Advances in

VIRUS RESEARCH

VOLUME 17

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VIRUS RESEARCH

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VIRUS-ERYTHROCYTE INTERACTIONS[1]

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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

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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.

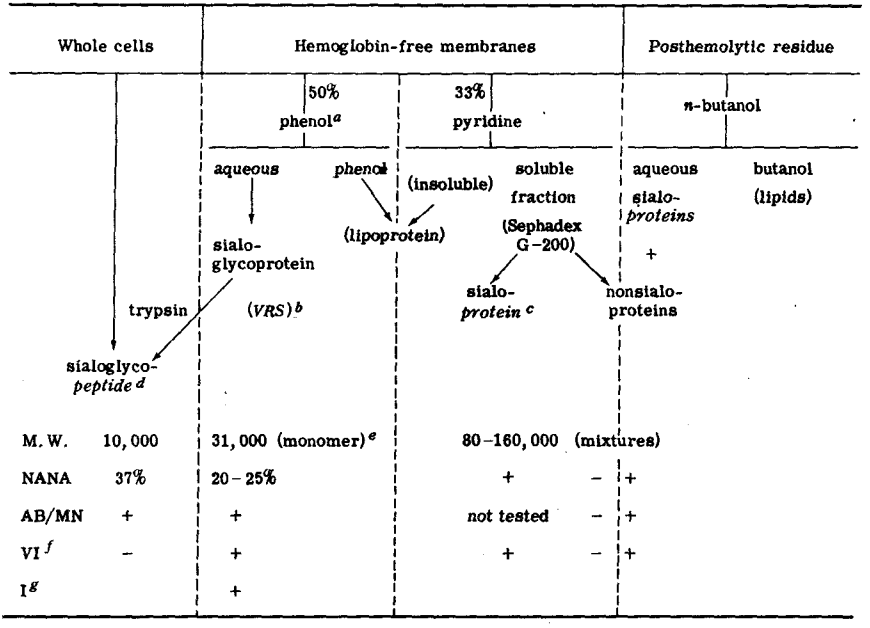
A direct approach to the chemical characterization of various receptor activities lies in attempting to render erythrocyte membranes soluble and then to separate components by standard methods of protein and carbohydrate chemistry. Much depends initially on the nature of the starting material which is 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.

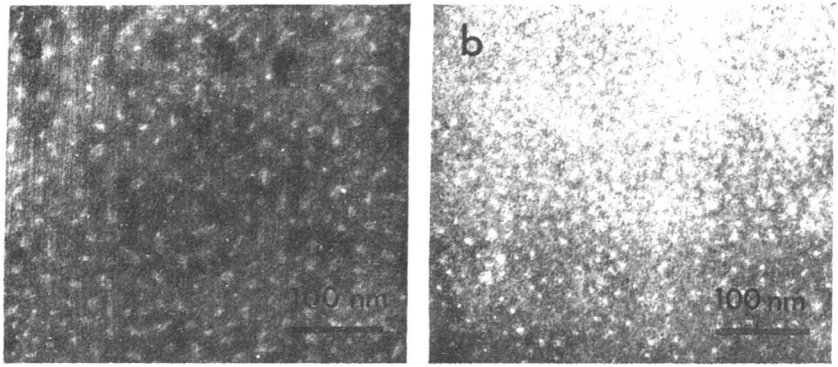


FIG. 1. Purified virus receptor substance (VRS) negatively stained with PTA. (a) VRS from human erythrocytes. (b) VRS from murine (Swiss) erythrocytes. (By Courtesy of Dr. James R. Harris.) × 125,000.

