution, could be eluted into isotonic saline. Antigenic analysis revealed clearly the relation of SDA to measles infection, and the data suggested that the particle might represent a virus-specific envelope precursor, or envelope components not included in released viral progeny.

Trypsin inactivates the hemagglutinin, suggesting that protein is involved in the interaction with erythrocytes. The same virus-coded proteins at the surface of infected cells may be held to account for hemadsorption, which can be demonstrated in monolayers infected with measles virus. In limited electron microscopic observations of this phenomenon (Baker *et al.*, 1965), erythrocytes were found to be in closely contoured fit with infected cells and their microvilli, but intact viral particles were rarely recognized. Moreover, there were none of the morphological changes characteristic of cells infected with paramyxoviruses, in which segments of cell membrane were converted to an antigenic specificity identical with that of the viral envelope (Howe *et al.*, 1967). These findings suggest that the mechanism of measles virus hemadsorption, and hence of hemagglutination by free viral components, differs perhaps significantly from that of the paramyxoviruses.

Concordant with this last supposition, evidence has been presented to suggest that the erythrocyte receptor also differs from that for orthomyxoviruses. Treatment of erythrocytes with formalin inactivated receptors for measles virus, leaving intact those for influenza virus (Peries

and Chany, 1962). Neuraminidase, whether myxoviral or bacterial, failed to inactivate measles virus receptors. It is of some interest in this connection that purified VRS prepared from rhesus erythrocytes by the phenol method did not inhibit measles hemagglutination (Howe, unpublished results). In one study rhesus erythrocytes treated with *Vibrio cholerae* neuraminidase displayed a 4- to 16-fold enhancement in agglutinability by measles virus and by certain adenoviruses (q.v.). Chymotrypsin, on the other hand, reduced or abolished the capacity of rhesus erythrocytes to be agglutinated by these viruses (Tischer, 1967). Taken together these facts suggest that the receptor activity is associated primarily with the protein of the erythrocyte surface rather than with carbohydrate residues. Animal sera contain nonspecific measles hemagglutinin inhibitors, which are probably lipoprotein and can be removed by adsorption with kaolin or by treatment of the serum with heparin–$MnCl_2$ (Feldman, 1968).

The hemolytic activity of measles virus is clearly related not only to the viral envelope, but also to the size of the hemagglutinating particle. Presumably the same erythrocyte receptors are involved, since attachment by hemagglutinin is required for hemolysis, and the conditions of pH and temperature parallel those for hemagglutination. While infectivity is not a prerequisite for hemolysis, it is clear now that the size of the hemagglutinating particle is critical. Thus, intact virion, large noninfective hemagglutinin, and the small hemagglutinin of low density are all hemolytic; all three are also capable of rapid cell fusion (Waterson, 1965). Hemagglutinating units of smaller size are nonhemolytic, as is also the hemagglutinin derived by Tween 80–ether extraction of virus. There would thus seem to be a parallel, in the relation between particle size and hemolytic activity, with the hemolytic action of Sendai and Newcastle disease viruses. A further parallel is suggested by results of studies on the inactivation of measles virus hemagglutinin and hemolysin (Neurath and Norrby, 1965) by compounds reacting specifically with protein. The results were similar to those reported with Sendai virus hemagglutinin and hemolysin (Neurath, 1964c), pointing to the aromatic amino acids as having a critical role in the mediation of these activities by peptides of the viral envelope. As with the paramyxoviruses, the exact mechanism of measles virus hemolysis remains to be elucidated.

D. Adenoviruses

Adenoviruses are divisible into at least three major groups depending on their capacity to agglutinate the erythrocytes of several mammalian species (Rosen, 1960a). This signal discovery not only afforded an additional basis for classification, but also pointed to important biological

differences between the several groups. Some of these differences are now explainable in part by the recent elucidation of the structure and function of adenoviral capsid components originally recognized by their antigenic specificities (Pereira, 1960; Pereira and Figueiredo, 1962). Reference to the summary table (Table III) will show that division into these groups is based on "complete" or "partial" agglutination of rhesus, rat, or human erythrocytes. Each group can be subdivided further according to the reactivity with erythrocytes of additional species (mouse, guinea pig, and grivet) (Wadell, 1970). Various morphological subunits associated with hemagglutinating and other properties of the viral capsid have been identified and form the basis for the nomenclature which is now widely accepted (Ginsberg *et al.*, 1966). In the original designation, "complete" hemagglutination referred to a smooth, uniform pattern produced under appropriate conditions by a given strain. In group 3, the "partial" pattern produced by the direct action of the virus preparation is seen as a button in the midst of agglutinated cells. This pattern can be rendered "complete," that is uniform, by the addition of heterotypic antibody (Rosen, 1960a; Pereira and Figueiredo, 1962). Other strains, showing little or no hemagglutination, could be made to agglutinate by the same procedure. Both "complete" and "partial" forms of agglutination are inhibited by type-specific antibody. The principal mediator of hemagglutination is the fiber antigen on the intact virion as well as on the various subunits recoverable as "soluble" hemagglutinins in fractions of infected cells. From the functional standpoint, these subunits may be either complete (bi- or multivalent) or incomplete (monovalent) hemagglutinins. The complete hemagglutinins are the intact virion and several structural forms of soluble hemagglutinin, namely the dodecon (12 pentons with intact fibers) and dimers of either pentons or fibers. Single pentons or fibers are incomplete hemagglutinins which, although monovalent, are capable of adsorbing to the erythrocyte surface. This was shown by the capacity of fiber antigen to inhibit agglutination of rat or human cells by purified virions (Wadell, 1969). The incomplete hemagglutinins are rendered bivalent through combination with heterospecific antibody. The latter is directed either to the vertex capsomere of pentons, or to an antigen in the proximal portion of the fiber (Fig. 4). The antigenic complexity of the fiber antigen increases with length, from one serotype to another. It is perhaps significant that, in general, fiber antigens of group 3 viruses are the longest, and are the most amenable to interaction with heterotypic antibody in the formation of complete agglutinin (E. Norrby, 1969a). It also appears that the several classes of capsid subunits are produced in greatest abundance in cells infected with group 3 viruses. The action of so-called "receptor modifying factor"

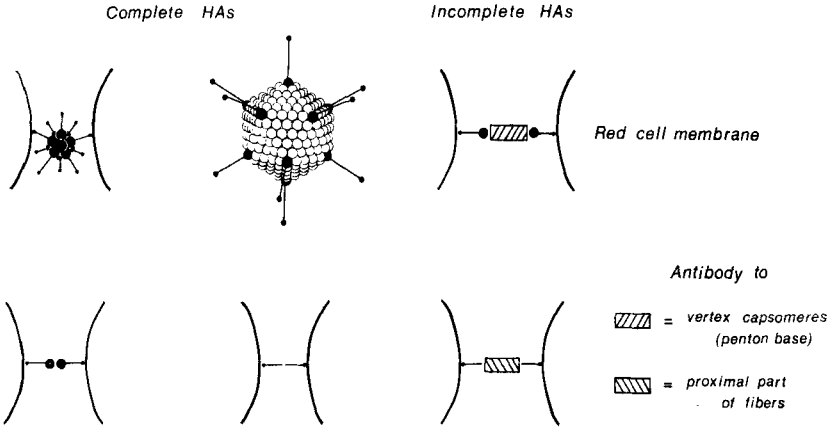


FIG. 4. Schematic representation of adenovirus hemagglutinins (HAs). Complete hemagglutinins: the intact virion, aggregates of complete (vertex) capsomeres, dodecons; dimerized pentons (vertex) capsomeres with fibers; dimerized fibers. Incomplete hemagglutinins: Monomeric pentons or fibers. Incomplete (monomeric) hemagglutinins on separate cells joined by heterotypic antibodies to penton base or to fiber. Redrawn from E. Norrby (1969a) by permission of the author and the publisher.

originally found in cell cultures of group 3 viruses (Kasel *et al.*, 1961; Kasel and Huber, 1964) can probably now be explained as competitive inhibition of the complete rat and human O cell agglutinins by fiber antigens elaborated by group 2 or group 3 viruses (Wadell, 1969). Type 4 viruses produce only the dodecon as a soluble hemagglutinin.

The evidence indicates that the distal knob on the adenovirus fiber is responsible for interaction of the virion or the soluble hemagglutinins with erythrocytes. The fiber knob is also the locus of attachment of hemagglutinin-inhibiting antibody. Nonterminal arginine residues on the viral capsid, presumably at this location on the fiber, and glutamic or aspartic carboxylic acid groups on the red cell membrane protein are required for the reversible interaction of adenovirus type 7 and erythrocytes (Neurath *et al.*, 1970). It is of some interest that treatment of human O cells with RDE from *Vibrio cholerae* as well as with influenza A2 virus reduced considerably the capacity of the cells to be agglutinated by type 9 adenoviruses. Likewise adenovirus 9 adsorbed to normal human O erythrocytes could be eluted with RDE. Apparently type 9 is unique among the adenoviruses thus far studied for this characteristic which suggests that the corresponding receptors differ from those for other types (Wadell, 1969).

