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.

ADVANCES IN VIRUS RESEARCH, VOLUME 23

NEUTRALIZATION OF ANIMAL VIRUSES *

Benjamin Mandel

Department of Virology, The Public Health Research Institute
of The City of New York, New York, New York

* The following abbreviations are used in this article: CMV, cytomegalovirus; DNP, 2,4-dinitrophenyl; EEE, eastern equine encephalitis; FMD, foot-and-mouth disease; JEV, Japanese encephalitis virus; LCM, lymphocytic choriomeningitis; LDH, lactic dehydrogenase; MLV, Moloney leukemogenic virus; MSV, murine sarcoma virus; NDV, Newcastle disease virus; VEE, Venezuelan equine encephalitis; WEE, western equine encephalitis; WN, West Nile.

I. Introduction

Edward Jenner's successful vaccination experiment at the end of the eighteenth century delineated the four elements of the immunological system: (1) the immunogen, (2) the animal host responding to the immunogen, (3) the product of the host's response, namely, antibody, and (4) the interplay of immunogen, antibody, and host. About 100

ISBN 0-12-039823-0

years later, in the late 1800s, the validity of Jenner's approach to combatting smallpox was reaffirmed by Pasteur when he succeeded in selecting an avirulent strain of rabies virus for use as a vaccine. At about the same time the studies of Iwanowski and Beijerinck began to reveal the nature of a then unknown form of infectious agent of plants, and somewhat later a similar revelation emerged from the studies of Loeffler and Frosch on infected cattle. The ubiquity of this kind of agent was revealed by the studies of Twort and d'Herrelle who showed that bacteria too were susceptible to what became known as filterable viruses.

As knowledge of the existence and the nature of viruses accrued, so did knowledge of the existence and the nature of antibodies through the studies of von Behring, Kitasato, and Bordet. In 1892 Sternberg (see Hahon, 1964) inferred that the blood of an individual recently recovered from an infection such as smallpox contained an "antitoxine which would neutralize the active poison of the disease." This inference proved to be correct when he showed that serum from a recently vaccinated calf neuralized cowpox virus. These observations presaged the start of experimental viral immunology.

The relative ease and simplicity of cultivating bacterial viruses led, in the early 1930s, to highly productive studies on their interaction with antibody. The introduction by Woodruff and Goodpasture of the use of embryonated chick eggs as an animal virus host greatly stimulated research on animal viruses. The usefulness of this host was extended when Burnet developed a technique for infecting the chorioallantoic membrane in a manner that resulted in discrete lesions (pocks) which could be readily recognized and enumerated. With the application of the plaque assay for bacterial viruses, and the pock-counting assay for animal viruses, the era of quantitative virology had its inception.

Various aspects of the interaction of bacterial viruses and antibody were studied by Andrewes and Elford in England. Similar studies, as well as studies on animal viruses, were carried out in Australia by Burnet and his colleagues. One result of their extensive studies, which were summarized in great detail (Burnet *et al.*, 1937), was the conclusion that, with respect to their interaction with antibody, bacterial and animal viruses were basically different. Specifically, the difference resided in the stability of the union of virus and antibody. Whereas bacterial viruses formed stable complexes, animal viruses formed complexes that tended to dissociate readily.

The introduction of animal cell cultures as host systems greatly aided in the study of animal viruses with respect to fewer and more readily controlled variables, and, by use of the plaque assay, in enhanced quantitative reliability. In 1956 Dulbecco *et al.* described the interaction of two animal viruses with their respective antibodies. The results of these

studies led these investigators to conclude, among other things, that animal viruses, at least the two they studied, reacted with antibodies to form complexes that did not dissociate spontaneously. This interpretation was challenged by Fazekas de St. Groth and Reid (1958). As more animal virus–antibody systems were studied by many investigators, there seemed to be greater accord for irreversible, rather than reversible, interaction. For this reason, in this article it is assumed that there are no differences between bacterial viruses, as one category, and animal viruses, as a separate category, concerning their interaction with antibody. Rather, differences, when they exist, are considered to be related to the viruses per se. Although this article is intended to survey the neutralization of animal viruses, occasional reference is made to studies on bacterial viruses when these studies are pertinent and illuminating to the topic at hand.

Previous Reviews

The subject was reviewed in great detail by Fazekas de St. Groth (1962) and, wherever feasible, relationships were reduced to mathematical terms. Subsequent reviews were presented by Svehag (1966, 1968) with emphasis on the biochemical aspects of virus–antibody interactions and the role of antibody diversity on these interactions. In 1971, Notkins and, in 1972, Majer reviewed the topic of viral sensitization by antibody, i.e., the formation of a virus–antibody complex that continues to be infectious but has acquired sensitivity to such reagents as complement, or antibody specific for the antibody that is bound to the virus. More recently, the same topic was surveyed by Oldstone (1975) who also reviewed the subject of immunopathology as it relates to virus-induced immunocomplexes. Other aspects of the general subject of viral immunology have been reviewed by Cowan (1973) who described the characteristics of the immune response to viral antigens, and by Burns and Allison (1975) who discussed the immune response, the mechanism of neutralization, and the nature of cell-mediated immunity. Methods for studying virus–antibody complexes have been reviewed by Mandel (1971a). Of particular relevance are the reviews of Daniels (1975) and Della-Porta and Westaway (1978) that deal with various aspects of the mechanism underlying the neutralization reaction.

II. General Considerations

Studies of the *in vitro* interaction of viruses with antibody have included a wide array of animal and bacterial viruses and antibody derived from a variety of animal species. In some instances antibody was obtained from animals responding to an active infection. In other instances

nonsusceptible animals were induced to synthesize antibody by inoculation of the viral immunogen. In the latter instance, rabbits were used most frequently. As indicated in Section II,C,1 antibodies are a diverse group of molecular species that vary both physicochemically as well as functionally. In the various studies of the *in vitro* reaction of virus with antibody, knowledge of the specific type of antibody was not always available, nor were sufficient details provided of the conditions under which antibody (i.e., the serum) was obtained. It has been reported (Petty and Steward, 1977) that the use of adjuvants to enhance the immune response may affect the level and the quality of antibody according to the composition of the adjuvant. Nonetheless, the general characteristics of the *in vitro* reaction have been found to be reasonably consistent to warrant generalizations.

When the concentrations of virus and antibody are appropriately adjusted, the rate of the reaction, i.e., loss of infectivity, can be measured. The quantitative expression of the rate is based on the early part of the reaction which can be mathematically described by the equation for first-order reactions:

$$C_t = C_0 e^{-kt} \tag{1}$$

where C_t and C_0 are the concentrations at times t and zero, respectively, and k is a constant. When adapted for use in the measurement of neutralization, the equation becomes (Adams, 1950):

$$\frac{V_t}{V_0} = e^{-kt/D} \tag{2}$$

where V_t and V_0 are the respective concentrations of virus, t most frequently is in minutes, and D is the final dilution of serum. The constant k is the rate constant (min^{-1}). When the information is available, the D term can be replaced by the known molar concentration of antibody, in which case k will have the dimensions of min^{-1} $mole^{-1}$.

A. Reaction Variables

The effects of several environmental conditions on the reaction of virus and antibody have been studied. The procedure is quite straightforward, involving measurement of the rate constant under specified conditions.

1. Temperature

Various studies, covering a range of temperatures consistent with the stability of the reactants, have indicated that the reaction is not activated by temperature. Activation energies of the order of 7–9 kcal/mole

have been reported for most animal and bacterial viruses. The interpretation is that the change in rate is dependent on thermal diffusion of the reactants, but the efficiency of the interaction is unaffected.

2. Ionic Strength

The concentration of salt has considerable influence. For example, Jerne and Skovsted (1953) reported that, when the salt concentration was reduced from 0.1 *M* to 0.001 *M*, the rate increased 1000-fold. Others (Svehag, 1968; Wallis *et al.*, 1973) have reported similar findings but not of the same order of magnitude. It is quite likely, as shown by Svehag (1968), that the degree of the salt effect varies with the quality (association affinity) of the antibody. Wallis *et al.* stated that the salt effect was seen with IgG but not IgM antibody, and that antiserum from baboon but not from rabbit or sheep showed this effect.

3. pH

Although not highly dependent on an optimum pH, the reaction becomes decreasingly efficient at either extreme. This undoubtedly reflects a decrease in the number of reactive complementary ionic groups due to their titration at low and high pH. It has been shown that restoration of the pH to neutrality restores the reactivity of virus and antibody, hence the reduced rate at pH extremes is not a consequence of denaturation.

B. Characteristics of Virus–Antibody Interaction

The binding of an antibody molecule to the antigenic site of a virus is mediated by noncovalent interactions. These are short-range interactions such as hydrogen bonding, electrostatic interactions, van der Waal's forces, and possibly nonpolar hydrophobic reactions. Thermodynamic studies have indicated that the forward reaction is accompanied by an increase in entropy and a change in mean free energy of the order of -10 kcal/mole. The reaction therefore is "downhill," i.e., is entropy-driven. Studies on the effect of temperature on the reaction rate have indicated that the system is not subject to activation and that the rate changes observed are related simply to changes in collision frequency induced by changes in thermal diffusion. Reaction rates of the order of 10^7 $mole^{-1}$ sec^{-1} have been reported for several systems, indicating a very rapid process. Hornick and Karush (1969) have suggested that the rate-limiting variable is collision efficiency rather than collision frequency. A successful collision requires that both reactive sites be in apposition at the time of approach. It has been indicated that, when the functional binding

affinity of virus–antibody interaction is examined, the effect of valence is extremely important (Karush, 1976). In studies with bacteriophage ϕX174 there was an increase of more than 10^4 for bivalent compared with monovalent binding. Blank *et al.* (1972) similarly stressed this aspect. In view of the fact that most virus–antibody interactions involve bivalent antibody and multivalent viral particles, it is likely that the majority of virus–antibody complexes are characterized by monogamous bivalent unions, hence by a very high binding affinity.

C. The Components of the System

Although the tendency may be to focus on the interactions of virus with antibody as the heart of the reaction, it has become clear that the host cell too must be scrutinized. The reason for this is twofold: (1) The outcome of a given reaction may vary with the host cell or host animal. (2) Since viruses are inert, antibody does not inhibit any viral function other than adsorptive behavior. Therefore, in instances in which neutralized virus adsorbs, the failure to infect is due to the inability of the cell to react to the virus as it would to an unneutralized particle.

1. Antibody

The diversity of antibodies in the serum of an animal at a given time is a function of several sources of variability. (1) Different classes and subclasses of antibodies exist that vary in their physicochemical as well as their functional characteristics. (2) The class of antibody produced may depend on the mass of immunogen. It has been shown (Svehag and Mandel, 1964a) that, with a relatively low amount of poliovirus, only the 19S class of antibody is induced in rabbits. With higher amounts both 19 and 7S antibodies are elicited. (3) It has been shown in many instances that the quality, i.e., the effectiveness, of antibodies improves with increasing time during the immune response. This characteristic implies a maturation process reflecting a progressive (either continuous or discrete) improvement in the combining characteristics of the antibody. (4) Viral immunogens that have more than one determinant induce antibodies to each one. Interaction of such an unfractionated serum with the respective virus can be expected to involve a complex set of interactions involving each respective antibody–antigen system as well as possible influences among the different systems due to steric complications. (5) Each of the technical aspects of the immunization procedure —dose of immunogen, route of inoculation, frequency of inoculation, use of adjuvants—influences the time course and the class of antibody responding to immunization.

2. Host

It was reported in the earlier literature (e.g., Burnet *et al.*, 1937) that the outcome of a neutralization reaction was not absolute. It may register as a positive or negative result depending on the host cell or animal used as indicator. The importance of this source of variation was reemphasized more recently by the results described by Kjellén and Schlesinger (1959), and subsequently by other investigators. This may be an important consideration, since the nature of the neutralization phenomenon may be an alteration in the viral capsid of lesser or greater subtlety. This alteration may, for one cell, represent a qualitatively unrecognizable particle but, for another, an altered particle albeit one that the cell still recognizes and can cope with, perhaps inefficiently. Andrewes and Elford (1933b), for example, discussed the basis for abnormally small plaques in terms of "incompletely neutralized" virus. It has also been shown that the rate of appearance is delayed when survivors of animal virus neutralization are assayed (Mandel, 1958; Westaway, 1965a; Yoshino and Taniguchi, 1965b). An unusual host variation was described by Hawkes (1964). Antiserum to some, but not all, togaviruses enhanced infectivity providing (1) antiserum was obtained from fowl, not rabbits or mice, and (2) the host was chick cells, not pig kidney cells. Enhancement resulted from interaction with low concentrations of serum, but neutralization was observed with higher concentrations. Subsequent studies (Hawkes and Lafferty, 1967) suggested that enhancement was the result of a modification of the protein capsid that rendered the virus more efficient in its interaction with chick cells.

3. Virus

Three aspects are of relevance—complexity, stability, and origin of viral antigens. With respect to stability, the genotype of viruses is usually very stable, and one can be assured that a given virus will display the same antigenic characteristics at all times, irrespective of the host in which it replicates. For enveloped viruses, the situation is complicated by the fact that the antigens are derived from two sources: (1) stable virus-coded proteins and (2) the host-coded lipoproteins that comprise the envelope. It has been thoroughly established that the viral envelope is acquired from the host cell during maturation (e.g., Haukenes, 1977). Although the envelope contains viral proteins, it also retains the antigenic characteristics of the host. Consequently, these viruses induce and react with two groups of antibodies with different specificities.

Nonenveloped viruses may be simple or complex. RNA bacteriophages, for example, are constructed of one kind of polypeptide (the one mole-

cule of A protein may be irrelevant). Although picornaviruses contain four polypeptides, certain members (poliovirus, rhinovirus) induce the synthesis of only one specific antibody, probably because the morphological unit functions as an immunogenic element.

With increasing complexity, the number of determinants increases. The interpretation of the effect of antibody on such viruses becomes difficult, since distinctions are required among critical, noncritical, and possibily quasi-critical sites. To illustrate, Madeley *et al.* (1971) showed that the hemagglutinin and neuraminidase antigens of influenza virus induced specific antibodies. The antihemagglutinin antibody neutralized infectivity, while the antineuraminidase antibody had no such effect. They reported that these antibodies did not interfere with each other, suggesting that the antigenic structures were so arranged that there was no steric interference. Majer and Link (1971) similarly observed specific antibody responses. In addition, they showed that the nonneutralizing interaction with neuraminidase antibody could lead to neutralization by the addition of antiglobulin serum. A reasonable explanation, in the opinion of these workers, was that steric interference with the hemagglutinin antigen followed the secondary antibody reaction.

For other viruses, the complexity is aggravated. Adenovirus has three protein antigens, hexon, penton, and fiber, and each elicits a specific antibody. For some strains the hexon antibody has neutralizing consequences. It has, however, been shown that this interaction is rather complicated (Kjellén and Pereira, 1968). The hexon consists of two antigens, one of which is subsurface. The interaction of antibody with the surface antigen results in a rearrangement of the hexon structure that exposes the second antigen. Neutralization is a consequence of the reaction of this antigen with antibody. Recently, Symington *et al.* (1977) studied the effect of antisera prepared specifically against the two (E1, E2) surface glycoproteins of Sindbis virus. Each antiserum alone had poor neutralizing activity. Antiserum versus the intact virion, however, was more active than the two specific sera, separately or in conjunction.

Brown and Smale (1970) described a complex interaction between FMD virus (a picornavirus) and viral antiserum that suggests three distinct antigenic binding sites. Two are associated with the vertex regions, and the third with the planar surfaces. There is some specificity with respect to the interactions of IgG and IgM with their respective combining sites. Since all studies were based on agar precipitin reactions and electron microscopy, the significance of these interactions cannot be assessed with respect to neutralization. Subsequent studies (Rowlands *et al.*, 1971) revealed that the antigens located at the vertexes were involved in stimulating the production of neutralizing antibody. Recently, Cavan-

agh *et al.* (1977) reported that the VP_1 capsid polypeptide of FMD virus induced neutralizing antibody. Studies on another picornavirus, poliovirus, showed that antigenic specificity was related to a structural requirement. Subviral 14S particles had the same specificity as the parent particle, however, the 5S substructure of the 14S particle was antigenically distinct.

D. Neutralization by Antibody Fragments

Proteins with antibody activity encompass a diverse group of molecular species that vary in several parameters: MW, net surface charge, conformation, number of antigen-binding sites (valence), propensity for reacting with antigen (avidity), and force for binding antigen (affinity). The numerically dominant class of antibody, the 7S IgG form, is composed of two heavy (H) and two light (L) polypeptide chains bound by interchain disulfide linkages. The two antigen-binding sites are located at the termini of two pairs of L-H chains.

The molecule can be dissected enzymically so as to obtain specific fragments. Pepsin cleaves the molecule into a 5S fragment which contains both combining sites but lacks a portion of the H chains. The 5S fragment, designated $F(ab')_2$, can be dissociated under reducing conditions into two monovalent 3.5S fragments designated Fab'. Papain cleaves the 7S molecule into two monovalent fragments designated Fab, and one fragment, designated Fc, which has no antibody activity. A third method for dissecting the molecule is based on reduction of the interpeptide disulfide bonds to yield free L and H chains. The different classes of antibody vary in valence (bi-, tetra-, decavalent) and, accordingly, in MW from about 150,000 to about 900,000 daltons. For a comprehensive discussion of this subject, the reader is referred to Spiegelberg's review (1974).