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

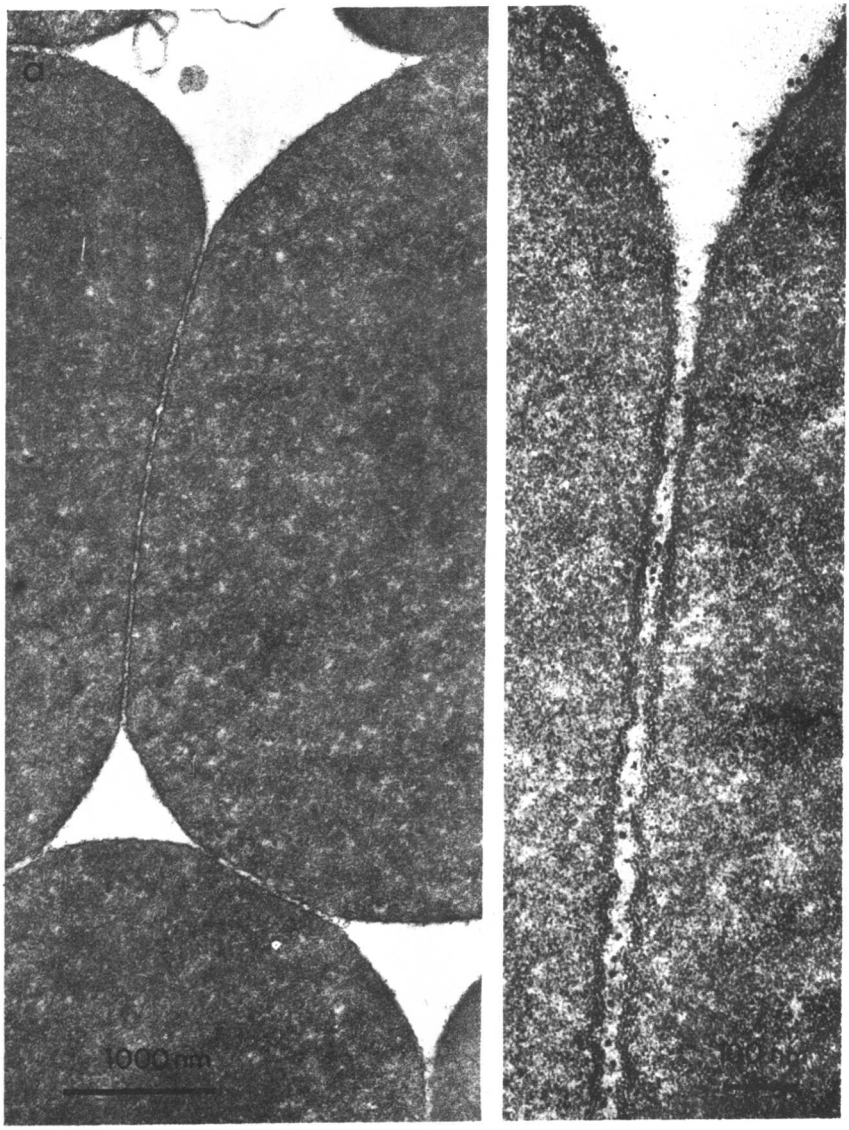


FIG. 2. Specific reaction of human group O erythrocytes with ferritin-labeled antibody to VRS. Glutaraldehyde and osmium tetroxide fixation after reaction with antibody and washing with phosphate-buffered saline, pH 7.2. (By courtesy of Dr. Thomas Bächi.) (a) × 20,000. (b) Detail from (a), × 100,000.

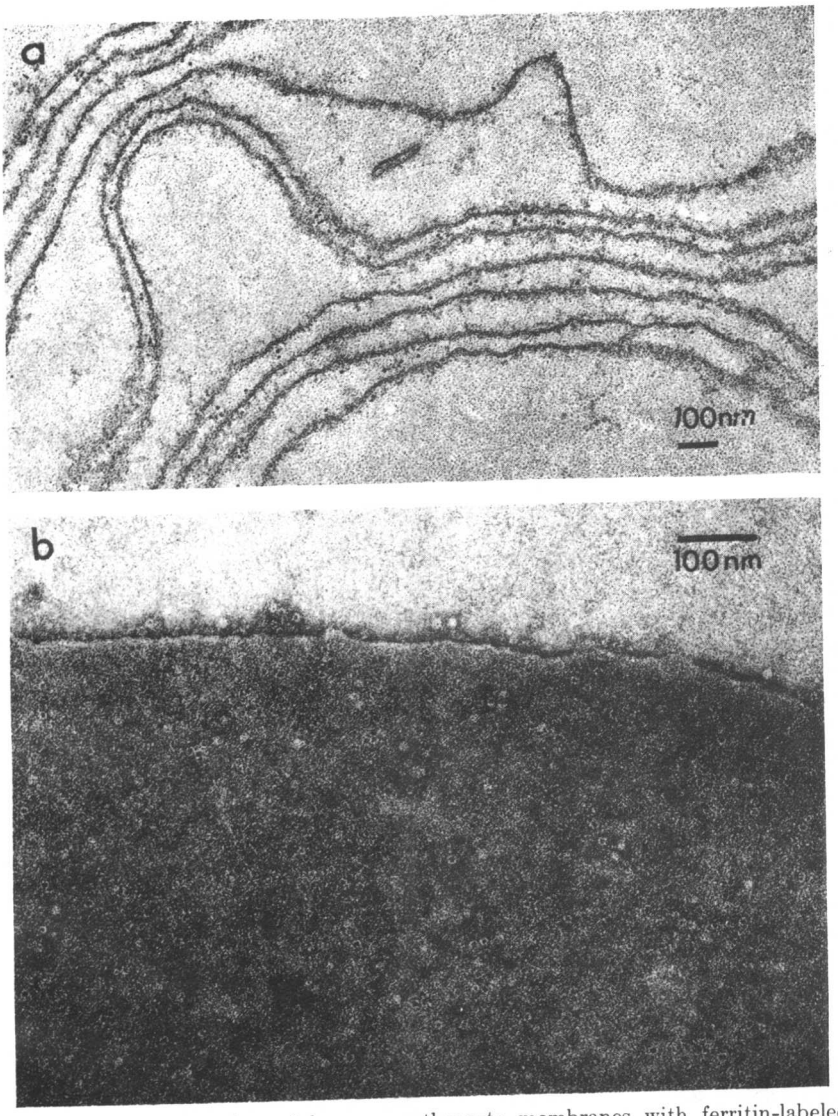


FIG. 3. Specific reaction of human erythrocyte membranes with ferritin-labeled antibody to VRS. (a) Erythrocytes were reacted with the antibody, washed free of nonspecific protein and ferritin, and lysed by the addition of complement. The cell membranes were washed free of hemoglobin, and fixed in glutaraldehyde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