Adenoviruses classified in group 2 give complete agglutination of rat

erythrocytes. Certain group 2 viruses, however, also agglutinate rhesus cells. Further investigation of several of these (types 9, 13, 15) showed that a portion of the monkey cell hemagglutinin was associated with the virion and, for types 9 and 15, could not be separated from the respective agglutinins for rat cells. On the other hand, the soluble rat cell agglutinins of types 9 and 15 were separable from one another on the basis of buoyant density and their differing affinities for monkey cells (Table II) (Wigand and Stöhr, 1965). In addition, monkey cell agglutinins were more resistant than were rat cell agglutinins to trypsin followed by heating to 56°C, a further indication of the diversity in physical and/or chemical configuration of the hemagglutinins. This diversity is reflected also in varying affinities for type-specific antibody.

Human and animal sera contain nonspecific inhibitors of hemagglutination occasionally in titer sufficient to interfere with detection of specific antibody (Rosen, 1960a; Schmidt *et al.*, 1966). These nonspecific inhibitors may be removed by adsorption to kaolin or inactivated with *Pseudomonas* filtrate (Schmidt *et al.*, 1964c). The latter treatment was thought to remove less antibody than kaolin, a particularly important consideration with sera containing low antibody titers. The same result may be achieved by adsorption with heparin–$MnCl_2$ (Mann *et al.*, 1967). What appears to be a different type of inhibitor for adenovirus type 5 was found in the α-1-globulin fraction of serum and, while also adsorbable to kaolin, had the characteristics of mucopolysaccharide with a molecular weight between that of orosomucoid (44,000) and albumin (69,000). Electron microscopic evidence suggested that, in flocculating whole viral

TABLE II
SUBDIVISION OF GROUP 2 ADENOVIRUSES[a]

RBC	Viral HA (serotype)	Soluble HA	
		High density	Low density
Monkey	9+	+	−
	13, 15+	+	−
Rat	9+	+[b]	+
	15+	+[b]	−
	13−[c]	+	−

[a] Data from Wigand and Stöhr (1965).

[b] Soluble HA (types 9, 15) solely for rat cells obtainable by absorption with monkey cells.

[c] Type 13 HA (viral and soluble) solely for monkey cells obtainable by absorption with rat cells.

particles with purified inhibitor, the latter attached directly to the hemagglutinating sites on the virion (Shortridge and Biddle, 1968).

Suggestive evidence as to the chemical nature of adenovirus hemagglutinin receptors has come from a direct analysis of rhesus erythrocyte ghosts (Neurath *et al.*, 1969). It was shown that phenol, *n*-butanol, and potassium periodate treatment of membranes rendered them incapable of inhibitory activity against adenovirus type 7 whereas RDE had no effect, although eliminating receptors for influenza virus. Inactivation of adenovirus 7 receptors was effected by treatment with trypsin, chymotrypsin, papain, pronase, and ficin. These findings were taken as evidence for the proteinaceous nature of the receptors which were considered not to involve glycoproteins.

E. Arboviruses

Since the original description of hemagglutination with Japanese B encephalitis virus (Sabin and Buescher, 1950), this property has been found to be associated with many, but not all of the more than 200 arboviruses now recognized. Certain arboviruses of groups A and B, in cell culture, are detectable by hemadsorption (Buckley, 1959). Hemagglutination and hemagglutination inhibition form the basis for the grouping of these viruses (Casals and Brown, 1954; Clarke and Casals, 1958), in the assay for which goose cells are used in preference to newly hatched chick cells. Methods used to prepare hemagglutinating antigens have been refined in recent years although they are still based on relatively empirical observations. The source of antigen is most often suckling mouse brain or, as in group C strains, mouse sera (Ardoin *et al.*, 1969; Casals and Whitman, 1961). The basic procedure involves the extraction of homogenized infected brain tissue with acetone in the presence of sucrose, the function of the latter being to protect the virus from the action of acetone. Acetone presumably separates viral particles from normal brain lipids which interfere with the attachment of hemagglutinin to erythrocytes. Aqueous extracts of brain tissue usually have lower hemagglutination titers due to the presence of these lipids. A recent study (Pedreira *et al.*, 1969), endeavoring to compare the utility of several preparative methods, demonstrated that simple genetron extraction of suckling mouse brain yielded antigens comparable to those derived by sucrose-acetone extraction. The hemagglutinating titer of group C viruses is enhanced by sonification and in certain instances by trypsin treatment (Ardoin *et al.*, 1969). Final antigen preparations, though partially inactivated, still retain a substantial measure of infectivity and hence must be handled with due precautions.

The conditions under which hemagglutination with arboviruses is per-

formed are critical. Thus, for most of the group A viruses the optimal temperature is 37°C, the optimal pH 6.4; the corresponding values for members of group B are 4–22°C and pH 7 (Casals and Brown, 1954). Spontaneous elution by certain group A viruses may be shown to occur outside the pH range optimal for hemagglutination (Salminen, 1960). With elution, there is no detectable alteration of the erythrocyte surface since erythrocytes from which virus has eluted are fully capable of absorbing more virus of the same or other types. RDE treatment of red cells fails to affect their agglutinability by certain arboviruses (Eastern and Western equine encephalomyelitis) (Karabatsos, 1963) thus indicating that receptors do not involve NANA. A difference in the character and/or disposition of group A and B receptors was suggested by the finding that trypsin, chymotrypsin, and papain inactivated group B viruses and not group A viruses with respect to both hemagglutinin and infectivity. The evidence indicated that the hemagglutinin of group B virus includes a protein on the viral capsid (Cheng, 1958).

A number of investigations have been directed toward establishing some analogy between lipids or lipoproteins on the one hand and, on the other, the "receptor" substance present on the erythrocyte surface. Thus, serum lipoproteins can be shown to have an inhibitory effect on arbovirus hemagglutination and their removal by absorption to kaolin or other means is often necessary in order to discover the presence of antibody (Clarke and Casals, 1958). A fraction extractable from ox brain and rich in phosphotidylinositol has also been shown to bind hemagglutinating arboviruses preferentially and thus to inhibit hemagglutination (Frisch-Niggemeyer, 1967). This led to the inference that the receptors on the erythrocytes were or included lipid. Suggestive evidence has also been adduced from the direct assessment of hemagglutination inhibition by human red cell phospholipid fractions with selected arboviruses of groups A and B (Porterfield and Rowe, 1960). The B viruses were inhibited by lipoproteins from either serum or erythrocytes; the single group A virus tested was not. A more recent attempt to explore the chemical nature of erythrocyte receptors for representative group A, B, and D viruses involved the assay, in hemagglutination inhibition tests, of crude goose red cell stromata which had been extracted with aqueous ether. The inhibitory material, as finally used, contained no detectable sialic acid, only traces of hexose, but substantial phospholipid and protein (Nicoli, 1965).

The evidence suggesting the similarity of these various lipid inhibitors to the actual receptor sites on erythrocytes is undermined by several considerations. Phosphotidylinositol is not present in the red cell membrane; the anionic character of erythrocyte surface can be ascribed

wholly to terminally bound NANA. An extensive study undertaken with mixtures of standard lipids showed that suitable steric arrangements were necessary in aqueous solution for interaction with arboviruses to take place (Gorman, 1970). This admits at least of the possibility of lipid–lipid interactions but does not justify the assumption that cell receptors are lipid or even that they require lecithin or cholesterol for interaction with viral hemagglutinin. The chemical nature of receptors for arboviruses thus remains obscure, although the most attractive possibility still remains that hemagglutination is a protein–protein interaction between viral capsid and membrane. The involvement of membrane polysaccharide is uncertain.

F. Reoviruses

The reoviruses, originally designated ECHO 10 viruses, were separated as a group on the basis of their larger size and their characteristic cytopathic effect, which differed from that of the enteroviruses. Reoviruses are found in many species besides man; but all, regardless of species, can be separated into three distinct serotypes based on hemagglutination inhibition reactions (Rosen, 1960b). Human erythrocytes are agglutinated by all three. Type 3, however, also reacts with bovine erythrocytes to somewhat higher titer. The agglutinin is neither strictly pH nor temperature dependent, taking place equally well at 4°C, 23°C, and in the pH range 6 to 8. Elution is variable and nonenzymatic in nature. The human erythrocyte receptors for reovirus are unaffected by *Vibrio cholerae* neuraminidase. However, bovine erythrocyte receptors for type 3 are inactivated by this enzyme (Eggers *et al.*, 1962; Gomatos and Tamm, 1962) suggesting that there may be a difference between receptors for type 3 and those for types 1 and 2 which is related to the presence of NANA residues. Trypsin reduces agglutinability of human O erythrocytes by all three types (Lerner *et al.*, 1963). Since human erythrocytes bearing the determinants of blood group A (i.e., cells of groups A or AB) are more reactive with all three serotypes than are cells of group O or B (Brubaker *et al.*, 1964), blood group substances may be implicated in the reovirus receptor. However, it appears that the main reactive site on the red cell is a protein sensitive to trypsin and chymotrypsin but insensitive to certain carbohydrate-splitting enzymes and to borohydride (Lerner and Miranda, 1968). This would minimize the role of polysaccharide components.