The functions of the various segments of the antibody molecule in the neutralization reaction have been studied by isolating the desired fragment and examining its behavior. Vogt *et al.* (1964) demonstrated that the Fab fragment of poliovirus antibody retained neutralizing activity but at reduced efficiency. Comparison of the Fab fragment with native antibody by kinetic analysis showed a reduced reaction rate for the monovalent Fab fragment. Vogt *et al.* (1964) also stated that the virus–Fab complex was stable when subjected to dilution. Although the kinetic reactions were not observed long enough to be sure, the data indicated that eventually both reactions approached the same final level, although at different rates.

Cremer *et al.* (1964) reported that poliovirus and WEE virus were

neutralized by the Fab fragments of their respective antibodies. These results were obtained with the metabolic inhibition test. When, however, neutralization of WEE virus was evaluated by mouse infectivity, there was no evidence of neutralization. These workers considered the possibility that dissociation had occurred or that the Fc moiety of the molecule was required.

Philipson *et al.* (1966) developed an interesting system for detecting virus–antibody interaction based on the distribution of virus between two phases of an aqueous polymer system. When poliovirus was neutralized by intact antibody, its distribution pattern was inverted. When, however, virus was neutralized by the Fab fragment, its distribution pattern remained characteristic of unneutralized virus (Philipson and Bennich, 1966). It was also reported that virus neutralized by the partially separated but otherwise intact IgG molecule (as a result of reduction and alkylation) showed a distribution pattern intermediate between that of virus neutralized by native IgG and that of virus neutralized by Fab. These results suggest that the distribution pattern of virus–antibody complexes is related to the charge characteristics of both virus and antibody, or antibody fragment, and that both species of the complex may undergo modifications as a consequence of interaction.

Neutralization reactions of poliovirus by 7S IgG, 5S $F(ab')_2$, and 3.5S Fab′ were compared (Keller, 1966). Neutralization was similar for all three forms of antibody. The stability of the virus–antibody complexes, however, varied when examined by dilution dissociation and by acid reactivation. Shortly after formation, the complex with 3.5S antibody was the least stable, and with 7S antibody the most stable. Given sufficient time, all complexes approached a uniform state of stability. Inasmuch as 7 and 5S antibodies are both bivalent, Keller considered the likelihood that the Fc fragment contributed to the stability of the virus–antibody complex. In light of this report, a subsequent article by Keller (1968) is puzzling. Poliovirus was neutralized with 7S antibody. Exposure of the virus–antibody complex to pepsin converted the 7S molecule to the 5S form without a change in the neutralized status of the virus. Further treatment with reducing agent converted the bivalent 5S molecule to the monovalent 3.5S form with restoration of infectivity. That the antibody fragment was still associated with virus was shown by the sensitivity of the complex to antibody specific for the attached 3.5S fragment. Keller had previously (1966) reported that the 3.5S fragment had neutralizing activity.

In a study of the effect of neutralizing antibody on poliovirus (Mandel, 1971b) it was seen that neutralization was accompanied by a change in the electrophoretic characteristics of the virus. A further analysis of this

phenomenon (Mandel, 1976) showed that the Fab fragment had the same effects as the native 7S antibody, namely, neutralization and electrophoretic modification.

The interaction of adenovirus with monovalent antibody fragments was examined by Kjellén (1964). Although the fragments inhibited hemagglutination, there was no reduction in infectivity. Antibody fragment was shown to have bound to virus by a blocking experiment. Possibly, Kjellén conjectured, the Fc portion was essential for neutralization, or the fragment had reacted with a site that was not critical. The latter possibility poses the question, Why, then, does it block the action of neutralizing antibody? In studies with influenza virus, Lafferty (1963b) reported that the Fab fragment neutralized infectivity and inhibited hemagglutination. However, in contrast to 7S antibody, the virus–monovalent antibody complex did not acquire stability. Lafferty attributed this deficiency in the antibody to its monovalency. Neutralization of herpesvirus by Fab fragments was reported by Ashe *et al.* (1969). It was also shown that the Fab fragment of anti-antibody neutralized herpesvirus that had been sensitized by viral 7S or Fab antibody. Shinkai and Yoshino (1975b) examined the reactivity of antibody fragments toward herpesvirus. At 2 weeks after initiation of the immune response, $F(ab')_2$ neutralized poorly, but at 4 weeks its neutralizing capability equaled that of IgG. At 4 weeks the monovalent Fab′ fragment failed to neutralize, per se, as well as in the presence of anti-antibody. Evidence that the fragment had combined with virus was provided by blocking experiments. After 9 weeks into the immune response, Fab′ displayed a moderate neutralizing capacity which could be enhanced by anti-antibody.

Several investigations of the interaction of bacterial viruses with antibody fragments have been reported. Klinman *et al.* (1967) used fragments to evaluate the role of antibody valence in the neutralization of an RNA bacteriophage, R17. A comparison of the neutralization rate constants for bivalent 7 and 5S molecules with those for monovalent 7, 5, and 3S molecules revealed a 30-fold higher rate for the bivalent molecules. The 7 and 5S monovalent antibodies were hybrid molecules containing one reactive site. These results stress the contribution of valence while minimizing a role for the Fc portion of the antibody molecule. These investigators attributed, speculatively, the lower reaction rate for the monovalent antibodies to a lower binding affinity and a concomitant higher dissociation rate compared with those of bivalent antibody. Rowlands (1967) questioned the role of antibody size and its steric effect on neutralization efficiency. Neutralization rate constants (molecule^{-1} ml min^{-1}) of 7, 5, and 3.5S antibodies were determined using

bacteriophage f2 (closely related to R17). For 7, 5, and 3.5S molecules the rate constants were 2.12×10^{-12}, 0.44×10^{-12}, and 0.16×10^{-12}, respectively. Since there was a considerable disproportionality between rate constant and molecular size, he concluded that size was a relatively minor determinant in efficiency of neutralization. In studies on the neutralization of two coliphages, T1 and T6, Goodman and Donch (1964) observed that the efficiency decreased as size and valence were reduced in going from a 7S to a 5S to a 3.5S molecule. In a later study Goodman and Donch (1965) analyzed the role of molecular size in neutralization. They examined the neutralizing ability of L and H chains and of the Fd fragment (the H-chain portion comprising the antigen-binding region of the antibody molecule). Neither L, H, nor Fd fragment neutralized bacteriophage T1. However, binding to virus occurred, since neutralization resulted when antibody specific for the respective fragments was added to the virus–fragment complex. These workers proposed that lack of neutralizing activity was related to an insufficiently large fragment size.

Stemke and Lennox (1967) compared the neutralization rates of two coliphages, T2 and T5, by bivalent and monovalent antibody. The bivalent 7S antibody rate was about 40-fold greater than that of the Fab I fragment, and about 10-fold greater than that of the Fab II fragment. They reported the interesting observation that, whereas the interaction of phage T2 was single-hit with the 7S antibody, it was multihit (about 2.5 average hits) with the Fab fragments. However, all reactions with phage T5 were of the single-hit form. When the reactions were enhanced by the addition of antibody directed against the viral antibody, it was observed that the degree of enhancement was inversely related to the neutralization efficiency. Stemke and Lennox proposed that several parameters such as size, valence, and binding affinity, separately or in conjunction, contributed to the activity of antibody. They also questioned the significance of the use of anti-antibody. Perhaps, they suggested, neutralization that required the secondary effect of anti-antibody was not a bona fide interaction with respect either to the antibody per se or to the viral site to which it binds. Stemke (1969) further corroborated the greater importance of valence over size of the antibody molecule.

Blank *et al.* (1972) evaluated the relative influence of antibody valence and affinity on the neutralization reaction. Based on their studies of the interaction of DNP–T4 (bacteriophage T4 coupled with the hapten DNP) with rabbit anti-DNP antibody, they concluded that valence was of greater significance. Bivalent antibody was found to be at least 1000-fold more efficient than monovalent antibody. Since the forward

reaction was extremely rapid, these investigators considered that the neutralization rate reflected the rate of dissociation of virus–antibody complexes.

Hornick and Karush (1969) studied the neutralization of DNP–ϕX174 bacteriophage. Among other observations, they reported that bivalent antibody was much more efficient (10^4) than monovalent antibody, again stressing the importance of valence.

E. The Neutralization Phenomenon

A survey of the literature, or of the reviews of the literature, indicates that several different phenomena are generically grouped as viral neutralization. Justification for this is found in the fact that there is a measure of consistency among all insofar as virus, antibody, and host indicator are, in all instances, part of the phenomenon, and that the criterion is, for all, loss of infectivity. A closer examination indicates that the underlying interactions have important differences. That there is an awareness of this consideration is reflected in the use of the plural form when Daniels (1975) and Burns and Allison (1975) refer to "mechanisms" of neutralization in their discussions. It has been shown that neutralization may require the contribution of mediators, e.g., complement, or may depend on secondary phenomena such as aggregate formation. It may therefore be useful for the purpose of analyzing reaction mechanisms to define the various situations.

1. Intrinsic Neutralization

a. Primary. At the simplest level, a virion composed of multiple copies of a single antigen is neutralized by one or more molecules of a single molecular species of antibody. This interaction is independent of any "third-party" mediation and of secondary events such as aggregation. The possibility that formation of the virus–antibody complex is the initial stage of sequential rearrangements within the complex is, however, recognized as a segment of a single event. The possibility that the neutralized state is conditional, namely, dependent on the host, is an unavoidable, but possibly explicable and informative, complication.

b. Mediated. A primary reaction that fails to go to completion may be activated by interaction of the bound antibody with complement, or interaction with an antibody to itself.

c. Biphasic. Two antigen–antibody reactions are involved. The first reaction enables the second reaction to take place, which culminates in neutralization.

2. Extrinsic Neutralization

a. Steric Hindrance. Involvement of a critical antigen does not occur when the virion is neutralized. Interaction of noncritical antigens with antibody results in the amassing of antibody that interferes, possibly by preventing adsorption, with viral replication.

b. Virolysis. Interaction of enveloped viruses with envelope-specific antibody (which is also host-specific) in the presence of the complete complement system results in irreversible traumatization of the envelope. Since these viruses can also be neutralized by antibodies that are specific for virus-coded antigens, virolysis represents an alternative neutralization pathway.

3. Pseudoneutralization

Since viruses are multivalent and antibody bivalent, it is possible to induce aggregate formation by appropriately adjusting the concentrations of the reactants. The interaction of antibody with noncritical sites does not lead to neutralization. However, if the concentrations are equivalent, secondary events will occur, resulting in the formation of a lattice structure. Reduction in infectivity may follow simply as a result of the polymerization of several monomeric units into a single infectious unit.

III. Reversibility

A. Experimental Results

One of the conclusions Burnet *et al.* (1937) arrived at in their studies of several bacterial and animal viruses was that the two groups differed fundamentally in their interaction with antibody. Whereas bacterial viruses formed irreversible complexes with neutralizing antibody, animal viruses (those examined by Burnet *et al.*) formed freely reversible complexes (Burnet, 1960). The assay methods employed were at least formally comparable, namely, the plaque method for bacteriophages and the then newly developed pock-counting method utilizing the chorioallantoic membrane of embryonated chick eggs for animal viruses. Subsequent studies (Dulbecco *et al.*, 1956) of two animal viruses, poliovirus and WEE virus, employing the plaque assay method with animal cell cultures, indicated that the reaction of these viruses with antibody was irreversible. Reversibility, in the present context, represents a dynamic equilibrium between reactants and product with the final equilibrium depending upon association and dissociation rates, which in turn are concentration-dependent. The demonstration of reversibility requires that

the sole variation in the reaction conditions be a change in the concentration of the reaction components, usually by dilution. Such a change should then drive the reaction toward dissociation until reestablishment of the equilibrium characteristic for the particular system.

This aspect of viral neutralization has been under examination from the time that neutralization was first subjected to experimental scrutiny. Interpretation of early studies indicated that reversibility was characteristic of the neutralization of fowl plague virus (Todd, 1928), vaccinia virus (Andrewes, 1928; Long and Olitsky, 1930), EEE virus (Pierce *et al.*, 1941), papilloma virus (Bryan and Beard, 1941), influenza virus (Taylor, 1941; Burnet, 1936, 1943), and poliovirus (Gebhardt and Bullock, 1931). However, the reaction was considered irreversible for papilloma virus (Friedewald and Kidd, 1940) and EEE virus (Labzoffsky, 1946), and partially reversible for influenza virus (Taylor, 1941; Burnet, 1943).

It has also been reported that the extent of dilution dissociation decreased with prolonged incubation (Andrewes, 1930; Burnet, 1943). This aspect of viral neutralization was examined by Gard (1955) using a particularly appropriate system, namely, a strain of Theiler's virus (FA strain of mouse poliomyelitis) which could be assayed in mice with or without dilution based on the incubation period. He observed that, whereas the undiluted virus–serum mixture indicated neutralization, the diluted mixture did not. However, after 24 hours at 37°C or 10 days at 4°C, dilution of the reaction mixture showed the expected decrease for a nondissociating complex. Gard proposed that the interaction between virus and antibody entailed two stages—a rapid but weakly cohesive union followed by enhancement of the forces binding virus and antibody. It may be relevant that the source of antibody in this study was serum from normal mice. It was shown by Olitsky (1940) that normal mice almost universally carry the TO strain of Theiler's virus. Antibody in the sera of these mice is TO-specific. The extent of cross-reactivity between the two strains may be a contributing factor to this phenomenon which Gard called "immunoinactivation." However, in subsequent studies with human poliovirus and homologous antibody, Gard (1957) observed the same immunoinactivation characteristics.

Using the plaque assay for animal viruses employing animal cell cultures, Dulbecco *et al.* (1956) analyzed the reaction of WEE virus and poliovirus with antibody. With respect to dilution dissociation, they reported that the phenomenon did not occur with these viruses. Studies of the reversibility of these and other viruses employing cell cultures as the test system indicated no spontaneous dissociation for NDV (Rubin, 1957; Rubin and Franklin, 1957; Granoff, 1965), poliovirus (Mandel,

1958, 1961; Ketler *et al.*, 1961; Keller, 1965; Wallis *et al.*, 1973), adenovirus (Kjellén, 1957, 1962), visna virus (Thormar, 1963), herpesvirus (Yoshino and Taniguchi, 1965b; Ashe and Notkins, 1967), or VEE virus (Hahon, 1969). The possibility that dissociation occurs but to a minor degree was considered for JEV (Hashimoto and Prince, 1963; Iwasaki and Ogura, 1968b) and was proposed for herpesvirus (Ide and Yoshino, 1974) and WN virus (Westaway, 1965a).

The data from which Dulbecco *et al.* (1956) concluded that the reactions were irreversible were reinterpreted by Fazekas de St. Groth and Reid (1958) to show that the results were consistent with a reaction which is freely reversible and subject to mass-action phenomena. Reaffirmation of the view that a freely reversible equilibrium characterizes virus–antibody interaction resulted from studies on neutralization of influenza virus (Fazekas de St. Groth and Webster, 1963). The complexity of the neutralization phenomenon has been stressed by Burnet (1960, p. 298), Dulbecco *et al.* (1956), Fazekas de St. Groth and Reid (1958), and Fazekas de St. Groth (1962). It is clear that neutralization consists of three domains—virus, antibody, and assay host—and each domain can be divided into subdomains of varying characteristics. To illustrate, Svehag (1965) compared the dissociability of poliovirus–rabbit antibody complexes when antibody was either early 19 or late 7S. In neither case was dissociation evident with simple dilution. When dilution was carried out in conjunction with increasing ionic strength, minor but significant dissociation occurred when virus was complexed to 19S antibody. With 7S antibody no dissociation occurred when salt molarity was increased from 0.14 *M* to 5.0 *M*. The bond strength of the complex is a function of antibody type. The results of Svehag (1965) indicate that with late antibody the affinity constant is exceedingly high.

In studies with poliovirus (Mandel, 1961) it was shown that, when centrifuged neutralized virus was suspended in antibody-free medium, no evidence for reversibility was obtained over a period of 50 days at 5°C. Both diluted and undiluted resuspended virus was monitored. Neutralized virus was prepared using varying antiserum multiplicities that resulted in surviving fractions as high as 10%. Potentially dissociable virus–antibody complexes were demonstrated by acid reactivation of infectivity.

An analysis of dissociability on a molecular level was described by Lafferty (1963a,b). The reaction of influenza virus with antibody was biphasic with respect to time. Shortly after initiation of the reaction, the virus–antibody complex was completely reversible by dilution. Given sufficient time, however, the complex became irreversible. When monovalent antibody fragments were used, virus–antibody complexes were

formed but failed to become irreversible. Lafferty (1963a,b) proposed that the transition to irreversibility represented the time required for the second combining valence of a bound antibody molecule to bind to an adjacent antigenic site on the same virion. That such monogamous bivalent binding occurred was demonstrated by electron microscopy (Lafferty and Oertelis, 1963). Reversibility therefore requires the simultaneous dissociation of both antibody–virus bonds. The probability of such an event is proportional to the square of the probability of the single event. Lafferty (1963a) also suggested that close scrutiny of the methodologies employed in studies of neutralization could reconcile the two views, i.e., that at least some features of a freely reversible system are characteristic of virus–antibody interaction.

B. Summary

The preponderance of recent evidence points to the virus–antibody (late) complex as a firmly bound structure. Under certain limited conditions, dissociable complexes can actually be demonstrated. Under other conditions dissociation is an artifact inherent in the experimental procedure.