The demonstrated sensitivity to trypsin and chymotrypsin indicated that an essential peptide linkage with aromatic amino acids is involved in the receptor activity. On the other hand, preincubation of type 2 reoviruses with sodium borohydride, as well as with carbohydrases (par-

ticularly beta glucosaminidase) substantially reduced their agglutinating potency. These findings suggested that polysaccharide residues on the virion might be essential to the hemagglutinating action. *N*-Acetylglucosamine has been reported to be inhibitory for agglutination of human O cells by all three types of reovirus (Gelb and Lerner, 1965). The inhibition was proved to be due to attachment of the sugar to the virion rather than to combination with the surface of the erythrocyte. This was interpreted to suggest that *N*-acetylglucosamine on the red cell surface might be involved in the union with capsid glycoprotein. However, no oligosaccharide residues have as yet been definitively recognized as forming a part of the reoviral capsid.

Hemagglutination is a property of both complete and incomplete, or coreless particles (Papadimitriou, 1966), for which —SH groups on the virion are essential. This conclusion was based on the finding that treatment of virus (types 2 and 3) with *p*-choloromercuribenzoate (PCMB) inactivated hemagglutinin and infectivity, the former being reactivable with thiol compounds, e.g., reduced glutathione (GSH) (Gomatos and Tamm, 1962). The removal of the capsid layer by proteolytic enzymes resulted in an increase in infectivity of all three types of reovirus. Hemagglutinating activity was retained by the enzyme-treated virus. Electron microscopic examination of these capsidless particles revealed the presence of spikelike components which protruded from the subcapsid layer and were thought to contain the hemagglutinin (Spendlove *et al.*, 1970). The exact nature of the bond between viral hemagglutinin and receptor on the erythrocytes remains unidentified, however. All that can be said is that —SH groups on the virion are necessary for the reaction and that one kind of receptor for type 3 may be glycoprotein. For the other two types, receptors are protein without essential —SH groups since pretreatment of erythrocyte with PCMB has no effect on hemagglutination with these viruses.

Nonspecific hemagglutination inhibitors show some characteristics which parallel those of erythrocyte receptors. Thus type 3 reovirus was found to bind to ovomucin or to inhibitors present in various animal sera, all of which were inactivated by RDE (Gomatos and Tamm, 1962). Likewise a salivary mucin was shown to have the same binding properties (Lerner *et al.*, 1966a) and to be inactivated by RDE. These findings further substantiated the affinity of reoviruses for glycoproteins but, except by analogy, gave relatively little direct information regarding the chemical nature of the erythrocyte receptor groupings. Nonspecific serum inhibitors of reovirus hemagglutination could be absorbed by treatment of the serum with kaolin or with rivanol (Styk *et al.*, 1968). These two reagents, however, removed substantial amounts of immuno-

globulin as well. Treatment of antisera with heparin–$MnCl_2$ resulted in only negligible loss of antibody while removing most of the nonspecific inhibitors. Inhibitory activity for the 3 serotypes was found in the β-lipoprotein fraction in addition to the mucoprotein inhibitor for type 3 (Mann *et al.*, 1967). This was consistent with the previous finding that nonspecific inhibition could be inactivated by phospholipase C (Schmidt *et al.*, 1964c) as well by lecithinase and proteases (Schmidt *et al.*, 1964b).

G. Enteroviruses

Among the picornaviruses it is predominantly the ECHO group and Coxsackie B3 virus which have been shown to hemagglutinate human O erythrocytes (Goldfield *et al.*, 1957). While hemagglutination appears to be a common property of many serotypes, all the strains of a given type may not hemagglutinate. However, with those strains that do hemagglutinate, the reaction can be inhibited with type-specific antibody. The optimum pH varies from strain to strain, as does also the optimum temperature. Indeed, on the basis of the latter variation, it is possible to group certain serotypes depending on their maximal activity at 4°C or 37°C with adult or newborn human erythrocytes (Kern and Rosen, 1964). To this extent, temperature dependence is evident with some strains. Some, but not all, of the ECHO viruses elute from the erythrocyte surface. Those which elute leave the red cell surface still capable of adsorbing fresh virus. The reaction can be considered, on this basis, to be nonenzymatic in nature (Podoplekin, 1964). In dissecting the hemagglutination reaction, some inferences may be made as to the nature of the responsible viral and cell receptor components, respectively. The hemagglutinating property resides in the capsid protein, since both complete and incomplete viral particles are antigenically identical and hemagglutinate, as shown with ECHO virus type 12. The incomplete particle, however, was found to be less stable to heat and ether treatment, from which finding the importance of the RNA core in maintaining icosohedral stability was inferred. Fragments of coreless particles, however, were without detectable hemagglutinating activity, suggesting a lower size limit for the manifestation of hemagglutination (Halperen *et al.*, 1964). While the thermal lability of different ECHO virus hemagglutinins varied in degree, it was shown in each case to parallel closely the inactivation of infectivity. The patterns of cationic stabilization for both hemagglutinin and infectivity were identical for those strains tested (Podoplekin and Ivanova, 1966). Moreover, the rate at which infectivity was inactivated was more rapid for whole virus than for the corresponding soluble RNA. These observations substantiated the hypothesis that stabilization by mono- and divalent cations involved primarily the capsid.

Further evidence as to the chemical nature of the viral component responsible for the hemagglutination reaction has been derived from studying the effects of various specific carbohydrases and proteases which reduce or eliminate the hemagglutinin. Complementary evidence was adduced by showing that several aldoses were capable of binding to the erythrocyte surface, thereby rendering the cells inagglutinable by ECHO viruses. The saccharides did not attach to viral particles. A relation was apparent between the number of carbon atoms in the aldose chains and their inhibitory potency (Lerner *et al.*, 1966b). The results suggested that hemagglutination may be effected by oligosaccharide residues in the viral capsid (Lerner *et al.*, 1965). As with the reoviruses, however, the validity of these explanations must await the direct demonstration of oligosaccharides in the viral capsid.

The role of —SH groups in the capsid has also been examined. Certain sulfhydryl reagents, notably PCMB, blocked the hemagglutinating activity of a number of enteroviruses (ECHO 7, 11, 12, 19 and Coxsackie B3) for human O cells. This effect was reversible by thiol compounds (Philipson and Choppin, 1960). With alkylating agents (e.g., iodoacetamide) inactivation of viral hemagglutination was greater at pH 9 than at pH 5. It seemed likely these reactions were taking place with dissociated mercaptides rather than with undissociated —SH groups. Since it is known that dissociation increases with pH, this would explain the parallel increase in inactivation with iodoacetamide. The inactivation with iodoacetamide, in contrast to PCMB, was irreversible; however, both reagents could be shown by blocking experiments to act at the same site on the viral particle. In contrast, the same sulfhydryl and alkylating reagents had no effect on the erythrocyte surface with respect to agglutinability by enteroviruses. Treatment with chymotrypsin or papain resulted in loss of agglutinability by ECHO viruses (Lerner and Miranda, 1968; Philipson, 1959). In addition, chymotrypsin altered both erythrocytes and erythrocyte membranes in such a manner that no virus was adsorbed, indicating further that adsorption and hemagglutinating activities involved the same protein(s) of the erythrocyte membrane. Receptors for ECHO 7, 11, and 19 and for Coxsackie B3 viruses were identical with one another (Philipson and Bengtsson, 1962).

Study of the dynamics of virus–erythrocyte receptor interaction revealed two stages in the attachment of enteroviruses to cells or membranes. The first was adsorption, which for noneluting enteroviruses (ECHO 7 and Coxsackie B3) was relatively independent of temperature. These noneluting viruses could be dissociated from erythrocytes by chymotrypsin at 37°C. This was followed in time by a second stage, characterized by resistance to chymotrypsin. Other strains could be

shown to adsorb at 4°C and elute spontaneously at 37°C. The time required for elution in the latter instance was the same as that required for the noneluting viruses to become resistant to dissociation from their receptors by chymotrypsin, i.e., attain the second stage. The pH optimum for adsorption to membranes was 5.5. Resistance of the receptor-virus complex to dissociation by chymotrypsin increased with pH above neutrality, and was maximal at pH 9, the pH optimum of chymotrypsin being 7.8. There were some parallels in this erythrocyte–virus system with the first stages of infection of a susceptible host cell, to which virus attached reversibly at first, with time showing a progressive resistance to dissociation from the receptor complex with chymotrypsin. This resistance was temperature dependent (Philipson and Bengtsson, 1962). Heat lability and temperature dependence indicated that this second stage might be an enzyme-mediated reaction, with both host cells and erythrocytes.

Attempts have been made to characterize the chemical nature of the erythrocyte receptor material and to determine the mechanism of interaction with enteroviruses (Philipson *et al.*, 1964). Crude membranes, prepared by freeze-thaw hemolysis, were treated with *n*-butanol in the presence of 2.5 *M* $CaCl_2$. Soluble inhibitory material was found in the aqueous portion of the mixture. This soluble material could be purified further by passage through Sephadex G-200. After removal of lipoprotein by precipitation with heparin and $MnCl_2$, the inhibitory material, eluting with the void volume, was assumed to have a molecular weight greater than 200,000. Analysis showed the presence of 60% protein, 31% lipid, and 9% carbohydrate. The major portion of the latter appeared to be bound to glycolipid. However, after lipid extraction of the soluble inhibitor, approximately 0.1% protein-bound NANA was found. A deoxynucleotide was also found in the receptor material. Double-stranded DNA was subsequently identified in hemoglobin-free membranes prepared from red cells which had been separated from leukocytes before lysis. Although the leukocytes were thereby excluded as a contaminating source, it was still possible that DNA from some other source was merely occluded to the erythrocytes (Philipson and Zetterqvist, 1964). In accord with the findings with whole membranes, the soluble inhibitor inactivated enteroviruses capable of agglutinating (ECHO 7, Coxsackie B3), but not those incapable (ECHO 9, poliovirus 1) of agglutinating human O cells. Similarly, hemagglutination inhibitory activity was destroyed by treating the red cell fraction with papain and chymotrypsin, but not with trypsin, venom phosphodiesterase, periodate, or sonication. It is of interest that the inhibitory activity of the soluble material was 1000 times greater against hemagglutinating enteroviruses than against in-

fluenza or Sendai viruses. This was consistent with its low content of NANA, and suggested that the receptor activity for enteroviruses depended on peptides other than those involved in glycosidic linkage with oligosaccharide residues. However, the overall purity of this soluble inhibitory material was difficult to assess. Previous experience with similar fractions suggests that several antigenically distinct proteins are involved, only one of which is glycoprotein (Table I). The macromolecular nature of the complex, moreover, can be ascribed to the tendency of these erythrocyte materials to aggregate spontaneously, especially in aqueous media of high ionic strength.

Some evidence has been presented to indicate that soluble "purified" erythrocyte receptor, after irreversible binding by ECHO 7 virus, caused the release of infective viral RNA. A significant amount of ribonuclease appeared to be present in the receptor preparation and could be inhibited by dextran sulfate. While it was readily shown that the labeled RNA released was infective, the mechanism of release from the virus–inhibitor complex remained obscure, although an enzymatic process was proposed (Philipson and Lind, 1964). The reaction of hemagglutinating enteroviruses with red blood cell components thus has some analogy with the first stages of enteroviral penetration into and eclipse by host cells. The red blood cell model, however, does not shed any particular light on the biochemical sequences involved, other than indicating that eclipse may be membrane mediated.

There is some degree of parallel between the receptor material on red blood cells, thought to be lipoprotein, and inhibitory substances demonstrable in fluids from both normal and infected cell cultures (Schmidt *et al.*, 1964a). The latter inhibitors, because of their susceptibility to chymotrypsin (not to trypsin), papain, and organic solvents, were thought to be lipoprotein in nature. The heat-stable nonspecific hemagglutination inhibitors present in many normal animal sera (Schmidt *et al.*, 1964b) may also be lipoprotein or phospholipid protein complexes, since they are inactivated by combinations of chymotrypsin and phospholipase C, or by filtrates of a psychrophilic pseudomonad (Schmidt *et al.*, 1964c). It would appear, insofar as both the tissue culture and serum inhibitors of enteroviral hemagglutination are concerned, that no polysaccharide constituents are involved, since neither inhibitor is affected by RDE or periodate oxidation. The general resemblance between soluble inhibitors and erythrocyte receptors for hemagglutinating enteroviruses thus holds. It is on this basis also that enteroviral receptors may be said to differ from those for myxoviruses, the latter being highly dependent on certain oligosaccharide and peptide portions of mucopolysaccharides at the cell surface. In sum, the evidence suggests that (1)

—SH groups on the virion, but not on the erythrocyte surface, are essential for hemagglutination to occur. Attachment is thus not simply the establishment of S—S bonds between virus and cells. (2) Reagents affecting sugars, such as glycosidases, KIO_4, and alkaline borohydride can inactivate viral hemagglutinin; comparable treatment of red cells does not interfere with the reaction. These facts suggest that oligosaccharide residues in the capsid may be the effectors of hemagglutinin; the presence of saccharides in the virion remains to be demonstrated. (3) The receptors on erythrocytes as well as on susceptible host cells are protein and/or possibly lipoprotein. The evidence suggests that polysaccharides of the membrane are not involved. The role of lipids is obscure. The character and behavior of nonspecific tissue culture and serum hemagglutinating inhibitors parallels to some extent those of the receptor material on erythrocytes insofar as this has been characterized.

H. Miscellaneous Hemagglutinating Viruses

1. Rubella Virus

In order to detect hemagglutinin in cell cultures infected with rubella virus, nonspecific inhibitors must be removed from the sera used in the maintenance medium (Stewart *et al.*, 1967; Furukawa *et al.*, 1967). This is accomplished by prior adsorption of the sera with kaolin (Clarke and Casals, 1958) or by precipitation with heparin-$MnCl_2$ (Mann *et al.*, 1967). Alternatively, infected cultures can be maintained in serum-free medium. Since the virus tends to remain cell associated, infected cells can be concentrated and hemagglutinin derived by disintegrating the cells. Various methods have been combined to enhance the hemagglutinin titer of rubella virus preparations. Virus dissociable from inhibitor with Versene is subsequently treated with Tween 80–ether (Furukawa *et al.*, 1967). These procedures result usually in loss of infectivity and, by analogy with measles virus, the fragmentation of hemagglutinin into smaller subunits.

The critical conditions necessary for hemagglutination by rubella are comparable to those for arboviruses. The diluent must include dextran or gelatin as well as divalent cations and the final pH after addition of erythrocytes must be 6.0 to 6.2 (Stewart *et al.*, 1967; Schmidt *et al.*, 1968). Hemagglutination takes place best at 4°C with avian erythrocytes, particularly those of the goose, newly hatched chickens or pigeons. Erythrocytes of mammalian species are nonreactive. Rubella virus adsorbed to erythrocytes does not elute spontaneously and at the pH of maximum agglutination is not capable of elution by RDE, myxoviruses or trypsin (Furukawa *et al.*, 1967). However, the addition of Versene

or elevation of the pH to 8.6 at 37°C cause elution of the virus. Erythrocyte receptors remain unaltered. The nonspecific hemagglutination inhibitor present in human and animal sera is thought to be β-lipoprotein, since it is inactivated by phospholipase C, is relatively heat stable and, as already noted, can be precipitated by heparin–$MnCl_2$ (Dold and Northrop, 1968) as well as by antibody to β-lipoprotein (E. Norrby, 1969b). There is evidence to suggest that the serum β-lipoprotein inhibitor may bear some analogy to erythrocyte receptors, particularly those on newly hatched chick cells which are more readily agglutinated than adult cells. Electron microscopic studies (Taylor-Dickinson *et al.*, 1969) showed the presence of "lanthanum staining material" as heavy electron-dense coating present on erythrocytes from newly hatched chicks. The same material was present in only scanty amounts on adult erythrocytes. Treatment of the former with phospholipase C not only stripped the cells of most of this material but rendered them inagglutinable by virus. No further evidence was forthcoming as to the chemical character of this material although it was suggested it might be lipoprotein acting as the receptor for viral hemagglutinin.