The initially formed monovalent complex is readily dissociable. With time, as bivalent binding occurs, the complex becomes stabilized. The degree of stabilization has been estimated to be several orders of magnitude increase in affinity constant. Even after stabilization a very small fraction of the neutralized population is readily dissociable by dilution, and the presumption is that virus has reacted with antibody of very low affinity.

The rate of neutralization depends on antibody concentration. When testing for dilution dissociation, the procedure is to prepare several dilutions of a virus–serum mixture and assay each for infectivity. If dilutions are made while the reaction is in progress, the rates will diminish in proportion to the dilution. After inoculation of the test host, the reaction continues until virus has interacted with susceptible cells. Moreover, the presence in the host of antiserum in different concentrations tends to bias the results additionally. The net result can be a disproportionality of surviving virus with dilution, which in fact is due to decreased neutralization rates rather than dissociation.

IV. The Nonneutralized Fraction

One result of the studies of Dulbecco *et al.* (1956) on the neutralization of animal viruses was to focus attention on a phenomenon they called the "persistent fraction." The observation that a fraction of a viral

population was refractory to neutralizing antibody had already been described by Andrewes and Elford (1933b) for several bacteriophages, and by Burnet *et al.* (1937) for several animal viruses. With respect to the phages they had examined, Andrewes and Elford proposed that failure to be neutralized was not attributable to genotypic resistance, nor to the tendency for virus–antibody complexes to dissociate. A reasonable explanation for this phenomenon, in the view of Andrewes and Elford, was that combination with antibody had occurred but without sufficient effect to negate infectivity. This view was supported by observations that (1) abnormally small plaques were produced, and (2) infectious particles were nonfilterable in contrast to the control virus. These surviving particles were designated "incompletely neutralized." As Andrewes and Elford interpreted this phenomenon, a small number of antibody molecules had combined with antigenic determinants of the virus which were distinct from the host combining determinants. The presence of antibody constituted a steric encumbrance, thereby retarding the initiation of replication, hence small plaques were produced.

The demonstration of a "persistent fraction" by Dulbecco *et al.* (1956) for animal viruses by means of a plaque assay method stimulated renewed interest in this phenomenon. Although Dulbecco *et al.* eliminated several explanations, e.g., dissociation, participation of nonantibody serum components, and hereditary heterogeneity, a positive explanation had yet to be found. Subsequent studies indicated that this phenomenon was characteristic of a wide variety of viruses (Wallis and Melnick, 1967), e.g., picornavirus (Dulbecco *et al.*, 1956; Mandel, 1958; Bradish *et al.*, 1962; Wallis and Melnick, 1965; Ozaki, 1968; Fiala, 1969; Lewenton-Kriss and Mandel, 1972), myxovirus (Lafferty, 1963a,b), paramyxovirus (Granoff, 1965), poxvirus (Lafferty, 1963a,b; McNeill, 1968; Majer and Link, 1970), togavirus (Dulbecco *et al.*, 1956; Hashimoto and Prince, 1963; Rawls *et al.*, 1967; Ozaki and Tabeyi, 1967; Westaway, 1968; Hahon, 1970b; Symington *et al.*, 1977), herpesvirus (Yoshino and Taniguchi, 1965b), equine arteritis virus (Hyllseth and Petterson, 1970), reovirus (Huggett *et al.*, 1972), and bacteriophages (Hale *et al.*, 1969; Rappaport, 1970). Kjellén (1962) observed a gradual increase in resistance to neutralization the longer adenovirus reacted with antiserum. He considered this result to be indicative possibly of a requirement for increased antibody multiplicity, but not because of dissociation or refractoriness.

The practical significance of this phenomenon was emphasized by several reports showing the presence of infectious virus–antibody complexes in the circulatory system of LDH virus-infected mice (Notkins *et al.*, 1966), visna virus-infected sheep (Gudnadóttir and Pálsson, 1965),

H-1 virus-infected hamsters (Toolan, 1965), LCM-infected mice (Oldstone and Dixon, 1967, 1969), mink infected with Aleutian disease (Porter and Larson, 1967), and MLV- and MSV-infected mice (Hirsch *et al.*, 1969).

The "persistent fraction" has also been referred to as the nonneutralizable fraction. Since, as discussed in Section IV, this fraction can be neutralized under certain conditions, it seems more appropriate to designate it the nonneutralized fraction.

Several basically divergent hypotheses have been proposed to explain the presence of a nonneutralized fraction (this designation is used in preference to "persistent fraction"). Fazekas de St. Groth and Reid (1958) ascribed it simply to a reversal of the neutralization reaction, i.e., dissociation. Wallis and Melnick (1967, 1970) considered that no unusual interaction between virus and antibody occurred. However, the formation of aggregates, either before or during interaction with antibody, resulted in the shielding of some particles from antibody. A somewhat similar view was proposed by Rappaport (1970) differing, however, in one respect, namely, that for a single virion, binding of antibodies may occur to most antigenic determinants, leaving others free and inaccessible, hence the particle retains infectivity. Bradish *et al.* (1962) proposed an "amphoteric" state for some virus–antibody complexes such that either viral or antibody function dominated, depending on various contributory factors, e.g., the type of host cell comprising the test system. A similar interpretation was proposed by Lafferty (1963a) for rabbit poxvirus.

Heterogeneous antibody populations in a given serum with the capacity to neutralize, or to induce the nonneutralized state, were considered a possibility (Lafferty, 1963a,b; McNeill, 1968; Lewenton-Kriss and Mandel, 1972; Ozaki *et al.*, 1974). All hypotheses, except that of Fazekas de St. Groth and Reid (1958), envision the nonneutralized fraction as consisting of virus complexed with antibody without absolute loss of infectious capability. Recently, Ide and Yoshino (1974) revised their previous interpretation of the nonneutralized state of herpesvirus and concluded, as did Fazekas de St. Groth and Reid, that a freely reversible equilibrium could account for their findings.

A. Hypothetical Mechanisms

1. Aggregation Hypothesis

Wallis and Melnick (1967) reported that the previously observed (Wallis and Melnick, 1965) resistance of a strain of echovirus to neutralizing antiserum could be overcome by eliminating viral aggregates.

In a monodispersed state, virus was readily neutralized. Extrapolation of this possibility to representatives of various groups of viruses revealed that in every case filtrates consisting of singly dispersed particles could be completely neutralized, whereas unfiltered virus showed nonneutralized fractions (Wallis and Melnick, 1967). Further analysis of this problem (Wallis and Melnick, 1970) disclosed that, when monodispersed herpesvirus was exposed to minimal concentrations of antibody, the resultant virus–antibody aggregates became nonneutralizable. Attempts by other investigators to eliminate the nonneutralized fraction by the method of Wallis and Melnick met with varying results. A reduction, but not complete elimination, was reported for VEE virus (Hahon, 1970b) and rhinovirus (Fiala, 1969). In other studies it was reported that filtration had no effect on the nonneutralized level or neutralization characteristics (Ashe *et al.*, 1969; Hyllseth and Petterson, 1970; Majer and Link, 1970; Huggett *et al.*, 1972; Lewenton-Kriss and Mandel, 1972), although elimination of a short lag period (Majer and Link, 1970) and a slight increase in neutralization rate (Lewenton-Kriss and Mandel, 1972) were observed. Baughman *et al.* (1968) described a complex result with respiratory syncytial virus. Filtration considerably reduced the nonneutralized fraction when horse antiserum was used. However, when guinea pig serum was used, no difference was seen between filtered and unfiltered virus.

2. Host Cell Hypothesis

Several studies focused on the role of the host cell, probably because of the striking illustration by Kjellén and Schlesinger (1959) that the outcome of virus–antibody interaction was affected by the host cell system serving as the indicator of neutralization. Bradish *et al.* (1962) observed that aliquots of FMD virus–antiserum mixtures assayed in pig kidney cell cultures and in mice showed 100-fold lower viral survival in the latter host. A similar observation was described by Lafferty (1963a) with rabbit poxvirus. Survival was considerably lower when tested in rabbit skin, compared with mouse brain or chorioallantoic membrane. In another study on the neutralization of poxvirus (McNeill, 1968), the neutralization rate as well as the nonneutralized level varied according to whether assays were done with monkey kidney or HEp2 cells, the latter showing a higher rate and lower final survival level. When the outcome of neutralization of poliovirus was assayed in HeLa or monkey kidney cell cultures, the results were the same (Dulbecco *et al.*, 1956; Lewenton-Kriss and Mandel, 1972). However, the interesting observation

was made (Lewenton-Kriss and Mandel, 1972) that virus propagated in HeLa cells yielded a considerably higher nonneutralized fraction than virus propagated in monkey kidney cells, both having been assayed in HeLa cells. It was also shown in the same study that results of mediated neutralization varied with the host cell, i.e., primary neutralization results were the same for HeLa and monkey kidney cells, but antiglobulin neutralization was seen only with HeLa cells. Hahon (1970b) surveyed five different cell lines and found the same level of nonneutralized VEE virus with each. These studies, as stressed by Bradish *et al.* (1962), strongly imply that nonneutralizability is a relative condition, a state of limbo, which is dependent on the host cell for the properties that the virus–antibody complex will manifest.

3. Antibody Heterogeneity Hypothesis

Considerable interest has centered on the role of antibody in establishing the nonneutralized state. Evidence was presented for poliovirus (Mandel, 1958) and LDH virus (Notkins *et al.*, 1966) that nonneutralized virus reacted with antibody, since antiserum prepared against the globulin fraction of the viral antiserum neutralized all or part of the nonneutralized fraction. It became of interest to determine (1) if virus that was not neutralized had reacted with a unique class of antibody, or (2) if the antibody was conventional but the antigen with which it combined was not essential for infectivity, or (3) if the characteristics of the interaction per se were unique. Recognition of an infectious virus–antibody complex was based on neutralization mediated by antibody specific for the antiviral serum globulin, namely, mediated neutralization.

A somewhat puzzling observation was described by Capstick *et al.* (1960) and Bradish *et al.* (1962). The size of the nonneutralized fraction of FMD virus was inversely related to serum concentration. A recent report (Symington *et al.*, 1977) described in more detail a similar observation, namely, at high- and low-antibody multiplicity the nonneutralized fraction of Sindbis virus was small, but at intermediate multiplicities the fraction was large. These workers considered the situation to be complex in that the serum contained antibodies of different avidities in unequal concentrations, and that the different antibodies either neutralized or protected against neutralization. With varying concentrations of antiserum one or the other kind of antibody had a competitive advantage. In studies with poliovirus evidence was presented that implicated antibody heterogeneity. Ozaki (1968), Svehag (1968), and Lewenton-Kriss and Mandel (1972) showed that the level of nonneu-

tralized virus was highest with the earliest collected antiserum and decreased the later this antiserum was collected during the immune response. McNeill (1968) suggested the possibility of a kind of antibody that interfered with neutralizing antibody but did not elaborate on the nature of this putative antibody.

In studies on rabbit poxvirus, Lafferty (1963a) concluded that the nonneutralized state was attributable to the interaction of virus with either of two kinds of protective antibody. In one case virus reacts with nonavid antibody. Such a complex is readily reversed by washing or by dilution. In the second case, virus binds firmly and retains its protected status. In each case, the complex is conditionally infectious, depending on the host system.

Ozaki (1968), Svehag (1968), and Lewenton-Kriss and Mandel (1972) showed that the level of the nonneutralized fraction of poliovirus decreased as antisera were collected at later times during the immune response. Westaway (1968) and Ozaki *et al.* (1974) reported similar observations for several togaviruses. It has been shown (Svehag, 1965) that the chronology of the immune response can be characterized by successive changes in the class of antibody produced, as well as qualitative changes within a class. Antibodies produced early are predominantly of the macroglobulin (19S) type with low binding affinity. These are succeeded by the 7S type which tends, with time, to increase in binding affinity. A similar relationship was shown to exist in the response of individuals actively infected with poliovirus (Brunner and Ward, 1959; Ogra *et al.*, 1968), arbovirus (Bellanti *et al.*, 1965), mumps virus (Brown *et al.*, 1970; Daugharty *et al.*, 1973), coxsackievirus (Schmidt *et al.*, 1968), or influenza virus (Brown and O'Leary, 1971). These observations tend to incriminate a specific type of antibody as the cause of the nonneutralized state.

Hahon (1970b) prepared nonneutralized VEE virus with human antiviral antiserum. For mediated neutralization he prepared anti-human IgG, IgA, and IgM in goats. The anti-IgG and anti-IgA sera were effective, the latter somewhat less. The anti-IgM had no secondary neutralizing activity. It was also shown that goat anti-human Fab and anti-Fc sera had secondary neutralizing activity, the latter quite weak. These results implicated IgG and IgA as nonneutralizing antibodies. The absence of anti-IgM activity indicated either that this class of antibody was not involved, or that it was absent in the virus-specific antiserum. A similar result was described by Notkins *et al.* (1968) in their studies on LDH. Virus was sensitized with mouse immune serum. Goat anti-mouse globulin neutralized the sensitized virus. Rab-

bit anti-mouse IgG, IgA, and IgF neutralized sensitized virus, but rabbit anti-mouse IgM was ineffective. In another aspect of these studies, it was shown that the monovalent Fab fragment of goat anti-mouse globulin partially neutralized and blocked the remainder against neutralization by the undigested anti-mouse globulin.

Ozaki *et al.* (1974) obtained antisera to JEV early (5 days) and late (28 days). Neutralization showed nonneutralized fractions in each case, the level being higher with the early serum. Neutralizing antibody in the early and late sera were IgM and IgG, respectively. Goat anti-rabbit IgM serum neutralized 99% of the nonneutralized fraction when early serum was used, whereas anti-IgG was without effect. When virus was neutralized with late serum, anti-IgG reduced the nonneutralized fraction slightly, but the anti-IgM not at all. The same specificity was seen when direct neutralization was carried out by fractionated antiviral IgM and IgG then challenged by the fractionated anti-antibodies.

The possible occurrence of a unique class of antibody that can specifically induce the nonneutralized state was investigated by attempting its isolation (Lewenton-Kriss and Mandel, 1972). An early (5-day) rabbit poliovirus antiserum was fractionated by three unrelated procedures: (1) gradient centrifugation, (2) chromatographic separation using Sephadex for molecular sieving, and (3) isoelectric focusing electrophoresis. In all instances in which antibody activity was detected, neutralization was accompanied by a nonneutralized fraction. The IgG and IgM fractions of a horse anti-rabbit γ-globulin each had secondary neutralizing activity, and the IgM fraction was the more active on a molar basis.

A further implication of the role of antibody in nonneutralizability was described by Hashimoto and Prince (1963). The resulting nonneutralized fraction when JEV reacted with rabbit antiserum in excess could be neutralized in part by guinea pig antivirus antiserum. However, the resulting nonneutralized fraction when guinea pig antiserum in excess was used was not affected by the rabbit antiserum. Perhaps the guinea pig antiserum contained a low level of functional complement. It has also been shown for poliovirus (Svehag, 1966; Lewenton-Kriss and Mandel, 1972) and for JEV (Ozaki *et al.*, 1974) that the nonneutralized fraction induced by an early rabbit immune serum cannot be neutralized by a late immune serum from the same rabbit. In all instances direct use of the late serum reduced infectivity to a considerably lower level than the level of the nonneutralized fraction resulting from the use of early serum. This observation is not consistent with a dissociation hypothesis, since the later appearing antibody with its greater binding affinity should be able to replace the weaker early antibody.

4. Unsaturation Hypothesis

Rappaport (1970) proposed that the nonneutralized condition was the result of incomplete binding of antibody to all antigenic determinants. He showed that the nonneutralized fraction of MS2 (an icosahedral RNA bacteriophage) represented particles which were about half-saturated with antibody. Hence these particles were protected against additional binding of antibody, yet they retained infectious capability. In their studies with herpesvirus, Yoshino and Morishima (1972) arrived at a similar interpretation.

5. Dissociation Hypothesis

Fazekas de St. Groth and Reid (1958) proposed that the data of Dulbecco *et al.* (1956) supported the dissociation hypothesis for the nonneutralized fraction. Ide and Yoshino (1974) reevaluated their interpretation of the nonneutralized state for herpesvirus in favor of the dissociation hypothesis. The experimental basis was the observation that the level of surviving virus diminished when the filtrate of a virus–serum mixture was incubated. These workers considered this evidence for reequilibration, since virus–antibody complexes, but not antibody or free virus, were retained on the filter. Failure to alter the nonneutralized fraction by altering the concentration of antibody was explained on the basis that equilibrium was established between antibody and the total number of critical reactive sites rather than the number of viral particles. Hence, for example, considerable dilution and possibly considerable time may be required for detectable signs of dissociation (i.e., increase in infectivity).

6. Abortive Reaction Hypothesis

The correlation between a high nonneutralized fraction and early serum, together with the known low affinity of early antibody, either IgM or IgG, suggested the possibility that the nonneutralized fraction of poliovirus was the result of a defective reaction (Lewenton-Kriss and Mandel, 1972). There is evidence that neutralization is the culmination of sequential events beginning with the union of virus and antibody. It has therefore been proposed (Lewenton-Kriss and Mandel, 1972) that, either because of a defect in antibody or because of improper binding of antibody to virus, the reaction does not go to completion. Addition of antibody specific for the virus-associated antibody activates the aborted reaction. A similar interpretation has been applied to mediated neutralization of the nonneutralized fraction of herpesvirus (Yoshino and Isono, 1978).