2. Coronaviruses

Members of this newly defined group of respiratory agents have in common a morphological resemblance to the viruses of avian infectious bronchitis (IBV-like agents) and mouse hepatitis. The name is descriptive of the electron microscopic appearance of the viral envelope which is endowed with regularly arrayed club-shaped rods. These peripheral subunits are larger and more widely spaced than are the spikes on the envelope of myxoviruses. Their precise function is unknown. Initially, coronaviruses were not found to agglutinate erythrocytes. However, treatment with trypsin or ether was found to render certain strains of IBV capable of hemagglutination (Corbo and Cunningham, 1969; Biswal *et al.*, 1966). More recently, two human strains have been adapted to growth in suckling mouse brain. Concomitant with this change in host species, virus-specific agglutinins were found to be associated with preparations of brain antigens of high infectivity (Kaye and Dowdle, 1969). Agglutinating activity was greatest with rat, mouse, and chicken erythrocytes at room temperature or at 37°C. Hemagglutination was inhibited by specific antibody and was unaffected by neuraminidase treatment of erythrocytes. Receptors were therefore considered to be different from those for myxoviruses. Disruption of viral particles with detergent and ether destroyed the hemagglutinating activity. Nonspecific serum inhibitors of coronavirus hemagglutination apparently do not occur.

3. Rhabdoviruses

Hemagglutination of goose erythrocytes by rabies virus has recently been reported. Hemagglutinating activity associated with tissue culture grown rabies virus (Kuwert *et al.*, 1968; Halonen *et al.*, 1968; Murphy *et al.*, 1968) depended on the integrity of the capsid protein. Agglutination was apparently greater with artificially released intracellular virus than with extracellular, i.e., spontaneously released, virus. No noninfective "soluble hemagglutinin" was found in cell cultures. Moreover, the hemagglutinin was sensitive to lipid solvents and to —SH reactive compounds. Maximum hemagglutination occurred at 4°C and at a pH below 6.8. Adsorbed virus eluted at 37°C without effecting any evident alteration of receptors. Treatment of erythrocytes with periodate or RDE had no effect on agglutinability of the cells by rabies virus. Proteolytic enzymes, lipid solvents, β-propiolactone and —SH reactive compounds inactivated the hemagglutinin. Because of the latter effect, it was inferred that —SH groups were involved in viral attachment to erythrocyte receptors. The nature of the latter, however, remains obscure.

High titers of virion-associated agglutinins for goose erythrocytes were obtained in suspension cultures of BHK21 cells infected with vesicular stomatitis virus (VSV) maintained in serum-free medium or containing BSA. In contrast to rabies, VSV hemagglutination, effected at 4°C and pH 5.8, was stable at higher temperatures (Arstila *et al.*, 1969). VSV also agglutinated erythrocytes of mammalian species, but to lower titer than avian erythrocytes. Disruption of the virion with detergents or ether destroyed hemagglutinating activity, indicating that, as with rabies virus, the intact capsid was required. Hemagglutinins with similar general properties, but exhibiting sharp strain specificity with respect to antibody inhibition, have also been described for Cocal and Kern Canyon viruses (Halonen *et al.*, 1968).

4. Oncogenic Viruses

Polyoma virus agglutinates erythrocytes of various species, both mammalian and avian, although consistently higher titers are obtained with guinea pig erythrocytes (Hartley *et al.*, 1959). Agglutination takes place over a wide range of pH, but most efficiently at 4°C at a pH between 7 and 8, and is neutralized by specific antibody. Erythrocytes exposed to RDE, or to various influenza viruses, are rendered inagglutinable by polyoma. Polyoma virus itself, however, although it elutes at 37°C, effects no alteration of erythrocyte receptors (Fogel and Sachs, 1959). These findings, along with the inhibition of polyoma hemagglutinin by

TABLE III

Major virus group	Subgroups, strains, types	Erythrocyte, species	Conditions for HA	
			pH	Temp.
Myxoviruses (influenza viruses)	A, B, C	HA, HAD fowl, various mammalian HAD: g.p.	7.2–4	Adsorb, 4°C; elute, 37°C
Paramyxoviruses	NDV, mumps, parainfluenza 1–5	HA, HAD fowl, various mammalian HL: mammalian	5.8–6.2	Adsorb, 4°C; HA: 4°C (fowl); 37°C (g.p.)
Pseudomyxoviruses	Measles only	HA, HL, HAD; primate only	7.2 (5–10)	37°C
		SDA	7.8 (High polyvalent anion concentration required)	37°C
Adenoviruses (human)	Group I	Monkey ("complete")	7–8	20°, 37°C
	II	Rat ("complete")		
	III	Rat, human ("partial") (mouse, g.p.)		
Arboviruses	Group A, B, C, TBE	HA: goose, chick hatchling, rooster HAD: (Groups A, B): goose	Critically narrow range	37°C (A); 4°, 22°C (B); 4°C (TBE)
Reoviruses		Human / Bovine		
Human	1	+ / −		
	2	+ / −	6–8	4°C = 23°C 37°C
	3	+ / +		
Avian		No HA		
Enteroviruses	ECHO, Coxsackie B[a]	Human O (adult, fetal)	Variable (7–8)	Various dependencies, 37°C

[a] Abbreviations: HA, hemagglutinin or hemagglutination, HAD, hemadsorption; receptor substance; g.p., guinea pig; SDA, salt-dependent HA; TBE, tick-borne

SUMMARY OF HEMAGGLUTINATING VIRUSES[a]

Elution effected by	Erythrocyte receptor		Viral HA site	Nonspecific inhibitors
	Alteration	Chemistry		
Viral and bacterial N-ase	NANA released by virus, trypsin (sialopeptide)	Sialoglycoprotein (VRS)	Envelope spikes	α (Francis); many sialoproteins. β (Chu) serum (A_1 strains). γ (horse serum) (A_2 "+" strains)
Viral and bacterial N-ase	NANA released by virus, trypsin (sialopeptide)	Sialoglycoprotein (VRS), HA = HL	Envelope spikes and subunits	Ovine α_1 glycoprotein (NDV)
None by virus, RDE, or proteases Into isotonic saline	None by virus, influenza, or bacterial N-ase; trypsin and formalin inactivate	? Protein	Envelope and subunits (noninfective) derived by Tween-ether treatment; SDA-protein	(HA for primate RBC in human sera)
None	None. RDE inactivates type 9 rat receptors	? Protein	Capsid and bi- or multivalent subunits ("soluble" HA); + heterotypic antibody in Group III	Serum, ? β lipoprotein
Nonenzymatic, outside optimum pH ranges (6.2–8)	None	? Protein	Capsid ? protein "soluble" subunits	Brain and other lipids; serum lipoproteins
Slight, nonenzymatic	None by virus. RDE inactivates type 3 receptors	Protein ? glycoprotein (type 3)	Capsid protein ? essential—SH; ? oligosaccharides	Serum, β lipoprotein; ?? glycoproteins (serum, saliva)
Some at 37°C (not ECHO 7, Coxsackie B[3])	None by virus RDE or myxoviruses; trypsin, formalin inactivate ECHO receptors	Protein or lipoprotein	Capsid protein; ? oligosaccharides; Cationic stabilization to heat	Serum, ? lipoproteins; infected cell culture fluids

HL, hemolysis; NANA, *N*-acetylneuraminic acid; *N*-ase, neuraminidase; VRS, virus encephalitis; RBC, erythrocyte; RDE, receptor-destroying enzyme (*V. cholorae*).

(*Continued*)

TABLE III

Major virus group	Subgroups, strains, types	Erythrocyte, species	Conditions for HA	
			pH	Temp.
Rubella		HA: avian (pigeon, goose, chick hatchling) (human- No HA) HAD: ? similar to arboviruses	7.3 (Veronal, dextran, gelatin, serum or other colloid required)	4°, 25°C
Coronaviruses (IBV-like)	("IBV MH-like")	Rat, mouse, chicken, human, vervet	7.2	Room temp., 37°C, 4°C
Rhabdoviruses	Rabies	Goose	6.2	4°C
	VSV	Goose (chick hatchling)	5.8	4°C
	(Cocal, Kern Kanyon)		(Bovine albumin borate saline)	
Polyoma		g.p. (avian, mammalian)	7–8	4°C
H-viruses	H-1, HT, H-3, RV, HB	g.p., hamster (variable with other species)	7.2	5°C
Mouse leukemia viruses	Rauscher, Gross	g.p.	6.4 (*N*-ase and phospholipase treatment of virus required)	4°C

ovomucin (Hartley *et al.*, 1959) and by an RDE-sensitive glycoprotein inhibitor in bovine serum (Borecky *et al.*, 1962), provide evidence that the erythrocyte receptor for polyoma hemagglutinin may be included in the virus receptor substance described in connection with myxoviruses.

(*Continued*)

Elution effected by	Erythrocyte receptor		Viral Ha site	Nonspecific inhibitors
	Alteration	Chemistry		
None by virus, EDTA, or change in pH to 8.6	None by RDE, paramyxoviruses, trypsin	? Lipoprotein, ?? RNA	Virion and subunits derived by Tween-ether; ? protein–lipid–RNA	Virus–serum complex (Ca^{2+}, Mg^{2+} dependent), dissociable with EDTA
Nonenzymatic, change to 37°C	None by RDE	Unknown	Suckling mouse brain adapted; destroyed by trypsin, Tween-80–ether	—
Nonenzymatic, change to toom temp.	None by virus or RDE	Unknown	Intact virion (capsid); inactivated by proteases, solvents, —SH compounds; propagate in serum-free medium	Normal human, burro, goat sera (R); normal rabbit sera (VSV)
Nonenzymatic at 37°C	By RDE, influenza viruses	Glycoproteins	Intact virion	Ovomucin, bovine serum glycoprotein, normal tissue, and tumor extracts
?	?	Unknown	Intact virion	Human and horse placental glycoprotein (phosphatase) inhibits H-1, HB
Nonenzymatic	None by *N*-ase	Unknown	HA in virion blocked by glycoprotein, phospholipid	?

Recent information on the structure of this material (VRS) may allow further elucidation of the interaction of polyoma and erythrocytes.

The small RNA viruses, called H viruses, have been found to agglutinate a wide variety of mammalian red cells. The pattern among the

different species was quite characteristic for each of 5 virus strains tested. H-1 virus had the widest range, and it alone agglutinated gerbil and goose cells. None of these viruses reacted with chicken erythrocytes (Toolan, 1967). A heat-stable glycoprotein inhibitor was found in horse and human placental fluids which was not a component of normal serum (Usategui-Gomez *et al.*, 1968). The inhibitor in human fluid was active against H-1 and HB viruses and was subsequently shown to possess alkaline phosphatase activity (Usategui-Gomez *et al.*, 1969). Inhibitory activity of the host placental material was destroyed by sialidase, metaperiodate, and papain, but not trypsin or chymotrypsin. These findings suggested by analogy that receptors on erythrocytes might be glycoprotein in nature.

The morphological resemblance of RNA leukemia viruses of mice to myxoviruses has prompted an unsuccessful search for hemagglutination by these agents. Recently, however, it has been shown that treatment of mouse leukemia viruses (Schäfer and Szántó, 1969) with neuraminidase and phospholipase C uncovered a hemagglutinin for guinea pig erythrocytes which was associated with the viral particle, and was neutralized by type-specific antiviral antibody. The masking of hemagglutinin in the untreated viral particle was ascribed to the presence of glycoprotein and phospholipid components acquired by the virion from the host cell membrane during the process of maturation and release. The receptors on erythrocytes, unlike those for myxoviruses, were resistant to neuraminidase. There was no evidence for an enzyme-mediated mechanism to explain the elution of virus.

V. Summary

The agglutination of erythrocytes by direct action of viral particles was first described in connection with myxoviruses. This led directly to the discovery of viral neuraminidase, a property unique to myxoviruses and paramyxoviruses. The discovery of hemagglutination by viruses of other taxonomic groups has since added new dimensions to the study of these agents and antibodies to them. In this review, we have attempted to summarize the current knowledge of these reactions as it applies to the virion and its subunits and to the erythrocyte membrane. The latter has been the subject of recent intensive investigation, not a small portion of which originated in connection with the analysis of myxoviral hemagglutination and hemolysis. Considerable insight has thus been gained into the structure and compositon of the erythrocyte membrane through the use of viral receptor activity and blood group antigens as biological surface markers. While the most precise information avail-

able to date regarding viral receptors concerns those for myxoviruses, the rapidly expanding knowledge of membrane structure and function should allow the elucidation of other viral receptors as well. Much has already been learned through a study of receptor analogs derived from nonerythrocyte sources. Table III (pp. 40–43) summarizes the principal features characterizing hemagglutination by various members of the principal taxonomic groups of viruses.

References

Ardoin, P., Clarke, D. H., and Hannoun, C. (1969). *Amer. J. Trop. Med. Hyg.* **18,** 592.

Arstila, P., Halonen, P., and Salmi, A. (1969). *Arch. Gesamte Virusforsch.* **27,** 198.

Baker, R. F., Gordon, I., and Stevenson, D. (1965). *Virology* **27,** 441.

Barclay, G. R., Flewett, T. H., Keller, E., Halsall, H. B., and Spragg, S. P. (1969). *Biochem. J.* **111,** 353.

Biswal, N., Nazerian, K., and Cunningham, C. H. (1966). *Amer. J. Vet. Res.* **27,** 1157.

Blumenfeld, O. O., Gallop, P. M., Howe, C., and Lee, L. T. (1970). *Biochim. Biophys. Acta* **211,** 109.

Borecky, L., Halperen, S., and Ackermann, W. W. (1962). *J. Immunol.* **89,** 752.

Brubaker, M. M., West, B., and Ellis, R. J. (1964). *Proc. Soc. Exp. Biol. Med.* **115,** 1118.

Buckley, S. M. (1959). *Ann. N.Y. Acad. Sci.* **81,** 172.

Buzzell, A., and Hanig, M. (1958). *Advan. Virus Res.* **5,** 289.

Campbell, B. J., Schneider, A. L., Howe, D. N., and Durand, D. P. (1967). *Biochim. Biophys. Acta* **148,** 137.

Carubelli, R., Bhavanandan, V. P., and Gottschalk, A. (1965). *Biochim. Biophys. Acta* **101,** 67.

Casals, J., and Brown, L. V. (1954). *J. Exp. Med.* **99,** 429.

Casals, J., and Whitman, L. (1961). *Amer. J. Trop. Med. Hyg.* **10,** 250.

Cascardo, M., and Karzon, D. T. (1965). *Virology* **26,** 311.

Cheng, P. (1958). *Virology* **6,** 129.

Choppin, P. W., and Tamm, I. (1960a). *J. Exp. Med.* **112,** 895.

Choppin, P. W., and Tamm, I. (1960b). *J. Exp. Med.* **112,** 921.

Chu, C. M. (1951). *J. Gen. Microbiol.* **5,** 739.

Chu, L., and Morgan, H. R. (1950). *J. Exp. Med.* **91,** 403.

Clarke, D. H., and Casals, J. (1958). *Amer. J. Trop. Med. Hyg.* **7,** 561.

Cook, G. M. W., Heard, D. H., and Seaman, G. A. F. (1961). *Nature (London)* **191,** 44.

Corbo, L. J., and Cunningham, C. H. (1969). *Amer. J. Vet. Res.* **20,** 876.

Danon, D., Howe, C., and Lee, L. T. (1965). *Biochim. Biophys. Acta* **101,** 201.

Dodge, J. T., Mitchell, C., and Hanahan, D. J. (1963). *Arch. Biochem. Biophys.* **100,** 119.

Dold, H. J., and Northrop, R. L. (1968). *Proc. Soc. Exp. Biol. Med.* **128,** 577.

Drescher, J., Hennessy, A. V., and Davenport, F. M. (1962). *J. Immunol.* **89,** 794.

Drzeniek, R. (1967). *Biochem. Biophys. Res. Commun.* **26,** 631.

Drzeniek, R. (1970). *Z. Med. Mikrobiol. Immunol.* **155,** 315; **156,** 1.