B. Bacterial Virus Studies

Studies by Stemke and Lennox (1967) on the neutralization of bacteriophage T2 disclosed that the Fab fragments derived from hyperimmune rabbit antiserum had neutralizing activity. Although the Fab II fragment had a higher neutralizing capacity than the Fab I fragment, the addition of antiglobulin (goat anti-rabbit globulin) increased neutralization of both to the same final level. It was therefore concluded that both fragments bound equally well quantitatively. However, the Fab I fragment had a lower neutralization potential. The possibility was considered that distinctive binding sites existed for the two fragments.

Antiglobulin was used by Goodman and Donch (1965) to demonstrate that L chains derived from rabbit anti-T1 serum reacted with T1 bacteriophage. Per se, L chains had questionable neutralizing activity, but activity was enhanced to an appreciable level by goat anti-rabbit globulin.

A kinetic examination of the effect of antiglobulin on bacteriophage neutralization (Dudley *et al.*, 1970) showed that the rate of neutralization of bacteriophage f2 (an icosahedral RNA virus) by 7S antibody, pepsin-derived 5S fragment, or 3.5S fragment derived from the 5S fragment was enhanced about three-fold by the addition of antiserum against the respective globulin fractions.

C. Summary

Evidence has been presented in support of the various hypotheses, although interpretations and attempted confirmations have not in some instances led to the same conclusions. In a broad sense, the hypotheses reduce to two: (1) the nonneutralized fraction is a result of the dissociation of virus–antibody complexes; and (2) virus has reacted with antibody (a) without losing infectivity and (b) acquiring a protected status. In support of the latter, evidence has been presented that clumping may be responsible. Evidence has also been presented that single particles are involved, since monovalent fragments of viral antibody as well as monovalent fragments of the anti-antibody participate in this phenomenon. Although the clumping hypothesis provides a possible explanation for the protected status, no explanation is evident when monodispersed virus is in the nonneutralized state. Possibly, on the abortive reaction hypothesis, a stage in the reaction sequence has been reached which is short of neutralization but renders the viral capsid nonantigenic.

Studies on bacterial viruses have shown that a nonneutralized fraction occurs. From the early studies of Andrewes and Elford (1933a) to recent studies (e.g., Hale *et al.*, 1969) the presumption has been that the non-

neutralized fraction is the result of an unusual virus–antibody reaction. As with animal viruses, the nonneutralized fraction is subject to neutralization by anti-antibody. Also, as with animal viruses, some reports indicate that IgM antibody is involved, whereas other reports state that IgG antibody is involved. However, there is unanimity in the observation that antibody synthesized early, rather than late, in the immune response is more responsible for the nonneutralized state. Since it has also been a common observation that early antibodies have a relatively weak binding capability, it is this characteristic that is most likely directly concerned with nonneutralization. Studies with antibody fragments (Stemke and Lennox, 1967) have shown that, although Fab II has a higher neutralizing capability than Fab I, both interact with virus to the same degree as shown by mediation with anti-antibody. The interaction of Fab I with antigen may be less likely to result in neutralization than the Fab II interaction. It is also worthy of note that aggregation cannot be the basis of this interaction, since these reactions involve monovalent fragments.

At the present time there are no experimental data that can account for the refractory state of the nonneutralized fraction. Possibly the infectious virus–antibody complex is sterically hindered, or the antigenicity of the capsid has been modified.

V. Sensitization and the Role of Accessory Neutralizing Factors

That virus could react with antibody without loss of infectious capability was suspected by Andrewes and Elford (1933a). Evidence in support of this presumption was reported for poliovirus (Mandel, 1958) and LDH virus (Notkins *et al.*, 1966). Poliovirus that survived exposure to rabbit antiserum, and LDH virus that survived exposure to mouse antiserum, were neutralized by anti-rabbit globulin and anti-mouse globulin sera, respectively. Notkins *et al.* (1966) referred to infectious virus–antibody complexes as "sensitized virus." The appropriateness of this designation is indicated by the observations that such complexes are sensitive to several other factors, sensitivity being manifested by loss of infectivity.

A. Background

The involvement of the complement system in viral neutralization was suspected as early as 1925. Gordon (1925) reported the loss of neutralizing activity when sera from rabbits immunized with vaccinia virus

were heated. However, the loss could be restored by the addition of fresh normal guinea pig serum. Similar observations were reported by Douglas and Smith (1930), Tanaka (1931), and Imagawa (1935), implicating the presence of a thermolabile accessory substance in sera derived from animals immunized with vaccinia virus. A more detailed investigation of this phenomenon was described by Mueller (1931). The presence of a thermolabile substance that participated in the neutralization of Rous sarcoma virus was shown to be present in the sera of geese, ducks, chickens, and rabbits that had been immunized with Rous virus. Neutralizing activity was lost as a consequence of heating (56°C for 15 minutes) but could be restored by the addition of guinea pig serum if fresh, but not if heated. These results prompted Mueller to suggest that the thermolabile substance was alexin (i.e., complement).

Aware of a probable role for complement in the neutralization of mumps virus by human sera, Leymaster and Ward (1948) stressed the need to preserve such activity by appropriate handling procedures. Of interest was their observation that sera from individuals as long as 40 years after mumps infection still showed a dependence on a thermolabile serum component. A similar caution was expressed by Pollikoff and Sigel (1952) with respect to sera with neutralizing activity for LCM virus (an arenavirus). Another instance of dependence of antiserum on a thermolabile serum factor was reported by Adams and Imagawa (1957) involving sera from ferrets immunized with a mouse-adapted strain of distemper virus.

Howitt (1934), however, was unable to detect a neutralizing accessory substance in sera of horses that had been immunized with equine encephalomyelitis virus. Neutralizing activity was affected neither by heat (56°C for 15 minutes) nor by addition of complement. These results do not necessarily challenge the credibility of previous observations since, in the light of more recent knowledge, the stage in the antibody response that is complement-dependent probably had been passed since, as Howitt stated, the animals had been hyperimmunized. The independence of neutralizing activity was also reported (Strong, 1936) for human sera capable of neutralizing a presumable herpes-like virus, virus W. Neither heat nor known complement-deactivating procedures had any effect on neutralization.

Another study that questioned the role of complement, but not the presence of an accessory substance, was reported by Ginsberg and Horsfall (1949). Sera from normal (i.e., not knowingly exposed or immunized to the viruses under study) humans, rabbits, guinea pigs, and mice with neutralizing activity for influenza virus, mumps virus, and NDV lost activity upon heating (56°C for 30 minutes), storage (4°C

for 2 weeks), depletion of Ca^{2+}, or decomplementation. Sera devoid of activity as a result of heat or storage were reactivated by the addition of fresh serum from a child with no prior history of mumps or influenza infection. Although these investigators were cognizant of the similarities in characteristics of complement and the accessory substance, they considered the latter to be distinct from complement because of certain quantitative discrepancies.

Whitman (1947) suspected the presence of a neutralizing accessory substance in serum from an individual convalescing from infection with WEE virus when neutralizing activity decreased disproportionately with serum dilution. Suspecting the presence of an accessory factor, he confirmed his suspicions by demonstrating its thermolabile character and the augmentation of neutralizing activity by the addition of guinea pig serum to heated (56°C for 30 minutes) immune serum. In spite of the similarities between complement and the accessory substance, Whitman conservatively eschewed a specific label for the active material. Somewhat earlier, Morgan (1945) had reported that discrepancies in the quantitative aspects of the neutralization of WEE virus by immune rabbit sera were related to the use of fresh or heated serum, and that the addition of complement to the latter tended to eliminate the discrepancies. Another togavirus (dengue) was reported (Sabin, 1950) to be neutralized by complement-dependent antibody present in the serum of a convalescent monkey.

Noting that previous studies had led to discrepant conclusions about the presence and nature of substances in immune sera that acted in conjunction with specific antibody, Leymaster and Ward (1949) attempted to refine the experimental system by using a virus, mumps, capable of being assayed in a host devoid of complement, i.e., an embryonated chicken egg. Sera from immunized monkeys, or from human cases of mumps infection either in the acute or convalescent stage, contained neutralizing activity that was nullified by heating or by decomplementation. The addition, in either case, of fresh sera obtained from children less than 3 years of age restored neutralizing activity. These workers attributed the restoration to complement.

In the studies cited thus far, the viruses under investigation were myxoviruses, togaviruses, or poxviruses, all of which are virions containing lipid-rich envelopes. That the presence of an envelope is not required for complement-dependent neutralization was shown by several studies involving envelope-free bacterial viruses. In 1947, Hershey and Bronfenbrenner described the participation of complement in the neutralization of two coliphages. An enigmatic aspect of this brief report indicated that in some instances a virus–serum mixture that had reacted to completion showed partial reactivation upon subsequent addition of complement.

Participation of complement in the neutralization of bacteriophages has also been reported by Barlow *et al.* (1958), Cowan (1962), Toussaint and Muschel (1962), Pernis *et al.* (1963), and Adler *et al.* (1971). Harris *et al.* (1962) reported the induction in humans and mice of complement-dependent antibody following immunization with *Bacillus megatherium* bacteriophage. Of interest was their observation that the addition of normal, fresh mouse serum in moderate concentration to heat-inactivated mouse antiserum restored activity. However, at a high concentration no reactivation ensued.

In order to establish more rigorously that the identity of the thermolabile accessory factor was complement, Dozois *et al.* (1949) fractionated a rabbit serum containing antibody to WEE virus as well as accessory factor. Removal of the complement system depleted the serum of neutralizing activity. By reconstituting the serum the neutralizing activity was restored, and the details of the reconstitution procedure indicated that components 2, 3, and 4 of the complement system were essential, whereas component 1 was either not required, or required in trace concentrations. Studies on two other togaviruses, Murray Valley encephalitis and WN encephalitis, were described by Westaway (1965a). Unlike WEE, Murray Valley encephalitis virus was neutralized only slightly more efficiently with added complement, whereas WN virus neutralization was perhaps slightly inhibited by the addition of guinea pig serum. Another instance of complement-independent neutralization of a togavirus, JEV, was reported by Hashimoto and Prince (1963). The addition of fresh guinea pig serum to either early (18-day) or late (34-day) immune rabbit sera affected neither the rate nor the degree of neutralization. An unusual observation in these studies was the spontaneous partial reactivation of neutralized virus relatively late in the kinetic analysis of the reaction.

Ozaki and Tabeyi (1967) observed marked enhancement of neutralizing actvity of JEV by fresh guinea pig serum. The rate and extent of neutralization were both increased. This result is in contrast to the results reported by Hashimoto and Prince (1963). Of possible relevance to the different results are the observations of Iwasaki and Ogura (1968a) that JEV grown in porcine cells induced complement-dependent antibodies specific for viral antigens as well as antibodies specific for host lipoproteins comprising the viral envelope. However, virus grown in mouse brain was insensitive to complement-dependent antibody. Whereas Ozaki and Tabeyi (1967) used virus cultivated in porcine cells, Hashimoto and Prince (1963) used virus derived from infected suckling mouse brain.

Baughman *et al.* (1968) reported that antibodies of guinea pigs and ferrets immunized with respiratory syncytial virus were markedly complement-dependent. Although antibody from horses appeared to be inde-

pendent by end point analysis, a complement effect was observed as an increase in the neutralization reaction rate.

It has been a common observation that sera obtained from normal individuals contain neutralizing substances, probably immature antibody, against an array of viruses. A rule of thumb to distinguish normal antibody from other inhibitory substances is the greater thermolability of the latter and the inability to replace the latter with complement. McCarthy and Germer (1952) examined normal sera from guinea pigs, rabbits, and humans for neutralizing activity against variola virus, herpesvirus, and cowpox virus. Positive sera lost some, but not all, activity after being heated at 58°C, and activity was regained upon the addition of fresh serum. These investigators proposed that the positive sera contained two separate neutralizing factors acting independently. Perhaps they envisioned two classes of antibody, one requiring complement and the other independent of complement. Howitt (1950) surveyed the sera from various animal species for the presence of normal neutralizing activity against NDV. The species examined included some known to have high levels of complement, e.g., human, monkey, rabbit, and guinea pig, and species with little or no complement, e.g., ferret, hamster, and chicken. Sera from human, monkey, rabbit, and guinea pig neutralized NDV when tested fresh, but lacked activity after heating (56°C for 30 minutes). The absence of neutralizing activity in fresh sera from ferret, hamster, and chick, and the ability to negate activity by heating the sera from complement-rich species, suggested a role for complement in the neutralizing activity of normal sera. Studies on the nature of the bacteriophage-neutralizing substance in normal human sera led Muschel and Toussaint (1962) to conclude that the substance was antibody which was qualitatively indistinguishable from antibody appearing early during the immune response. In both instances, neutralizing antibody was complement-dependent.

More recently, Bendinelli *et al.* (1974) reported on the distribution of normal antibody among various species of animal. In the presence of complement (endogenous or added) the sera from various species, but not from mouse, rat, and sheep, neutralized Friend leukemia virus. Because of sensitivity to heat (56°C for 30 minutes), a requirement for divalent cation, and restoration of deactivated sera by the addition of complement, these workers considered the neutralizing activity the result of complement-dependent antibody.

Several studies on rubella and equine arteritis virus (togaviruses of the alphavirus subgroup) have shown that antibodies to these viruses may be complement-dependent (Neva and Weller, 1964; Parkman *et al.*, 1964; Leerhoy, 1966). Rawls *et al.* (1967) examined sera from an infant with rubella infection, from the mother of the infant 1 year after the infection,

and from an adult more than 5 years after infection. In each instance the addition of complement to the heated sera increased the degree of neutralizing activity. Decomplementation resulted in reduced activity. It was of interest to these investigators that, unlike herpesvirus, rubella virus induced complement-dependent antibody long after infection.

Leerhoy (1968) observed that, whereas guinea pig and horse sera effectively enhanced the neutralizing activity of human serum for rubella virus, rabbit and calf sera were relatively poor. Since horse sera lack C2 (Leerhoy, 1968), this component of complement does not participate in this system. However, Maess (1971) considered C2 the limiting factor in the complement-dependent neutralization of equine arteritis virus. This conclusion was based on the distribution of activity following dialysis of a guinea pig serum with complement-effected activity. Hyllseth and Petterson (1970) also reported complement-dependent neutralizing antibody for equine arteritis virus in the serum of immunized rabbits as well as in the postinfection serum of horses. Kinetic studies, using horse serum as the source of antibody, showed a very slow rate of neutralization. The addition of complement (i.e., fresh guinea pig serum) 0, 1.5, or 3 hours after the start of the homologous reaction resulted, in each instance, in rapid extensive neutralization. All reactions, homologous as well as complement-effected, were kinetically single-hit.

Radwan and Burger (1973a,b) demonstrated that antibody from horses, guinea pigs, rabbits, hamsters, and mice immunized with equine arteritis virus was complement-dependent. Such dependent antibody was found to be late-appearing IgG. Early IgG and IgM were nonneutralizing, either per se or with complement. Further studies (Radwan *et al.*, 1973) showed that the addition of complement to virus complexed with dependent antibody eventually resulted in lysis of the viral membrane. However, through the use of trypsin it was shown that neutralization preceded viral lysis. In previous studies on the neutralization of a coronavirus, avian infectious bronchitis (Berry and Almeida, 1968), it was shown that complement-dependent neutralization occurred in the absence of viral lysis if the antiserum was devoid of antibody to the lipoprotein envelope. In the presence of such antibody, lysis was observed by electron microscopy.

B. Complement

1. The Complement Systems

Recent studies on complement-effected neutralization have focused on the role of the various components of the complement system. It is perhaps appropriate at this point to outline the salient features of complement. For a very comprehensive recent exposition of this subject, the

reader is referred to Osler's review (1976) or, for a briefer discussion more oriented to the subject at hand, to Oldstone (1975).

Two reaction pathways have been defined—classic and alternative. The classic system consists of 11 glycoproteins, 3 of which are functional only when aggregated, for which Ca^{2+} is required. In the presence of an antigen–antibody complex the first component, C1, (a conglomerate of C1q, C1r, and C1s), binds to the antibody of the complex. Thereupon, components C4, C2, C3, C5, C6, C7, C8, and C9, in that order, bind successively to the forerunner complex. Some of the components exist in a precursor state and are activated as a result of binding (e.g., C1 acquires esterase activity) or by an activated component (e.g., C4 undergoes partial cleavage by the activated C1 esterase). For complement-induced cell lysis, the entire sequence of reactions is required after the initial reaction between antibody and a cellular surface antigen. As shown below, the entire complement system may not be required for complement-effected neutralization.

The alternative pathway involves activation of properdin (a basic glycoprotein) and several additional cofactors present in serum. The final product of the alternative system is C3 and therefore is functional in the absence of C1, C4, and C2. Biochemical studies have elucidated the characteristics and isolation methods of the individual components, thus allowing an analysis of their individual roles. The discovery of specific inhibitors has added to the investigative versatility of these systems. For example, zymosan or cobra venom can destroy C3, thus the classic pathway is nonfunctional. Differential thermolability allows for selective inactivation of the alternative pathway (50°C for 20 minutes) and the classic pathway (56°C for 30 minutes). Finally, sera can be obtained from animals with genetic defects, so that a specific component is absent.