Duc-Nguyen, H. (1968). *J. Virol.* **2,** 494.
Duc-Nguyen, H., Rose, H. M., and Morgan, C. (1966). *Virology* **28,** 404.
Dzierzkowa-Borodej, W., Lisowska, E., and Seyfriedowa, H. (1970). *Life Sci.* **9,** 111.
Eckert, E. A. (1967). *J. Virol.* **1,** 920.
Eggers, H. J., Gomatos, P. J., and Tamm, I. (1962). *Proc. Soc. Exp. Biol. Med.* **110,** 879.
Fazekas de St. Groth, S., and Gottschalk, A. (1963). *Biochim. Biophys. Acta* **78,** 248.
Feldman, H. A. (1968). *J. Immunol.* **100,** 1353.
Finter, N. B. (1964). *Virology* **24,** 589.
Fogel, M., and Sachs, L. (1959). *Brit. J. Cancer* **13,** 266.
Fontanges, R., Garrigue, J., and Colobert, L. (1964). *Ann. Inst. Pasteur, Paris* **106,** 894.
Francis, T. (1947). *J. Exp. Med.* **85,** 1.
Frisch-Niggemeyer, W. (1967). *Arch. Hyg. Bakteriol.* **151,** 585.
Funahashi, S., and Kitawaki, T. (1963). *Biken's J.* **6,** 73.
Furukawa, T., Plotkin, S. A., Sedwick, W. D., and Profeta, M. L. (1967). *Proc. Soc. Exp. Biol. Med.* **126,** 745.
Gelb, L. D., and Lerner, A. M. (1965). *Science* **147,** 404.
Ginsberg, H. S., Pereira, H. G., Valentine, R. C., and Wilcox, W. C. (1966). *Virology* **28,** 782.
Goldfield, M., Srihongse, S., and Fox, J. P. (1957). *Proc. Soc. Exp. Biol. Med.* **96,** 788.
Gomatos, P. J., and Tamm, I. (1962). *Virology* **17,** 455.
Gorman, B. (1970). *J. Gen. Virol.* **6,** 305.
Gottschalk, A. (1965). *Aust. J. Exp. Biol. Med. Sci.* **43,** 391.
Gottschalk, A. (1966a). *Glycoproteins, 1966,* pp. 543–547.
Gottschalk, A. (1966b). *In* "The Amino Sugars: The Chemistry and Biology of Compounds Containing Amino Sugars" (R. W. Jeanloz and E. A. Balazs, eds.), Vol. 2, pp. 337–359. Academic Press, New York.
Graham, E. R. B., and Gottschalk, A. (1960). *Biochim. Biophys. Acta* **38,** 513.
Halonen, P. E., Murphy, F. A., Fields, B. N., and Reese, D. R. (1968). *Proc. Soc. Exp. Biol. Med.* **127,** 1037.
Halperen, S., Eggers, H. J., and Tamm, I. (1964). *Virology* **23,** 81.
Hanig, M. (1948). *Proc. Soc. Exp. Biol. Med.* **68,** 385.
Harboe, A. (1963). *Acta Pathol. Microbiol. Scand.* **57,** 448.
Hartley, J. W., Rowe, W. P., Chanock, R. M., and Andrews, B. E. (1959). *J. Exp. Med.* **110,** 81.
Haukenes, G., Harboe, A., and Mortensson-Egnund, K. (1966). *Acta Pathol. Microbiol. Scand.* **66,** 510.
Hermodsson, S., Dinter, Z., and Bakos, K. (1961). *Acta Pathol. Microbiol. Scand.* **51,** 75.
Hirst, G. K. (1941). *Science* **94,** 22.
Hirst, G. K. (1942). *J. Exp. Med.* **76,** 195.
Hirst, G. K., and Pickels, E. G. (1942). *J. Immunol.* **45,** 273.
Hjerten, S., Höglund, S., and Ruttkay-Nedecký, G. (1970). *Acta Virol. (Prague), Engl. Ed.* **14,** 89.
Hosaka, Y. (1970). *J. Gen. Virol.* **8,** 43.
Howe, C., and Lee, L. T. (1969). *J. Immunol.* **102,** 573.

Howe, C., and Morgan, C. (1969). *J. Virol.* **3,** 70.
Howe, C., Lee, L. T., and Rose, H. M. (1961). *Arch. Biochem. Biophys.* **95,** 512.
Howe, C., Avrameas, S., de Vaux St. Cyr, C., Grabar, P., and Lee, L. T. (1963). *J. Immunol.* **91,** 683.
Howe, C., Morgan, C., de Vaux St. Cyr, C., Hsu, K. C., and Rose, H. M. (1967). *J. Virol.* **1,** 215.
Howe, C., Milliken, S. A., and Newcomb, E. W. (1970a). *Arch. Gesamte Virusforsch.* **92,** 50.
Howe, C., Spiele, H., Minio, F., and Hsu, K. C. (1970b). *J. Immunol.* **104,** 1406.
Howe, C., Blumenfeld, O. O., Lee, L. T., and Copeland, P. C. (1971). *J. Immunol.* **106,** 1035.
Howe, C., Lloyd, K. O., and Lee, L. T. (1972). *In* "Methods in Enzymology," Vol. 28: Complex Carbohydrates (V. Ginsburg, ed.) In press.
Hoyle, L. (1968). *Virol. Monogr.* **4,** 1.
Hoyle, L. (1969). *J. Hyg.* **67,** 289.
Hoyle, L., and Hana, L. (1966). *J Pathol. Bacteriol.* **92,** 447.
Imagawa, D. T. (1968). *Progr. Med. Virol.* **10,** 160.
Ishida, N., and Homma, M. (1960). *J. Exp. Med.* **73,** 56.
John, T. J., and Fulginiti, V. A. (1968). *Arch. Gesamte Virusforsch.* **23,** 138.
Karabatsos, N. (1963). *J. Immunol.* **91,** 76.
Kasel, J. A., and Huber, M. (1964). *Proc. Soc. Exp. Biol. Med.* **116,** 16.
Kasel, J. A., Rowe, W. P., and Nemes, J. L. (1961). *J. Exp. Med.* **114,** 717.
Kathan, R. H., Winzler, R. J., and Johnson, C. A. (1961). *J. Exp. Med.* **113,** 37.
Kaye, H. S., and Dowdle, W. R. (1969). *J. Infec. Dis.* **120,** 576.
Kendal, A. P., and Apostolov, K. (1970). *Nature (London)* **226,** 167.
Kern, J., and Rosen, L. (1964). *Proc. Soc. Exp. Biol. Med.* **115,** 536.
Kilham, L. (1949). *Proc. Soc. Exp. Biol. Med.* **71,** 63.
Kisseljov, F. L., Mazzarelli, M., Varitch, N. L., and Tikchonenko, T. I. (1969). *Arch. Gesamte Virusforsch.* **27,** 138.
Kohn, A., and Fuchs, P. (1969). *J. Virol.* **3,** 539.
Kuwert, E., Wiktor, T. J., Sokol, F., and Koprowski, H. (1968). *J. Virol.* **2,** 1381.
Laver, W. G., and Valentine, R. C. (1969). *Virology* **38,** 105.
Lee, L. T., Howe, C., Meyer, K., and Choi, H. U. (1969). *J. Immunol.* **102,** 1144.
Lenard, J. (1970). *Biochemistry* **9,** 1129.
Lennette, E. H., and Schmidt, N. J. (1969). "Diagnostic Procedures for Viral and Rickettsial Infections," 4th ed. Amer. Pub. Health Ass., New York.
Lerner, A. M., and Miranda, Q. R. (1968). *Virology* **36,** 277.
Lerner, A. M., Cherry, J. D., and Finland, M. (1963). *Virology* **19,** 58.
Lerner, A. M., Bailey, E. J., and Tillotson, J. R. (1965). *J. Immunol.* **95,** 1111.
Lerner, A. M., Bailey, E. J., and Kofender, M. (1966a). *J. Immunol.* **96,** 59.
Lerner, A. M., Gelb, L. D., Tillotson, J. R., Carruthers, M. M., and Bailey, E. J. (1966b). *J. Immunol.* **96,** 629.
Levine, S., Puck, T. T., and Sagik, B. P. (1953). *J. Exp. Med.* **98,** 521.
Levinson, B., Pepper, C., and Belyavin, G. (1969). *J. Virol.* **3,** 477.
McClelland, L., and Hare, R. (1941). *Can. Pub. Health J.* **32,** 530.
Maddy, A. H. (1964). *Biochim. Biophys. Acta* **88,** 448.
Mann, J. J., Rossen, R. D., Lehrich, J. R., and Kasel, J. A. (1967). *J. Immunol.* **98,** 1136.
Marchesi, V. T., and Andrews, E. P. (1971). *Science* **174,** 1247.