The complexity of virus–antibody–complement interaction is further compounded by the molecular heterogeneity of the different classes of antibody, their diverse reactions with viral antigens, and their differential capacity to activate the complement system. The first stage in complement activation is the binding of the C1q component to the Fc hinge region of the antibody molecule. IgM, being a pentamer of IgG, has a far greater potential for activating complement. Variation in capacity to activate complement exists among the subclasses of IgG, e.g., IgG2, but not IgG1, from the same rat is capable. A study of human myeloma proteins (Spiegelberg, 1974) revealed that IgG1 and IgG3 are more active than IgG2, whereas IgG4 is incapable of activating complement. The complexity of this system is further ramified by the variable sensitivity of a given complement system to antibodies of different origins. For example, guinea pig complement reacts maximally with homologous

antibody, half as well with rabbit antibody, feebly with dog antibody, and not at all with chicken antibody. Furthermore, the homologous reaction is not necessarily optimal, as shown by the threefold greater reactivity of rabbit complement with guinea pig antibody than with rabbit antibody (Gigli and Austen, 1971). In view of the complex nature of complement activation, failure to effect viral neutralization by complement may be due as much to an inappropriate system as to real insensitivity.

In several of the previously cited studies, it was shown that the ability to effect viral neutralization with complement was related to the antibody developmental stage in the immune response. Antibody that appeared early reacted with virus either without neutralizing or neutralizing poorly, but in either case the addition of complement enhanced the degree of neutralization. In contrast, late antibody was independent of complement. Studies on the chronology of appearance of the different classes of antibody (Uhr *et al.*, 1962; Uhr, 1964; Uhr and Finkelstein, 1967; Svehag and Mandel, 1962, 1964a,b; Graves *et al.*, 1964; Brown *et al.*, 1964; McKercher and Giordano, 1967; Ogra *et al.*, 1968) have indicated that IgM (19S) antibody precedes IgG (7S). However, Osler (1976) indicated that, by the use of appropriate methods, the response to a nonviral immunogen (human serum albumin) in rabbits and guinea pigs within the first week was predominantly IgG antibody. Although the IgG antibody exceeded by 50-fold the amount of IgM (on a molar basis), the IgG antibody was not detectable by the more conventional serological reactions. It is likely therefore that the affinity of the very early IgG antibody is of a very low order of magnitude. Cowan (1973) has also questioned the interpretation of such data. In some studies, detection of the early IgM was based on its sensitivity to 2-mercaptoethanol. It has, however, been recognized recently that early IgG has a similar sensitivity. In other studies recognition of the type of antibody was based on separation by density gradient centrifugation or by molecular sieving.

2. Recent Studies and Mechanism

Yoshino and Taniguchi (1964) reported that early antibody in rabbits elicited by immunization, or after corneal infection, with herpesvirus could neutralize only when complement was present. Antibody appearing later was complement-independent. Further studies (Yoshino and Taniguchi, 1965a) showed that dependent and independent antibodies were of the IgG class, that both resisted thermal inactivation at 56°C, or 70°C for 30 minutes, and resisted inactivation by reduction (2-mercapto-

ethanol). It was also shown (Yoshino and Taniguchi, 1966) that antibodies induced in guinea pigs by immunization, and in humans following herpes infection, were initially dependent and later independent of complement for neutralizing activity. The additional observation was made that herpes antibody in normal individuals (i.e., not currently, or recently infected) is complement-independent. Hence the presence of complement-dependent neutralizing antibody is of diagnostic significance.

A more detailed characterization of the antibody response was described by Shinkai and Yoshino (1975a). Early sera from herpes-immunized rabbits and guinea pigs were fractionated. Rabbit sera contained complement-dependent antibody of the IgM, fast IgG, and slow IgG classes. The dependent antibody in guinea pig serum was principally slow IgG (i.e., IgG2). Late rabbit sera contained very little IgM, and the IgG antibody was no longer complement-dependent, unlike the guinea pig serum which continued to synthesize dependent IgG. Reimmunization of rabbits elicited a brief response of dependent IgM and a burst of independent IgG.

Following primary herpes infection in humans, three categories of antibody were described (Heineman, 1967)—totally complement-dependent, potentiated, and independent. The data do not permit an interpretation of "potentiated," but conceivably it refers to the presence of antibodies of both other categories.

Basically similar results have been described for bacterial viruses. Hájek (1968, 1969) immunized newborn rabbits with bacteriophage T2. Sera collected 7, 15, and 28 days later contained complement-dependent, thermolabile 19S antibody on day 7, complement-dependent 7S antibody on day 15, and partially dependent 7S antibody on day 28. This investigator suggested that the transition indicated the gradual enhancement of binding strength of antibody with time after immunization. In related studies Hájek (1966) and Hájek and Mandel (1966) showed that, whereas normal or early immune antibody to T2 was complement-dependent, normal or early immune antibody to bacteriophage ϕX174 was independent. As a possible explanation for the difference in behavior of the two viruses, it was proposed that binding affinity of antibody to antigen was the determinant characteristic, and that affinity for the relatively simple, small, icosahedral ϕX174 bacteriophage was sufficiently high not to require augmentation. These investigators stated that, when the source of normal serum was newborn pigs that had not been allowed to receive colostrum, the sera contained no neutralizing activity against either T2 or ϕX174. Pernis *et al.* (1963) compared antibody to bacteriophage T2 obtained from normal newborn, normal adult, and immunized adult rabbits. Neutralization with newborn serum required complement,

unlike both adult sera. Irreversible (by addition of fresh serum) loss of activity by heating (56°C for 30 minutes) occurred with newborn and adult normal sera. Treatment with EDTA or zymosan eliminated activity from both normal sera. These workers attributed complement-dependent neutralization to thermolabile 19S antibody. The results of treatment implicate the early components (C1, C4, and C2) of the classic pathway in complement-effected neutralization.

A recent study by Schrader and Muschel (1975) on phage T2–rabbit antibody interaction indicated that 7S antibody (collected over a period of 7–21 days after a single immunization) was dependent on the C1 component only. Interestingly, they observed that the addition of both C1 and C4 was less effective than the addition of C1 alone. Neutralization by 19S antibody was not activated by C1 but only by the complete complement system (i.e., whole guinea pig serum).

Studies on the complement dependence of herpes antibody induced in rabbits (Hampar *et al.*, 1968) revealed the interesting observation that 7S antibody obtained after hyperimmunization was independent of complement when tested for end point titer. However, the same antibody when assayed by kinetic analysis showed an increase in reaction rate constant in the presence of complement.

In their studies on the serological relationship between human and simian herpesviruses, Stevens *et al.* (1968) observed, as others have, the dependence on complement of early 7 and 19S antibodies. Late antibody of the 7S type "matured" to independence, unlike late 19S antibody. Previously, Westaway (1965b) had examind the serological relationships among group-B arboviruses (flaviviruses). Inclusion of fresh normal rabbit or guinea pig sera in the neutralization reactions enhanced the quantitative results. However, Westaway questioned the desirability of this procedure, in this case, inasmuch as the effect of enhancement on the specificity of the reaction was not known.

Several studies on neutralization of CMV (currently classified as herpesvirus) have yielded divergent results. In rabbits immunized with a strain specific for humans, Andersen (1971) observed the early appearance of complement-fixing antibody which was incapable of neutralizing even in the presence of complement. Although late sera contained neutralizing antibody, complement was required as late as 18 weeks after hyperimmunization. Pursuing these studies, Andersen (1972) described the failure to detect neutralizing antibody, either complement-dependent or independent, in early sera of infected human subjects, although complement-fixing antibodies were present. Antisera in rabbits were prepared with two human strains (AD169 and C87) and two simian strains (Davis and T27). With the human strains, late sera contained complement-

independent antibody, but with the simian strains antibody was dependent on complement for neutralization. In another study Graham *et al.* (1971) prepared antisera in primates (baboons and monkeys) to two human strains (C87 and AD169) and two monkey strains (GR2598 and GR2757). The hyperimmune sera to the human viruses contained complement-dependent antibody (of 19 and 7S classes), whereas antibody to the monkey strains was independent—the reverse of the results obtained by Andersen (1972). Graham *et al.* (1971) have also indicated that antibody from goats hyperimmunized with the human strains was complement-dependent, as with the primate antisera. These workers have stressed the need for inclusion of Freund's adjuvant to obtain reasonably high levels of circulating antibody. Whether the use of adjuvant had any effect on the quality or class of antibody was not discussed. Minamishima *et al.* (1971) characterized CMV antibodies obtained from subhuman primates in the wild. Complement-independent antibody was detected against a simian strain of CMV. When the sera were tested for neutralization of human CMV strains, one strain (C87) was neutralized only when complement was present, while a second strain (AD169) was not neutralized at all. Finally, Ablashi *et al.* (1969) described the successful immunization of rabbits with a simian strain (SA6). Success, in this case, was indicative of the production of 7 and 19S antibodies, both of which were complement-independent.

With the elucidation of the nature and mode of action of complement, attempts were undertaken to determine the basis for complement participation in neutralization. Shortly after the discovery in 1954 of properdin, but before its role in the alternative complement pathway was understood, Van Vunakis *et al.* (1956) attempted to determine its function in the neutralization of phage $T2r^+$ by normal human sera. Heating (56°C for 30 minutes), the addition of EDTA, or the addition of zymosan nullified neutralizing activity. Zymosan-treated sera, however, retained hemolytic activity. In the light of more recent knowledge, these data implicate one or more of the early-acting components (C1, C4, or C2) in neutralization, inasmuch as the alternative pathway was functional. The early suspected implication of the role of properdin in viral neutralization was discussed by Ginsberg and Wedgwood (1959).

Taniguchi and Yoshino (1965) examined the role of complement in the neutralization of herpesvirus by early rabbit antibody. The immune serum was deprived of neutralizing activity by heating (56°C for 30 minutes), zymosan, and ammonium hydroxide treatments. Individual components of complement (i.e., C1, C2, C3, and C4), obtained by fractionation of guinea pig sera, were added singly and in combination to the deactivated rabbit serum. Only when all four components, in optimal

concentrations, were added did the rabbit serum reacquire neutralizing activity. An earlier study of this phenomenon (Dozois *et al.*, 1949), in which rabbit antibody to WEE virus was examined, resulted in a somewhat different conclusion; whereas components C2, C3, and C4 were required, component C1 was either dispensable or required in trace amounts. At least one difference in the methodologies employed in the two studies involved the source of the complement components, namely, guinea pig sera and rabbit sera, respectively. Yet another interpretation of this phenomenon was proposed by Daniels *et al.* (1969, 1970). Virus was allowed to react with isolated IgM antibody from early rabbit antiserum. Virus–antibody complexes were isolated by centrifugation and shown to be infectious and neutralizable by complement. The addition of either activated C1 or C4 was without effect, but the addition of both resulted in neutralization. The C2 and C3 components manifested activity when C1 was present at an optimal concentration and C4 at a suboptimal concentration.

A dependence on the first four complement components for neutralization of NDV by early rabbit IgM antibody was described by Linscott and Levinson (1969). Neutralization enhancement by fresh guinea pig serum was negated by such treatments as heat, divalent cation depletion, and cobra venom. Activity was restored when all of the first four components were added to the treated serum. A similar conclusion with an interesting ramification was reported by Oldstone *et al.* (1972, 1974). Antibody to polyoma, a nonenveloped virus, was induced in rabbits with the aid of Freund's incomplete adjuvant. Antibody of the IgG class was isolated from heated antisera. Dependence on complement was a function of the multiplicity of bound antibody: below 10 to 20 molecules per virion required complement, above did not. At a multiplicity of 2, virus was sensitized and therefore neutralizable by fresh serum. Neutralization was also effected by a C6-deficient serum but not by a C4-deficient serum. Neutralization was effected by C1q alone, or by C1, C2, C3, and C4 in combination, but not by combinations of C1 and C4, or C1, C4, and C3. The positive result with C1q is attributed to its ability to aggregate the virus–antibody complexes. As these investigators indicate, this effect is of academic interest only, since C1q is not normally found in the free state in serum but rather as the conglomerate C1q-C1r-C1s, i.e., C1.

In the various studies on complement-effected neutralization, several mechanisms to account for this phenomenon have been proposed. (1) The accumulation of complement on a virion–antibody complex produces a shield that, for steric reasons, prevents viral function. (2) Complement components cross-link juxtaposed virion–antibody complexes, resulting in the formation of aggregates. (3) For enveloped viruses, interaction of

a virion–antibody complex with the complete complement system results in lytic lesions in the membrane. (4) Based on the hypothesis that neutralization is the terminal stage of a multistage phenomenon, complement supplements a reaction which in its absence fails to go to completion. (5) Complement stabilizes a virus–antibody complex which otherwise is readily dissociated by dilution.

Daniels *et al.* (1969, 1970) observed that complement component C1 failed to effect neutralization of herpesvirus that had combined with dependent antibody. However, the subsequent addition of C4 resulted in neutralization. These workers proposed therefore that the additive accumulation of both components was sufficient to interfere sterically with viral infectivity. A similar interpretation was proposed by Linscott and Levinson (1969) for complement-effected neutralization of NDV. The latter workers stressed the exponential manner in which the successive components of complement accumulate, e.g., one molecule of bound C1 may initiate the binding of several thousand molecules of C3. Direct support for this proposal of the amassing of complement on virion–antibody complexes was provided by electron microscopy. Berry and Almeida (1968) demonstrated the accretion of material on the surface of avian infectious bronchitis virus when complement was included in the reaction of virus and antiserum. Radwan *et al.* (1973) observed a similar consequence of including complement in the reaction of equine arteritis virus with complement-dependent antibody.

Evidence to substantiate the aggregation hypothesis was based on filtration and sedimentation analyses. In studies on herpesvirus (Wallis and Melnick, 1971; Wallis, 1971) it was shown that filterable complexes of virus and dependent antibody became nonfilterable after reacting with complement. This was attributed to the formation of relatively large aggregates. If, however, the virion–antibody complexes were highly diluted at the time of addition of complement, neutralization without loss of filterability resulted. Hence, under the latter conditions, complement exerted its effect through steric hindrance. In speculating on the nature of complement-dependent antibody, Wallis and Melnick suggested it was, or functioned as, a monovalent molecule. Oldstone *et al.* (1972, 1974) demonstrated the aggregation of polyoma virus–antibody complexes by the increase in sedimentation rate. The addition of an average of two molecules of antibody per virion increased its sedimentation rate from 240S to 260S. When the first four components of complement were added, the sedimentation rate increased to 450S, indicating aggregation through the cross-linking action of complement. In these studies virus was doubly labeled radioisotopically, thereby allowing individual tracking of capsid and genome. Although the sedimentation rate

was increased and the infectivity was neutralized, the virion remained intact based on the cosedimentation of the capsid and genome radioactivity markers. It should be noted that polyoma is a simple nonenveloped virus. Notkins *et al.* (1971) examined the effect of complement on herpesvirus complexed with IgM complement-dependent antibody. Although the addition of complement (pooled guinea pig sera) reduced infectivity by 99%, these investigators observed "little or no effect" on the sedimentation rate. The reasons for the different results for presumably similar systems (i.e., herpes–antibody–complement) are not discernible.

Yoshino and Taniguchi (1965b) demonstrated the stability of complexes of herpesvirus and complement-dependent antibody. Neither extensive dilution nor sonication promoted dissociation. It was also shown that complexes that had adsorbed to host cells continued to show sensitivity to complement, hence cellular induced dissociation did not occur.

Yoshino and colleagues reject steric hindrance as the basis for complement-effected neutralization based on filtration studies (Yoshino and Kishie, 1973) and on kinetic analyses in which single-hit kinetics were observed for both sensitization and complement-effected neutralization (Yoshino *et al.*, 1977).

It has been shown for avian infectious bronchitis virus (Berry and Almeida, 1968) and for equine arteritis virus (Radwan *et al.*, 1973) that under appropriate conditions irreversible damage to the viral envelope accompanies neutralization in the presence of complement. However, it has also been shown that neutralization can occur in the absence of envelope damage.

As the culmination of extensive studies of the complement-effected neutralization of herpesvirus by Yoshino and colleagues, the following hypothesis has been proposed (Yoshino and Isono, 1978). Antibodies are distinguishable functionally, if not physicochemically, by their neutralizing or sensitizing effect. Neutralizing antibody per se is capable of initiating the sequential reactions that terminate in the neutralization of infectivity. In contrast, the reactions initiated by dependent antibody fail to go to completion. They proposed that, whereas the earliest stages of the neutralization pathway cannot be influenced by complement, the later stages require progressively decreasing amounts of complement. In their studies, Yoshino and Isono (1978) discerned differences in antibodies with respect to how far the antibody could drive the reaction; complement-independent antibody (e.g., late IgG) drove the reaction to completion, and the decreasing order of capability was: dependent late and early IgG, late IgM, and early IgM. The above proposal was based on a concept (Mandel, 1976) of neutralization in which one molecule of

antibody initiates conformational transitions between neighboring capsid subunits. The net result is an overall capsid alteration. Evidence underlying this concept was obtained with a picornavirus. Yoshino and Isono (1978) recognized the risk in extending this concept to an enveloped virus such as herpesvirus. However, they considered the possibility that the reactive surface antigens were protrusions of an underlying protein continuum.

The role of complement in the *in vivo* interaction of herpesvirus and antibody was recently examined by Strunk *et al.* (1977). Guinea pigs genetically deficient in C4 synthesis were compared with genetically competent guinea pigs. Following infection with herpesvirus, both groups showed the same C1 and C3 through C9 levels before and after infection. The normal animals showed no utilization of C4 during infection. These investigators suggested therefore that the classic pathway was not involved in viral clearance, and possibly the alternative pathway was activated.

C. Other Serum Factors

1. Cofactor

In 1958 Styk *et al.* reported the presence in normal sera of a substance that acted in conjunction with antibody to inhibit influenza virus. Subsequent investigations by Styk and colleagues (1958, 1961, 1964; Styk, 1961, 1962, 1965; Hana *et al.*, 1961; Kociskova, *et al.*, 1961; Styk and Hana, 1961a,b, 1964, 1965a,b) were carried out to characterize the substance which they called cofactor. An A2 strain of influenza virus was used, and antibody activity was based primarily on inhibition of hemagglutination, and occasionally on neutralization. As to the nature of cofactor, it was found to be distinct from any of the complement components, although the distinction between it and C1 was in some instances equivocal. Electrophoretically, it migrated as a β-globulin. Based on exclusion chromatographic analysis it was a macroglobulin. It was found to be thermolabile and resistant to trypsin but not to periodate, and was considered a lipoprotein. Normal sera of humans, mice, horses, pigs, and cattle contain cofactor. Unlike cofactor from these sera, cofactor from guinea pigs requires divalent cation.

The proposed mode of action resembles that of complement and antiglobulin, namely, it combines with antibody that has reacted with antigen. In doing so, cofactor reduces the probability of dissociation of the virus–antibody complex. Additionally, its presence increases the degree of steric hindrance to normal viral function. There was no evidence that cofactor reacts directly with virus. The relationship of co-

factor to antibody class was studied. Antibody appearing early in the immune response was more sensitive to cofactor than late antibody. Characterization of antibody class showed that 19S antibody, but not late 7S antibody, was sensitive. These investigators considered the interesting possibility that the early immune response may consist of a 7S antibody that is cofactor-dependent. However, the procedures usually employed for separation of antibody types also segregate cofactor with the 19S antibody. Hence, although 7S antibody may be present, the demonstration of its presence requires cofactor from which it has been separated. Based on this conjecture, these investigators found that in some instances a very early response consisted of 19 and 7S antibodies, both requiring cofactor. Because of this dependence, these antibodies were considered "imperfect" in contrast to "classic" antibodies.

Polyak *et al.* (1961) confirmed the conclusion of Styk and his colleagues that cofactor and complement were different entities. Smorodintsev and Yabrov (1963) extended the observation to other strains of influenza virus, as well as to Sendai virus. In their interpretation, a nonspecific thermolabile stimulator of antibody functions by stabilizing complexes of virus and nonavid antibody.

2. Rheumatoid Factor

The involvement of rheumatoid factor in mediated neutralization has been described recently for herpesvirus (Ashe *et al.*, 1971), vaccinia virus (Gipson *et al.*, 1974), and hepatitis virus (Markenson *et al.*, 1975). Rheumatoid factor is a circulatory IgM antibody that appears in response to a variety of pathological conditions. It is considered specific for the host's own IgG which may have undergone alteration as a result of binding to a foreign antigen. It was shown by Ashe *et al.* (1971) that, when herpesvirus was sensitized by IgG antibody, the complex bound rheumatoid factor. However, the tripartite complex was still infectious but had acquired sensitivity to complement or to anti-human IgM with loss of infectivity. It was also shown through the use of Fab fragments of viral antibody that the Fc portion of the antibody molecule was the binding region for rheumatoid factor.

3. Staphylococcal Protein Factor

It has also been reported (Austin and Daniels, 1974) that staphylococcal protein A can effect secondary neutralization or mediate secondary neutralization of herpesvirus and vaccinia virus. Like rheumatoid factor, protein A binds to the Fc region of IgG that is complexed to virus. In some instances neutralization is effected, or in other instances requires

the addition of anti-protein-A antibody. Failure of rheumatoid factor and protein A to effect neutralization has been attributed (Austin and Daniels, 1974) to their MWs, about 900,000 and about 40,000, respectively. A lower-MW preparation of protein A, i.e., about 20,000 daltons is effective. It was therefore suggested that too few molecules of the higher-MW substances bind to the infectious virus–antibody complex. A survey of other physicochemical parameters indicated to Austin and Daniels (1974) that net charge and shape were irrelevant. This interpretation is somewhat puzzling, since these workers consider steric hindrance to be the basis for neutralization. It seems that, if fewer molecules of the higher-MW substance bind to the virus–antibody complex, it is because the complex has been saturated and therefore is as completely sterically hindered as by the lower-MW compounds.

4. Uncharacterized Factor

Way and Garwes (1970) have reported that neutralization of Semliki Forest virus by guinea pig antiserum can be enhanced by the addition of serum from various animal species. The nature of the enhancing factor and its mode of action were not characterized.

D. Antiglobulin

In studies on the neutralization of poliovirus by rabbit antiserum (Mandel, 1958) it was observed that the degree of neutralization was enhanced by the subsequent addition of an antiserum against rabbit γ-globulin. Antiserum induced in one species of animal (e.g., goat) to whole globulin, or a specific fraction thereof, of another animal (e.g., rabbit) is designated goat anti-rabbit globulin, or goat anti-rabbit Fab, and so on. In their studies on mice chronically infected with LDH virus, Notkins *et al.* (1966) suspected the presence of infectious virus–antibody complexes in the sera of these mice. Neutralization by goat anti-mouse globulin confirmed this suspicion. The generality of this phenomenon was extended when it was seen that herpesvirus could be neutralized by an antiglobulin serum (Ashe and Notkins, 1966). After exposure of herpesvirus to a hyperimmune rabbit antiserum, a considerable proportion of virus survived. Goat anti-rabbit globulin neutralized more than 99.9%. The same general phenomenon has been described for adenovirus (Kjellén and Pereira, 1968), poliovirus in human stool specimens (Keller and Dwyer, 1968), vaccinia virus (Majer and Link, 1970), influenza virus (Majer and Link, 1971), VEE virus (Hahon, 1970a), equine arteritis virus (Radwan and Burger, 1973a), NDV (B. Mandel, unpublished

observation), and bacteriophages T1 and T6 (Goodman and Donch, 1965), f2 (Dudley *et al.*, 1970), and T2 (Adler *et al.*, 1971).

Following the suggestion of Notkins *et al.* (1966), the term "sensitization" indicates infectious virus–antibody complexes. The neutralization of sensitized virus by anti-antibody is designated "mediated neutralization."

Notkins *et al.* (1968) compared the effectiveness of antisera against unfractionated as well as fractionated globulin for mediated neutralization. Viral antiserum was obtained from mice chronically infected with LDH virus and was used for sensitization. Rabbit anti-mouse globulin and anti-mouse IgG were capable of inducing neutralization, unlike anti-IgM which was inactive. It was also shown that the Fab fragment derived from a goat anti-mouse globulin serum had secondary neutralizing activity, albeit weak, and also had blocking activity against intact globulin. The Fc fragment, however, had no activity. These studies suggest that IgM either did not participate in sensitization, or could sensitize but was not accessible for mediated neutralization. There is the third possibility that the antiviral serum had little or no IgM. Inasmuch as monovalent Fab was able to mediate neutralization, its mode of action could not have been due to the aggregation of sensitized virus.

Poliovirus obtained from human stool specimens was neutralized by anti-human IgA, but not by anti-human IgM, as reported by Keller and Dwyer (1968). These workers suggested that virus was complexed with the L-chain moiety of antibody. In another report, Keller (1968) demonstrated sensitization of poliovirus "in reverse." Virus that had been neutralized by rabbit antiserum was reactivated by peptic digestion and sulfhydryl reduction. The presence of fragmented antibody (Fab) complexed to virus was ascertained by mediated neutralization with goat anti-rabbit globulin. Keller (1968) proposed that neutralization was a consequence of an altered structural configuration, rather than aggregation.

Hampar *et al.* (1968) examined the sensitizing potentialities of the different classes of antibody appearing early or late in rabbits immunized with herpesvirus. Early 7 and 19S antibodies lacked appreciable neutralizing activity. Although complement neutralized 7 or 19S-complexed virus, goat anti-rabbit globulin neutralized only 7S-bound virus. Late 19S antibody had neutralizing activity which was enhanced by anti-antibody. Neutralization of late 7S-bound virus was enhanced by anti-antibody but not by complement.

Ashe *et al.* (1968) investigated the possibilities for sensitization and mediated neutralization using various combinations of antibody fragments. Herpesvirus-specific fragments were derived from hyperimmu-

nized rabbits and anti-antibody fragments from sheep anti-rabbit globulin serum. Virus-specific Fab I and Fab II fragments neutralized, and survivors where shown to have been sensitized. The Fc fragment did neither. Virus, sensitized by intact antibody (class not indicated) was neutralized by sheep anti-rabbit globulin as well as by the Fab II and Fab I fragments therefrom. Virus, sensitized by either Fab I or II, was effectively neutralized by antiglobulin. When virus was sensitized by Fab I, anti-Fab I was moderately effective but anti-Fab II was not. When sensitized by Fab II, neither anti-Fab I nor anti-Fab II was effective. Hence virus that had been sensitized by univalent antibody fragments could be neutralized by univalent anti-antibody fragments. Basically similar findings were reported for LDH virus (Notkins *et al.*, 1968). In the same study (Ashe *et al.*, 1968), it was shown that, when virus was sensitized with antibody of a specific allotype, it could be neutralized only by an antiserum directed against the same allotype. A similar result was described by Adler *et al.* (1971) for bacteriophage T2. Virus-specific and antiglobulin sera were induced in rabbits of the appropriate allotypes. Mediated neutralization required that the anti-antiserum be induced in a rabbit that lacked one allotype determinant present in the virus-specific serum. For example, antibody from rabbit of allotype 1,1/4,4 was sensitive to mediated neutralization by antiserum from rabbit of allotype 1,1/5,5 that had been immunized with the 1,1/4,4 globulin. These workers attribute particular significance to this result because these allotypic determinants specify the characteristics of the variable regions (i.e., the combining site) of the antibody molecule.

Shinkai and Yoshino (1975b) recently examined the sensitizing capabilities of antibodies produced at various stages in the immune response, and the fragments derived from these antibodies. Antibody to herpesvirus was elicited in rabbits and collected 2 weeks (early) and >9 weeks (late) after immunization. Antiglobulin was prepared with chromatographically isolated normal rabbit globulin. Immune γ-globulin was purified and fractionated by appropriate procedures to yield $F(ab')_2$, Fab' Fab I, and Fab II fragments. Tests to determine sensitization and neutralization showed the following results. Early IgG, IgM, and late IgM had little or no neutralizing activity, unlike late IgG. Of these, only early IgG had sensitizing activity. All fractions [i.e., $F(ab')_2$, Fab, Fab I, and Fab II] of early IgG neither neutralized nor sensitized. Of the late IgG fractions, $F(ab')_2$ neutralized almost as well as IgG itself, Fab' and Fab II neutralized moderately but could be enhanced to near full activity by anti-antibody. Fab I neutralized weakly and sensitized moderately. Although Fab' derived from early IgG neither sensitized nor neutralized, it was shown to have reacted with virus in blocking experiments.

VEE virus showed a strong susceptibility for sensitization by human

antiserum (Hahon, 1970b). Goat anti-human globulin, specific for IgG or IgA, neutralized sensitized virus, while IgM was ineffective. It cannot be decided if the ineffectiveness was real or if it was due to the absence of IgM antibody in association with virus. Further studies (Hahon, 1970b) revealed that the Fab fragment of the antiglobulin had mediated neutralizing activity but was less effective than the intact IgG. Hahon (1970a) expressed the opinion that assaying for neutralization by the use of specific serum and antiglobulin when carried out by the quenching of fluorescence of infected cells was more sensitive than by the quenching of infectivity in mice.

Majer and Link (1970) studied the kinetics of neutralization of vaccinia virus. Neutralization by human antiviral IgG showed a slight lag, followed by an exponential decline and leveling off. When sheep anti-human globulin was included in the reaction, the lag and leveling off were eliminated. Filtration of virus to exclude aggregates eliminated the lag but not the tendency for the reaction to level off.

Radwan and Burger (1973b) presented data indicating that the Fab region of the bound antivirus antibody is the target site for mediated neutralization. They sensitized equine arteritis virus and then cleaved the bound antibody with trypsin. Virus complexed to the fragmented antibody was insensitive to neutralization by complement but retained its sensitivity to antiglobulin, hence this evidence indicated absence of the Fc region.

Notkins *et al.* (1971) attributed neutralization of sensitized virus to aggregation of the complexes by antiglobulin. Herpesvirus complexed with hyperimmune antibody was isolated by gradient centrifugation and then treated with antiglobulin. Compared with untreated complexes, treatment caused an increase in sedimentation rate.

Influenza virus has two discrete surface antigens, hemagglutinin and neuraminidase. The interaction of hemagglutinin with antibody results in neutralization, unlike the reaction of neuraminidase with antibody. A study of each antigen–antibody reaction (Majer and Link, 1971) showed that the hemagglutinin reaction was insensitive to antiglobulin. However, addition of antiglobulin to the neuraminidase reaction resulted in neutralization. It was theorized that the sites were, respectively, critical and noncritical for infectivity. Neutralization via the noncritical site mediated by antiglobulin could be explained on the basis of steric interference with the adjacent hemagglutinin. Or, less likely in the opinion of Majer and Link, the antiglobulin stabilized the neuraminidase–antibody reaction. Presumably, these workers considered that stabilization could have an effect on the neighboring hemagglutinin structures; the term "stabilization" was not defined.

Sensitization of adenovirus and neutralization by anti-antibody was

described by Kjellén and Pereira (1968) in studies to determine which of the viral antigens induced neutralizing antibody. Guinea pig antibody to the hexon antigen of type-5 adenovirus had neutralizing activity. Antibody specific for other adenovirus types, e.g., 1 and 2, reacted with type 5 but without neutralizing. Addition of goat anti-guinea pig globulin resulted in neutralization.

VI. The Mechanism of Neutralization

There is no evidence that antibody can influence the viral replicative process after viral uncoating has occurred. Hence the focus of interest on how antibody functions is restricted either to the direct effect of antibody on the integrity of the virion, or to the subversion of one of the early stages in the replication cycle. As indicated previously (Section II,E), neutralization can be the final manifestation of several phenomena that are fundamentally different. At the most elementary level, one can envision a morphologically simple virion (with several copies of a single antigen uniformly distributed throughout the capsid) at a very low concentration interacting with one or several molecules of a homogeneous antibody population, and as a result losing its infectious capability. At the other extreme, one can envision a morphologically complex virus, composed of several different antigens in a topographically complex distribution, some antigens being virus-specific and others host-specific, some reacting with one class of antibody and others with a different class (e.g., herpesvirus; see Miyamoto *et al.*, 1971). In addition, if the relative concentrations of virus and antibody are adjusted appropriately, secondary interactions leading to the formation of large aggregates may occur. Which of the concurrent reactions represents the critical neutralizing event cannot readily be isolated from those that may also lead to neutralization but for superficial reasons.

A systematic analysis of the intrinsic neutralization mechanism should entail an examination of the direct effect of antibody on the virion, and an examination of the distinct early stages of the viral growth cycle.

A. Effect of Antibody on the Virion

1. Irreversible Neutralization (Virolysis)

It has been shown that irreversible damage may ensue when certain viruses are exposed to antisera in the presence of complement (Almeida and Waterson, 1969). In all instances, including more recent studies (Oroszlan and Gilden, 1970; Radwan *et al.*, 1973), the viruses were enveloped. Analysis of the interacting antigen–antibody systems revealed

that the host-specific envelope antigens, reacting with antibody in the presence of the complete complement system, resulted in the formation of lesions in the envelope (Berry and Almeida, 1968). Similar results were described by Radwan *et al.* (1973). Visible holes and leakage of internal genomic material clearly represent irreparable damage. However, the interaction of such viruses with antibody to nonenvelope antigens resulted in neutralization that could be reversed (Radwan *et al.*, 1973).

2. Reversible Neutralization

It has been shown that exposure of neutralized poliovirus to acidic conditions restored infectivity (Mandel, 1958, 1961). Similarly, acid treatment of neutralized tobacco mosaic virus resulted in reactivation (Rappaport, 1961). Subsequent studies with many viruses, both simple and complex, have shown that reversal of neutralization can be achieved by a variety of procedures (for a review of this topic, see Mandel, 1971a). These results indicate that any alteration in the capsid induced by antibody binding to it is fully reversible. It has been reported that reactivation by acid did not necessarily entail dissociation of the virus–antibody complex when adenovirus was neutralized by rabbit antibody (Kjellén, 1965a,b).

3. Effect of Antibody on Viral Adsorption

Although it has been reported that under certain conditions neutralized virus is incapable of adsorbing to host cells, this is not the basis for neutralization in every case. Adsorption of neutralized virus has been described for several bacterial and animal viruses (Hultin and McKee, 1952; Nagano and Oda, 1955; Tolmach, 1956; Rubin and Franklin, 1957; Mandel, 1958; Dales and Kajioka, 1964; Joklik, 1964; Silverstein and Marcus, 1964). Moreover, it has been reported that virus is still susceptible to neutralization after adsorption, providing penetration has not yet occurred (Nagano and Mutai, 1954; Ishida and Ackermann, 1956; Mandel, 1958, 1962; Yoshino and Taniguchi, 1965b; Stinski and Cunningham, 1970).

The above observations, particularly the latter, implicate a stage in the replication cycle subsequent to adsorption as the antibody-sensitive stage.

The capability of neutralized poliovirus to adsorb to HeLa cells was found to vary according to several conditions (Mandel, 1967a). At low multiplicities of antibody, and soon after virus–antibody interaction, the virus–antibody complexes adsorbed as well as unneutralized virus. At high multiplicities, adsorption was impaired but not totally negated.