Marchesi, V. T., Tillack, T. W., and Scott, E. W., (1971). *In* "Glycoproteins of Blood Cells and Plasma" (G. A. Jamieson and T. J. Greenwalt, eds.), p. 94. Lippincott, Philadelphia, Pennsylvania.
Matsumoto, T., and Maeno, K. (1962). *Virology* **17**, 563.
Meyer, K., Bhavanandan, V. P., Yung, D., Lee, L. T., and Howe, C. (1967). *Proc. Nat. Acad. Sci. U.S.* **58**, 1655.
Morawiecki, A., and Lisowska, E. (1965). *Biochem. Biophys. Res. Commun.* **18**, 606.
Morgan, H. R., Enders, J. F., and Wagley, P. F. (1948). *J. Exp. Med.* **88**, 503.
Murphy, F. A., Halonen, P. E., Gary, G. W., and Reese, D. R. (1968). *J. Gen. Virol.* **3**, 289.
Neurath, A. R. (1963). *Acta Virol. (Prague), Engl. Ed.* **7**, 490.
Neurath, A. R. (1964a). *Acta Virol. (Prague), Engl. Ed.* **8**, 154.
Neurath, A. R. (1964b). *Acta Virol. (Prague), Engl. Ed.* **8**, 191.
Neurath, A. R. (1964c). *Acta Virol. (Prague), Engl. Ed.* **8**, 143.
Neurath, A. R., and Norrby, E. C. J. (1965). *Arch. Gesamte Virusforsch.* **15**, 651.
Neurath, A. R., Hartzell, R. W., and Rubin, B. A. (1969). *Nature (London)* **221**, 1069.
Neurath, A. R., Hartzell, R. W., and Rubin, B. A. (1970). *Virology* **42**, 789.
Nicoli, J. (1965). *Ann. Inst. Pasteur, Paris* **109**, 472.
Norrby, E. (1962). *Proc. Soc. Exp. Biol. Med.* **111**, 814.
Norrby, E. (1966). *Proc. Soc. Exp. Biol. Med.* **121**, 948.
Norrby, E. (1969a). *J. Gen. Virol.* **5**, 221.
Norrby, E. (1969b). *Virol. Monogr.* **7**, 115.
Norrby, E., Chiarini, A., and Marusyk, H. (1970). *In* "The Biology of Large RNA Viruses" (R. D. Barry and B. W. J. Mahy, eds.), pp. 141–153. Academic Press, New York.
Norrby, E. C. J., Magnusson, P., Falksveden, L. G., and Gronberg, M. (1964). *Arch. Gesamte Virusforsch.* **14**, 462.
Papadimitriou, J. M. (1966). *Aust. J. Exp. Biol. Med. Sci.* **44**, 701.
Pedreira, F., Tauraso, N. M., Klutch, M. J., and Shelokov, A. (1969). *Amer. J. Trop. Med. Hyg.* **18**, 614.
Pereira, H. G. (1960). *Nature (London)* **186**, 571.
Pereira, H. G., and Figueiredo, M. V. T. (1962). *Virology* **18**, 1.
Peries, J. R., and Chany, C. (1962). *Proc. Soc. Exp. Biol. Med.* **110**, 477.
Philipson, L. (1959). *Arch. Gesamte Virusforsch.* **9**, 251.
Philipson, L., and Bengtsson, S. (1962). *Virology* **18**, 457.
Philipson, L., and Choppin, P. W. (1960). *J. Exp. Med.* **112**, 455.
Philipson, L., and Lind, M. (1964). *Virology* **23**, 322.
Philipson, L., and Zetterqvist, O. (1964). *Biochim. Biophys. Acta* **91**, 171.
Philipson, L., Bengtsson, S., Brishammar, S., Svennerholm, L., and Zetterqvist, O. (1964). *Virology* **22**, 580.
Podoplekin, V. D. (1964). *Acta Virol. (Prague), Engl. Ed.* **8**, 254.
Podoplekin, V. D., and Ivanova, T. K. (1966). *Acta Virol. (Prague), Engl. Ed.* **10**, 89.
Porterfield, J. S., and Rowe, C. E. (1960). *Virology* **11**, 765.
Rebel, G., Fontanges, R., and Colobert, L. (1962). *Ann. Inst. Pasteur, Paris* **102**, 137.
Reeve, P., and Alexander, D. J. (1970). *Cytobios* **5**, 55.
Reeve, P., and Poste, G. (1971). *Nature (London)* **229**, 157.

Rosen, L. (1960a). *Amer. J. Hyg.* **71,** 120.
Rosen, L. (1960b). *Amer. J. Hyg.* **71,** 242.
Rosenberg, S. A., and Guidotti, G. (1969). *J. Biol. Chem.* **244,** 5118.
Rott, R., Drzeniek, R., and Frank, H. (1970). *In* "The Biology of Large RNA Viruses" (R. D. Barry and B. W. J. Mahy, eds.), pp. 75–85. Academic Press, New York.
Sabin, A. B., and Buescher, E. L. (1950). *Proc. Soc. Exp. Biol. Med.* **74,** 222.
Sagik, B. P., and Levine, S. (1957). *Virology* **3,** 401.
Salk, J. E. (1944). *J. Immunol.* **49,** 87.
Salminen, A. (1960). *Ann. Med. Exp. Biol. Fenn.* **38,** 267.
Sato, S. (1958). *Virus (Kyoto)* **8,** 48.
Schäfer, W., and Szántó, J. (1969). *Z. Naturforsch. B* **24,** 1324.
Schluederberg, A. (1968). *Proc. Soc. Exp. Biol. Med.* **127,** 804.
Schluederberg, A., and Nakamura, M. (1967). *Virology* **33,** 297.
Schmidt, N. J., Dennis, J., Hoffman, M. N., and Lennette, E. H. (1964a). *J. Immunol.* **93,** 367.
Schmidt, N. J., Dennis, J., Hoffman, M. N., and Lennette, E. H. (1964b). *J. Immunol.* **93,** 377.
Schmidt, N. J., Dennis, J., and Lennette, E. H. (1964c). *J. Immunol.* **93,** 140.
Schmidt, N. J., Lennette, E. H., and King, C. J. (1966). *J. Immunol.* **97,** 64.
Schmidt, N. J., Dennis, J., and Lennette, E. H. (1968). *Arch. Gesamte Virusforsch.* **25,** 308.
Schulman, J. L., and Kilbourne, E. D. (1969). *Proc. Nat. Acad. Sci. U.S.* **63,** 326.
Sever, J. L. (1962). *J. Immunol.* **88,** 320.
Segrest, J. P., Jackson, R. L., Andrews, E. P., and Marchesi, V. T. (1971). *Biochem. Biophys. Res. Commun.* **44,** 390.
Shelokov, A., Vogel, J. E., and Chi, L. (1958). *Proc. Soc. Exp. Biol. Med.* **97,** 802.
Shortridge, K. F., and Biddle, F. (1968). *Arch. Gesamte Virusforsch.* **25,** 148.
Sokol, F. (1963). *Virology* **21,** 126.
Sokol, F., and Neurath, A. R. (1962). *Acta Virol. (Prague), Engl. Ed.* **6,** 122.
Sokol, F., Blaskovic, D., and Krizanova, O. (1961). *Acta Virol. (Prague), Engl. Ed.* **5,** 153.
Sokol, F., Neurath, A. R., and Vilcek, J. (1964). *Acta Virol. (Prague), Engl. Ed.* **8,** 59.
Spendlove, R. S., McClain, M. E., and Lennette, E. H. (1970). *J. Gen. Virol.* **8,** 83.
Springer, G. F. (1963). *Bacteriol. Rev.* **27,** 191.
Springer, G. F., Nagai, Y., and Tegtmeyer, H. (1966). *Biochemistry* **5,** 3254.
Springer, G. F., Schwick, H. G., and Fletcher, M. A. (1969). *Proc. Nat. Acad. Sci. U.S.* **64,** 634.
Stewart, G. L., Parkman, P. D., Hopps, H. E., Douglas, R. D., Hamilton, J. P., and Meyer, H. M. (1967). *N. Engl. J. Med.* **276,** 554.
Stone, J. D., and Ada, G. L. (1952). *Brit. J. Exp. Pathol.* **33,** 428.
Styk, B., Schmidt, N. J., and Dennis, J. (1968). *Amer. J. Epidemiol.* **88,** 398.
Suttajit, M., and Winzler, R. J. (1971). *J. Biol. Chem.* **246,** 3398.
Takatsy, G., Barb, K., and Farkas, E. (1959). *Acta Virol. (Prague), Engl. Ed.* **3,** 79.
Taylor-Dickinson, P. C., Chang, T. W., and Weinstein, L. (1969). *Proc. Soc. Exp. Biol. Med.* **132,** 55.

Tischer, I. (1963). *Arch. Gesamte Virusforsch.* **12,** 89.
Tischer, I. (1967). *Zentralbl. Bakteriol., Parasitenk., Infektionskr. Hyg., Abt. 1B Orig.* **204,** 466.
Toolan, H. W. (1967). *Proc. Soc. Exp. Biol. Med.* **124,** 144.
Usategui-Gomez, M., Morgan, D. F., and Toolan, H. W. (1968). *Proc. Soc. Exp. Biol. Med.* **128,** 287.
Usategui-Gomez, M., Ghosh, N. K., and Toolan, H. W. (1969). *Proc. Soc. Exp. Biol. Med.* **130,** 1128.
Wadell, G. (1969). *Proc. Soc. Exp. Biol. Med.* **132,** 413.
Wadell, G. (1970). "Structural and Biological Properties of Capsid Components of Human Adenoviruses." D. Broberg, Stockholm.
Waterson, A. P. (1965). *Arch. Gesamte Virusforsch.* **16,** 57.
Webster, R. G. (1970). *In* "The Biology of Large RNA Viruses" (R. D. Barry and B. W. J. Mahy, eds.), pp. 53–74. Academic Press, New York.
Whitehead, P. H., and Winzler, R. J. (1968). *Arch. Biochem. Biophys.* **126,** 657.
Whittemore, N. B., Trabold, N. C., Reed, C. F., and Weed, R. I. (1969). *Vox Sang.* **17,** 289.
Wigand, R., and Stöhr, M. (1965). *Z. Naturforsch. B.* **20,** 560.
Winzler, R. J. (1969a). *In* "Red Cell Membrane, Structure and Function" (G. A. Jamieson and T. J. Greenwalt, eds.), p. 157. Lippincott, Philadelphia, Pennsylvania.
Winzler, R. J. (1969b). *In* "Cellular Receptors" (R. T. Smith and R. A. Good, eds.), p. 11. Appleton, New York.