When neutralized virus was held at 5°C, the capability to adsorb improved until it exceeded that of the control about three-fold. The time-dependent improvement was seen with 7S but not with 19S antibody.

4. Elution and Penetration

It was first shown (Joklik and Darnell, 1961) that poliovirus particles, adsorbed to HeLa cells at low temperature, have about an equal chance either to penetrate or to elute at a higher temperature. The possibility that anybody might bias these alternatives in favor of elution was examined (Mandel, 1967b). It was found that neutralized virus had a decreased probability for elution—about 5% compared with 25–35% for unneutralized virus. This observation, together with the enhanced adsorptive capacity, implies that when virus has reacted with antibody the virion has acquired a greater binding affinity for the receptor. With neutralized avian infectious bronchitis virus, there was no effect on elution (Stinski and Cunningham, 1970), but with NDV (Silverstein and Marcus, 1964) and rabbit poxvirus (Joklik, 1964) elution was greater for neutralized virus.

Several studies have been concerned with microscopic observation of the fate of adsorbed neutralized virus (Dales and Kajioka, 1964; Joklik, 1964; Silverstein and Marcus, 1964). Where penetration had occurred, the uncoating process appeared to be unusual, leaving the impression that the intracellular particles were being randomly degraded or incompletely uncoated. In biochemical studies of poliovirus (Mandel, 1967b), it was observed that the fraction of cell-associated virus that was uncoated was smaller for neutralized than for unneutralized virus—about 10% compared with about 50%. In the uncoated unneutralized virus, the viral RNA was distributed between intact RNA and degraded (i.e., acid-soluble) RNA. In the uncoated neutralized virus about half as much degraded RNA was seen, but no intact RNA was detected. Basically similar results were described by Stinski and Cunningham (1970) for a quite different virus—avian infectious bronchitis, an enveloped virus.

5. Antibody-Induced Alterations in the Viral Capsid

Several studies have indicated that the presence of antibody as a ligand to the antigenic binding site causes an alteration in the biochemical characteristics of the capsid. A DNA coliphage, ϕX174, is incapable of infecting bacterial protoplasts. Bowman and Patnode (1963) reported that, when virus was treated with highly diluted antiserum, infectivity was not demonstrable with intact bacteria but was with bacterial protoplasts. Since DNase did not affect the phage, infectivity could not be

attributed to released DNA. However, an anti-DNA serum (lupus erythematosus) neutralized infectivity for protoplasts. These workers suggested that the capsid had undergone a rearrangement rendering the DNA accessible to interaction with the external environment.

Kjellén and Pereira (1968) have proposed that the hexon structure of adenovirus is composed of two antigens, one of which is internal. Interaction of the external antigen with antibody causes a rearrangement that exposes the internal antigen, thus enabling it to react with neutralizing antibody.

As a possible interpretation of the enhanced infectivity of arboviruses by antibody, Hawkes and Lafferty (1967) suggested that the effect may be attributed to a "particle that has, in effect, a new protein coat" as a result of binding antibody.

A study of the electrophoretic characteristics of poliovirus (Mandel, 1971b) showed that the capsid structure was in a metastable conformation, oscillating between two distinguishable isoelectric states. When neutralized, irrespective of antibody multiplicity, the capsid was stabilized in one of the conformational states.

Studies on the topographic arrangement of the structural elements of picornavirus capsids have been carried out by labeling procedures. It has been shown (e.g., Carthew, 1976; Beneke *et al.*, 1977) that the VP1 polypeptide is most highly labeled, and VP2 and VP3 are relatively inaccessible. VP4 appears to be entirely shielded, although in the free state it can bind the labeling compound. Carthew (1976) showed that, after reacting with antibody, the VP4 polypeptide became accessible, indicating that a rearrangement in the spatial organization of the capsid had occurred. In light of this observation, it is of interest to consider the results described by Breindl (1971). After neutralization of poliovirus, the virus–antibody complex was fractionated, yielding a fragment consisting of the atibody bound to the VP4 polypeptide. This result, together with the labeling results, suggest the interesting possibility that antibody initially combines with the reactive site which may not include the VP4 polypeptide. Conformational rearrangements may ensue that allow the antibody to transfer its allegiance to a more compatible receptor, the VP4 polypeptide. A schematic proposal of how such a rearrangement might occur has been suggested by Lonberg-Holm and Yin (1973).

An *in vitro* system for uncoating poliovirus has been developed (De Sena and Mandel, 1976, 1977). When neutralized poliovirus was examined in this system, it was seen to be resistant to uncoating. In the previous studies on the intracellular fate of neutralized poliovirus (Mandel, 1967b), evidence for a low level of uncoating was seen in the form of degraded viral RNA. However, a similar result was seen with native virus

but, unlike neutralized virus, undegraded RNA was also seen. The degraded RNA possibly reflects an aberrant, abortive uncoating process to which neutralized virus as well as native virus is susceptible.

B. Quantitative Aspects of Neutralization

Perhaps the most basic problem of the neutralization phenomenon concerns the mechanism whereby antibody deprives a virion of its infectious capability. The second most basic problem is perhaps the quantitative requirement for antibody to exert its effect. In all likelihood, the solution of either problem will point to the solution of the other. The early studies of Andrewes and Elford (1933a) and Burnet *et al.* (1937) began to suggest some ways of thinking about the latter problem.

1. The Percentage Law

In their studies of the kinetic characteristics of the neutralization reaction, Andrewes and Elford (1933a) summarized their findings as the "percentage law." In essence they observed that a given concentration of antiserum neutralized the same fraction of virus per unit time, irrespective of the concentration of the initial viral input. The law, however, had to be qualified: The concentration of antibody had to be in considerable excess over the concentration of virus. The law has been found to apply to virus-antibody interactions in general. The basis for this relationship can be understood in terms of collision frequency and relative concentrations of the reactants. The number of successful collisions is a function of the concentrations of virus and antibody. If the concentration of antibody is in great excess, the number of collisions per unit time will not decrease as a result of removal of antibody by binding to virus. Since the collisions are indiscriminate, the number involving unneutralized particles will diminish by the same proportion for each unit of time. The result is a reaction of the first order. If virus concentration is increased, the number of collisions will increase accordingly, but the fraction of virus particles undergoing neutralization will be the same as for the lower concentration. From these observations it follows that the rate of neutralization is a function of the concentration of free antibody, and the rate will be constant, providing the decrease in free antibody concentration during the course of the reaction is extremely small relative to the total concentration.

When, under these conditions, the reaction is plotted on a semilogarithmic plot, a linear relationship is seen of the kind that can be described by the expression for a first-order reaction. It is, however, clear that the

reaction in actuality is second-order, involving two reacting molecules. As indicated in Section II,E it has been proposed that neutralization is the result of sequential bimolecular interactions culminating when a critical number of antibody molecules are associated with the virion.

2. Single-Hit Versus Multihit

In most instances a plot of the logarithm of the surviving fraction of virus as a function of time is linear for the early phase of the reaction. In their analyses of the neutralization reaction, Dulbecco *et al.* (1956) interpreted this observation as evidence for a single-hit phenomenon. There has been criticism of this interpretation on purely speculative grounds, and occasionally a reaction has been recorded that shows evidence, based on a time-dependent delay of neutralization, for a multihit reaction (e.g., Lafferty, 1963a; Philipson, 1966). In accordance with the target theory of inactivation, the proportion of surviving virus can be expressed by the equation

$$\frac{V_t}{V_0} = 1 - (1 - e^{-kt})^n \tag{3}$$

where V_t and V_0 are the concentrations of virus at times t and zero, k is a constant, and n is the number of targets per virion that must be hit in order for the virion to be inactivated. In the case in which $n = 1$ the equation reduces to

$$\frac{V_t}{V_0} = e^{-kt} \tag{4}$$

A semilogarithmic plot of a reaction that fits Eq. (4) shows a linear course from time zero, indicating that one successful encounter inactivates that particle. Where more than one successful encounter is required, a period of time has to elapse to allow for the total necessary encounters before the first particle will be inactivated. The period of time is indicative of the number of hits required, which is the value of n in Eq. (3). When portrayed graphically, this period is seen as a lag or shoulder on the curve preceding the linear course of inactivation. A single-hit mechanism does not rule out the possibility that more than one molecule combines with a particle prior to inactivation. It does rule out the *participation* of these molecules in the inactivating event. Otherwise their participation would be manifested by a lag in the time course of inactivation. The data of Rappaport (1970), which showed four or five molecules of antibody per inactivated virion, indicated that all but one

molecule were not involved in causing neutralization. Other studies (Yoshino and Taniguchi, 1965b; Ashe and Notkins, 1967; Krummel and Uhr, 1969; Dudley *et al.*, 1970; Hahon, 1970b; Lewenton-Kriss and Mandel, 1972) showed that several molecules of antibody reacted with one virion (animal or bacterial) prior to inactivation. Their presence was detected by the use of antiglobulin to mediate neutralization. In several of these studies the mediated inactivation showed a more rapid rate but, nonetheless, also characteristically a single-hit mechanism. For example, in the studies of Dudley *et al.* (1970) and Krummel and Uhr (1969), the reaction rate of the mediated reaction was three- to fourfold more rapid than the direct reaction, suggesting that the earliest binding molecules were noneffective but could be made effective by the antiglobulin.

In instances in which multihit kinetics were observed (e.g., Lafferty, 1963a,b; Philipson, 1966) either low temperature or antibody of the IgM class, or sera at very low dilutions, were involved. Conceivably, an effective interaction under these conditions might require the cooperation of several molecules of antibody binding to neighboring antigens. A further possibility for a multihit phenomenon with single-hit kinetics would require that the necessary hits all occur simultaneously, not sequentially.

Recently, Della-Porta and Westaway (1978) reviewed the question of the minimum number of molecules of antibody required for neutralization. Their view, as previously proposed by Westaway (1965b), is that neutralization results from a multihit reaction. The conclusion is based on several phenomena: (1) Several examples of multihit inactivation have been reported in which a lag is seen in the kinetic analysis. (2) The existence of infectious virus–antibody complexes that can be neutralized by complement or antiglobulin has been reported. (3) The outcome of virus–antibody interaction is variable, depending on the host or cell indicator. (4) Neutralization of infectious virus–antibody complexes may ensue after prolonged incubation. (5) Synergistic effects, either within a native virion composed of several distinct antigens, or particles with hybrid antigenicities resulting from mixed infection that produces phenotypically mixed particles (i.e., capsids with specificities of both parents) have been observed.

Until more definitive knowledge about neutralization is available, some of the above illustrations can as well be used to fortify the single-hit hypothesis. At the outset, if these investigators consider the few instances in which a shoulder was evident (in kinetic analyses) as support for a multihit mechanism, the same logic can support the single-hit mechanism where no shoulder was evident. There can be no argument that on the average several molecules of antibody may combine with a virion when

it is neutralized. Whether all, or only one, have a function in neutralization is at present undetermined. Possibly all but one may be functionally deficient, or they may have reacted with sites that do not respond appropriately. A relevant observation has been made in several studies; neutralization of infectious virus–antibody complexes by antiglobulin also shows a single-hit mechanism, and at a rate that exceeds the rate of the homotypic reaction. If a virus requires n hits to be neutralized but has received $n - x$ hits, it should not be neutralizable by antiglobulin. It is also pertinent that monovalent fragments of the antiglobulin can mediate neutralization. These observations can also be explained on a single-hit basis, if it is assumed that a virus–antibody interaction may be ineffective but subject to activation.

The view that neutralization is dependent on a multihit reaction was also expressed by Daniels (1975). He indicated that neutralization was the outcome of sequential reactions involving initially free virus and then infectious virus–antibody complexes. Not until the final required molecule reacted did the virion lose its infectivity. However, short of the full requirement, anti-antibody could achieve the final effect. It is difficult to envision an explantory mechanism, since the anti-antibody does not bind to the virion but only to the antiviral antibody. In essence, the number of virus-bound antibodies has not been increased by the antiglobulin reaction.

A single-hit mechanism implies either that there is only one critical site for neutralization, or that one molecule of antibody can cause neutralization at any of several sites. An infectious phenotypically mixed particle is in a sense two particles, since the capsid is composed of the antigens of both progenitors. It is not unexpected that the reaction shows the need for more than one molecule. It is relevant that an excessive amount of antiserum to either parent has little neutralizing effect, whereas a mixture of antisera to both parents neutralizes effectively.

C. Hypothetical Mechanisms

1. Multiple Dispositions

As a result of extensive studies of the serological reactions of togaviruses, Della-Porta and Westaway (1978) have proposed a multihit model to account for neutralization. As previously reported (Westaway, 1965b), neutralization requires that several antigen–antibody complexes be strategically disposed over the viral surface. Since these viruses also hemagglutinate, and since neutralization is not absolute but is host-

dependent, the topology of the bound antibodies determines for which hosts the complex is neutral or infectious, and whether the virion retains its ability to hemagglutinate. Further, these viruses contain two antigens of type and group specificity. The extent to which cross-reactions occur, based on neutralization or hemagglutination–inhibition, depends on how the cross-reacting antibodies are disposed on the viral surface.

2. Conformational Alteration

Recently, a hypothesis was proposed to account for the neutralization of poliovirus and to suggest a mechanism whereby one molecule of antibody achieved this end (Mandel, 1976). In this hypothesis the assumption is made that neutralization is the result of one effective collision. Justification for this assumption is based on the interpretation of kinetic data. An effective collision is dependent either on reaction with a critical site, or on reaction with any antigenic site but in a critical manner. Previous studies (Mandel, 1971b) showed that the poliovirus capsid undergoes spontaneous oscillations between two conformational states characterized by distinct isoelectric points. Inactivation by antibody stabilized the capsid in one of the conformational states. Subsequent studies (Mandel, 1976) with highly diluted antiserum showed the redistribution of the viral population from the normal to the neutralized conformational state by an all-or-none mechanism. The two states differed by more than 2 pH units. Under no conditions were particles of intermediate isoelectric point seen. Moreover, the proportion of virus neutralized and the proportion of virus conformationally stabilized were equal. It was also shown that, in an *in vitro* system capable of carrying out the early stage of viral uncoating, neutralized virus was refractory.

The above observations have been integrated into the following hypothesis. The metastable nature of the viral capsid suggests intersubunit cooperative transitions. Effective attachment of one molecule of antibody to an antigenic subunit stabilizes that subunit in a specific nonfunctional conformation. Through cooperative transitions all neighboring units are similarly stabilized until the entire capsid becomes conformationally homogeneous. These expectations are based on the similarities of the structure of the viral capsid and the structure of proteins subject to allosteric transitions (see Monod *et al.*, 1965, for a discussion of allosterism). Particles in this state are incapable of undergoing normal interactions with plasma membrane of susceptible cells. It was pointed out earlier that neutralized virus not only retained its capability to adsorb, but did so with increased affinity, probably because of the more highly charged nature of the abnormal conformation.

3. Antibody Competence and Conformational Alteration

After extensive studies of the neutralization of herpesvirus, with particular emphasis on sensitization and mediation by complement, Yoshino and his colleagues have proposed a hypothesis to account for neutralization and sensitization (Yoshino and Isono, 1978). Crucial to this hypothesis is an appreciation of the variability inherent in antibodies with respect to their class and to their binding affinity. According to their hypothesis the most efficient antibody is capable of initiating a series of physicochemical changes in a virion that culminates in neutralization. Less efficient antibody can induce some, but not all, of the changes. In this case, complement (and presumably antiglobulin) can mediate the reaction. Very inefficient antibody—"slow-reacting" in their terminology —can bind to virus but cannot drive the reaction to the stage where complement can complete the reaction. In terms of how far the various antibodies can alter the virion toward the neutralized state, these workers classify the antibodies as slow-reacting, early IgM, late IgM, early and intermediate IgG (all incapable per se of neutralizing, and, finally, late IgG antibody that is independent of complement. They have also subdivided the essential changes in the virion leading to neutralization with respect to (1) dependence on time (the early stages) and (2) dependence on temperature (late stages). They propose that one molecule of antibody is sufficient to initiate all reactions whether they fail or succeed in neutralizing. The satisfying feature of this hypothesis is that it allows an interpretation of several distinct phenomena that characterize neutralization: (1) single-hit inactivation, (2) role of antibody diversity, (3) mediated neutralization and, possibly, (4) the nonneutralized state.

4. Minimum Number of Critical Sites

In order to understand the neutralization phenomenon, Trautman has stressed the necessity for ascertaining two parameters: the minimum number of critical sites required for infectivity, and the various small virus-antibody complexes that are infectious. To derive this information, Trautman (1976) has developed a computer assisted program. Based on data for FMD virus, Trautman and Harris (1977) have determined that at least two or three critical sites (the number varies according to the host assay system) that react with IgG are required, whereas only one that reacts with IgM is required for infectivity. The total number of either type of critical site is as yet not known. A reasonable summation of available data have led Trautman and Harris (1977) to propose that FMD virus contains one unique vertex involved in infectivity. This structure consists of one IgM, and five to ten IgG reactive sites. The

authors have indicated that "complications" may arise that would require investigation in order to derive valid interpretations. They have discussed six possible complications.

VII. Concluding Comments

In 1930, Smith reported data that clearly showed the *in vitro* interaction of viruses (vaccinia and herpes) with homologous antibody. Somewhat later, Sabin (1935) was unable to demonstrate such *in vitro* interaction and questioned the basic nature of antibody action. However, subsequent studies established beyond reasonable doubt that virus and homologous antibody do combine. The only other aspect of viral neutralization about which there is a similar degree of unanimity is that the mechanism is not yet understood. This state of uncertainty has recently been deplored: ". . . remains undefined" (Della-Porta and Westaway, 1978), ". . . remains unknown" (Yoshino and Isono, 1978), and ". . . questions remain to be clarified" (Daniels, 1975).

When consideration is given to the known variables of this system (i.e., when each component is recognized as a population within which there is broad variation) and to the realization that neutralization encompasses several distinct phenoma, it is not unexpected that the fundamental nature of the neutralization reaction has not yet been elucidated. Burnet's eloquent assessment of this situation (1960, p. 298) is as true today as then. However, the ability to cope with this difficulty has improved, at least technologically.

Daniels (1975) has summarized the neutraliztion phenomenon as representing three pathways: (1) saturation of viral antigens, (2) aggregation by cross-linking of multivalent virions by bivalent antibodies, and (3) virolysis. Although the net result in each case is loss of or reduction in infectivity, the underlying mechanisms may be basically different. Virolysis, for example, is the result of an enveloped virus reacting with antibodies specific for the envelope proteins and depends on participation of the complete complement system. Aggregation may reduce the number of infectious units without literally neutralizing the individual virions. Saturation may or may not involve intrinsic neutralization.

In order to understand the fundamental mechanism, it seems preferable to select as a paradigm the simplest available system—a simple virus (e.g., one composed of several copies of one antigen directly accessible to antibody) reacting with a homogeneous population of high-affinity antibody (e.g., late IgG) capable of neutralizing without the aid of accessory substances. Interaction of virus at a very low concentration with the

minimum multiplicity of antibody is most likely to reveal why virus has been neutralized. Since under some circumstances neutralized virus can still adsorb to cells, or conversely, since adsorbed virus can still be neutralized, the implication is that the viral capsid has undergone an alteration that subverts subsequent interaction with cells. The nature of the alteration is most likely to be understood on the basis of protein–protein interactions. Some clues have already been described indicating that each reactant has undergone some change from its native state. Antibodies can interact with complement, or bind to cells, only after they have reacted with antigen. Feinstein and Rowe (1965) have proposed that antibody undergoes a conformational rearrangement upon binding to antigen (for an extensive discussion of this proposal see Feinstein *et al.*, 1971). Several examples have been cited that indicate conformational or degradative changes in virion capsids following interaction with antibody. It is of interest that, whereas early antibody reacts with virus but requires mediation, late antibody can neutralize independently. It has also been shown that one difference between early and late antibody is the greater binding affinity of late antibody. The greater the binding affinity, the greater the stress, involvement, and disorganization in the capsid. Such disorganization may be reversible (e.g., by causing dissociation) but in such a state the capsid may not be acceptable to a cell.

An attempt has been made in this article to delineate some of the problems: (1) the mechanism of intrinsic neutralization, (2) the single-hit versus multihit requirement for neutralization, (3) the nature of the nonneutralized or sensitized state, (4) the nature of mediated neutralization by complement or antiglobulin, (5) the differences in the characteristics of virus–antibody complexes according to antibody type and affinity. It hardly needs to be stated that solution of these problems will represent the groundwork for considerations of problems at the next level—*in vivo* interactions.

Acknowledgments

I express my gratitude to Dr. K. Yoshino and to Drs. E. G. Westaway and A. J. Della-Porta for the opportunity to review their manuscripts prior to publication. Also, I extend my thanks to Miss Katherine Kelly for secretarial assistance.

References

Ablashi, D. V., Martos, L. M., Gilden, R. V., and Hampar, B. (1969). *J. Immunol.* **102,** 263.

Adams, J. M., and Imagawa, D. T. (1957). *Proc. Soc. Exp. Biol. Med.* **96,** 240.

Adams, M. H. (1950). *Methods Med. Res.* **2,** 1–73.

Adler, F. L., Walker, W. S., and Fishman, M. (1971). *Virology* **46,** 797.

Almeida, J. D., and Waterson, A. P. (1969). *Adv. Virus Res.* **15,** 307.

Andersen, H. K. (1971). *Arch. Gesamte Virusforsch.* **33,** 187.
Andersen, H. K. (1972). *Arch. Gesamte Virusforsch.* **36,** 133.
Andrewes, C. H. (1928). *J. Pathol. Bacteriol.* **31,** 671.
Andrewes, C. H. (1930). *J. Pathol. Bacteriol.* **33,** 265.
Andrewes, C. H., and Elford, W. J. (1933a). *Br. J. Exp. Pathol.* **14,** 367.
Andrewes, C. H., and Elford, W. J. (1933b). *Br. J. Exp. Pathol.* **14,** 376.
Ashe, W. K., and Notkins, A. L. (1966). *Proc. Natl. Acad. Sci. U.S.A.* **56,** 447.
Ashe, W. K., and Notkins, A. L. (1967). *Virology* **33,** 613.
Ashe, W. K., Mage, M., Mage, R., and Notkins, A. L. (1968). *J. Immunol.* **101,** 500.
Ashe, W. K., Mage, M., and Notkins, A. L. (1969). *Virology* **37,** 290.
Ashe, W. K., Daniels, C. A., Scott, G. S., and Notkins, A. L. (1971). *Science* **172,** 176.
Austin, R. M., and Daniels, C. A. (1974). *J. Immunol.* **113,** 1568.
Barlow, J. L., Van Vunakis, H., and Levine, L. (1958). *J. Immunol.* **80,** 339.
Baughman, R. H., Fenters, J. D., Marquis, G. S., Jr., and Holper, J. C. (1968). *Appl. Microbiol.* **16,** 1076.
Bellanti, J. A., Russ, S. B., Holmes, G. E., and Buescher, E. L. (1965). *J. Immunol.* **94,** 1.
Bendinelli, M., Nardini, L., and Compa, M. (1974). *J. Gen. Virol.* **22,** 207.
Beneke, T. W., Habermehl, K.-O., Diefenthal, W., and Buchholz, M. (1977). *J. Gen. Virol.* **34,** 387.
Berry, D. M., and Almeida, J. D. (1968). *J. Gen. Virol.* **3,** 97.
Blank, S. E., Leslie, G. A., and Clem, L. W. (1972). *J. Immunol.* **108,** 665.
Bowman, B. U., Jr., and Patnode, R. A. (1963). *Virology* **21,** 506.
Bradish, C. J., Farley, J. O., and Ferrier, H. E. N. (1962). *Virology* **18,** 378.
Breindl, M. (1971). *Virology* **46,** 962.
Brown, F., and Smale, C. J. (1970). *J. Gen. Virol.* **7,** 115.
Brown, F., Cartwright, B., and Newman, J. F. E. (1964). *J. Immunol.* **93,** 397.
Brown, G. C., and O'Leary, T. P. (1971). *J. Immunol.* **107,** 1486.
Brown, G. C., Baublis, J. V., and O'Leary, T. P. (1970). *J. Immunol.* **104,** 861.
Brunner, K. T., and Ward, R. (1959). *J. Immunol.* **83,** 405.
Bryan, W. R., and Beard, J. W. (1941). *J. Infect. Dis.* **68,** 133.
Burnet, F. M. (1936). *Aust. J. Exp. Biol. Med. Sci.* **14,** 247.
Burnet, F. M. (1943). *Aust. J. Exp. Biol. Med. Sci.* **21,** 231.
Burnet, F. M. (1960). "Principles of Animal Virology," 2nd ed., p. 295. Academic Press, New York.
Burnet, F. M., Keogh, E. V., and Lush, D. (1937). *Aust. J. Exp. Biol. Med. Sci.* **15,** 227.
Burns, W. H., and Allison, A. C. (1975). *In* "The Antigens" (M. Sela, ed.), Vol. 3, pp. 479–574. Academic Press, New York.
Capstick, P. B., Sellers, R. F., and Stewart, D. L. (1960). *Arch. Gesamte Virusforsch.* **9,** 606.
Carthew, P. (1976). *J. Gen. Virol.* **32,** 17.
Cavanagh, V., Sangar, D. V., Rowlands, D. J., and Brown, F. (1977). *J. Gen. Virol.* **35,** 149.
Cowan, K. M. (1962). *J. Immunol.* **88,** 476.
Cowan, K. M. (1973). *Adv. Immunol.* **17,** 195.
Cremer, N. E., Riggs, J. L., Fujimoto, F. Y., Hagens, S. I., Ota, M. I., and Lennette, E. H. (1964). *J. Immunol.* **93,** 283.
Dales, S., and Kajioka, R. (1964). *Virology* **24,** 278.

Daniels, C. A. (1975). *In* "Viral Immunology and Immunopathology" (A. L. Notkins, ed.). pp. 79–97. Academic Press, New York.
Daniels, C. A., Borsos, T., Rapp, H. J., Snyderman, R., and Notkins, A. L. (1969). *Science* **165,** 508.
Daniels, C. A., Borsos, T., Rapp, H. J., Snyderman, R., and Notkins, A. L. (1970). *Proc. Natl. Acad. Sci. U.S.A.* **65,** 528.
Daugharty, H., Warfield, D. T., Hemingway, W. D., and Casey, H. L. (1973). *Infect. Immun.* **77,** 380.
Della-Porta, A. J., and Westaway, E. G. (1978). *J. Gen. Virol.* **38,** 1.
De Sena, J., and Mandel, B. (1976). *Virology* **70,** 470.
De Sena, J., and Mandel, B. (1977). *Virology* **78,** 554.
Douglas, S. R., and Smith, W. (1930). *Br. J. Exp. Pathol.* **11,** 96.
Dozois, T. F., Wagner, J. C., Chemerda, C. M., and Andrew, V. M. (1949). *J. Immunol.* **62,** 319.
Dudley, M. A., Henkens, R. W., and Rowlands, D. T., Jr. (1970). *Proc. Natl. Acad. Sci. U.S.A.* **65,** 88.
Dulbecco, R., Vogt, M., and Strickland, A. G. R. (1956). *Virology* **2,** 162.
Fazekas de St. Groth, S. (1962). *Adv. Virus Res.* **9,** 1.
Fazekas de St. Groth, S., and Reid, A. F. (1958). *J. Immunol.* **80,** 225.
Fazekas de St. Groth, S., and Webster, R. G. (1963). *J. Immunol.* **90,** 140.
Feinstein, A., and Rowe, A. J. (1965). *Nature (London)* **205,** 147.
Feinstein, A., Munn, E. A., and Richardson, N. E. (1971). *Ann. N.Y. Acad. Sci.* **190,** 104.
Fiala, M. (1969). *J. Immunol.* **103,** 107.
Friedewald, W. F., and Kidd, J. G. (1940). *J. Exp. Med.* **72,** 531.
Gard, S. (1955). *Acta Pathol. Microbiol. Scand.* **37,** 21.
Gard, S. (1957). *Arch. Gesamte Virusforsch.* **7,** 449.
Gebhardt, L. P., and Bullock, I. T. (1931). *J. Immunol.* **21,** 171.
Gigli, I., and Austen, K. F. (1971). *Annu. Rev. Microbiol.* **25,** 309 (p. 315).
Ginsberg, H. S., and Horsfall, F. L. (1949). *J. Exp. Med.* **90,** 475.
Ginsberg, H. S., and Wedgwood, R. J. (1959). *Ann. N.Y. Acad. Sci.* **83,** 528.
Gipson, T. G., Daniels, C. A., and Notkins, A. L. (1974). *J. Immunol.* **112,** 2087.
Goodman, J. W., and Donch, J. J. (1964). *J. Immunol.* **93,** 96.
Goodman, J. W., and Donch, J. J. (1965). *Immunochemistry* **2,** 351.
Gordon, M. H. (1925). *Med. Res. Coun. (G.B.), Spec. Rep. Ser.* **SRS-98.**
Graham, B. J., Minamishima, Y., Dreesman, G. R., Haines, H. G., and Benyesh-Melnick, M. (1971). *J. Immunol.* **107,** 1618.
Granoff, A. (1965). *Virology* **25,** 38.
Graves, J. H., Cowan, K. M., and Trautman, R. (1964). *J. Immunol.* **92,** 501.
Gudnadóttir, M., and Pálsson, P. A. (1965). *J. Immunol.* **95,** 1116.
Hahon, N. (1964). "Selected Papers on Virology." Prentice-Hall, Englewood Cliffs, New Jersey.
Hahon, N. (1969). *J. Gen. Virol.* **4,** 77.
Hahon, N. (1970a). *J. Gen. Virol.* **6,** 285.
Hahon, N. (1970b). *J. Gen. Virol.* **6,** 361.
Hájek, P. (1966). *Folia Microbiol. (Prague)* **11,** 290.
Hájek, P. (1968). *Folia Microbiol. (Prague)* **13,** 557.
Hájek, P. (1969). *Folia Microbiol. (Prague)* **14,** 165.
Hájek, P., and Mandel, L. (1966). *Folia Microbiol. (Prague)* **11,** 282.
Hale, E. M., Hirata, A. A., Brusenback, R. A., and Overby, L. R. (1969). *J. Immunol.* **102,** 206.

Hampar, B., Notkins, A. L., Mage, M., and Keehn, M. A. (1968). *J. Immunol.* **100,** 586.
Hana, L., Krizanova, O., Styk, B., and Sokol, F. (1961). *Acta Virol. (Engl. Ed.)* **5,** 325.
Harris, J. E., Siminovitch, L., McCulloch, E. A., and Cinader, B. (1962). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **21,** 17.
Hashimoto, N., and Prince, A. M. (1963). *Virology* **19,** 261.
Haukenes, G. (1977). *Acta Pathol. Microbiol. Scand., Sect. B* **85,** 125.
Hawkes, R. A. (1964). *Aust. J. Exp. Biol. Med. Sci.* **42,** 465.
Hawkes, R. A., and Lafferty, K. J. (1967). *Virology* **33,** 250.
Heineman, H. S. (1967). *J. Immunol.* **99,** 214.
Hershey, A. D., and Bronfenbrenner, J. (1947). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **6,** 428.
Hirsch, M. S., Allison, A. C., and Harvey, J. J. (1969). *Nature (London)* **223,** 739.
Hornick, C. L., and Karush, F. (1969). *Isr. J. Med. Sci.* **5,** 163.
Howitt, B. F. (1934). *J. Infect. Dis.* **55,** 138.
Howitt, B. F. (1950). *J. Immunol.* **64,** 73.
Huggett, D. O., Rodriguez, J. E., and McKee, A. P. (1972). *Infect. Immun.* **6,** 996.
Hultin, J. V., and McKee, A. P. (1952). *J. Bacteriol.* **63,** 437.
Hyllseth, B., and Petterson, U. (1970). *Arch. Gesamte Virusforsch.* **32,** 337.
Ide, K., and Yoshino, K. (1974). *Jpn. J. Microbiol.* **18,** 397.
Imagawa, R. (1935). *Acta Med. Hokkaidonensia* **13,** 523 (based on citation in Iwasaki and Ogura, 1968a).
Ishida, N., and Ackermann, W. W. (1956). *J. Exp. Med.* **104,** 501.
Iwasaki, T., and Ogura, R. (1968a). *Virology* **34,** 46.
Iwasaki, T., and Ogura, R. (1968b). *Virology* **34,** 141.
Jerne, N. K., and Skovsted, L. (1953). *Ann. Inst. Pasteur, Paris* **84,** 73.
Joklik, W. K. (1964). *Virology* **22,** 620.
Joklik, W. K., and Darnell, J. E., Jr. (1961). *Virology* **13,** 439.
Karush, F. (1976). *Contemp. Top. Mol. Immunol.* **5,** 217.
Keller, R. (1965). *J. Immunol.* **94,** 143.
Keller, R. (1966). *J. Immunol.* **96,** 96.
Keller, R. (1968). *J. Immunol.* **100,** 1071.
Keller, R., and Dwyer, J. E. (1968). *J. Immunol.* **101,** 192.
Ketler, A., Hinuma, Y., and Hummeler, K. (1961). *J. Immunol.* **86,** 22.
Kjellén, L. (1957). *Arch. Gesamte Virusforsch.* **7,** 307.
Kjellén, L. (1962). *Virology* **18,** 448.
Kjellén, L. (1964). *Arch. Gesamte Virusforsch.* **14,** 189.
Kjellén, L. (1965a). *Arch. Gesamte Virusforsch.* **17,** 398.
Kjellén, L. (1965b). *Virology* **27,** 580.
Kjellén, L., and Pereira, H. G. (1968). *J. Gen. Virol.* **2,** 177.
Kjellén, L., and Schlesinger, R. W. (1959). *Virology* **7,** 236.
Klinman, N. R., Long, C. A., and Karush, F. (1967). *J. Immunol.* **99,** 1128.
Kociskova, D., Styk, B., and Hana, L. (1961). *Acta Virol. (Engl. Ed.)* **5,** 19.
Krummel, W. M., and Uhr, J. W. (1969). *J. Immunol.* **102,** 772.
Labzoffsky, N. A. (1946). *J. Infect. Dis.* **79,** 63.
Lafferty, K. J. (1963a). *Virology* **21,** 61.
Lafferty, K. J. (1963b). *Virology* **21,** 76.
Lafferty, K. J., and Oertelis, S. (1963). *Virology* **21,** 91.

Leerhoy, J. (1966). *Acta Pathol. Microbiol. Scand.* **67,** 158.
Leerhoy, J. (1968). *Acta Pathol. Microbiol. Scand.* **73,** 275.
Lewenton-Kriss, S., and Mandel, B. (1972). *Virology* **48,** 666.
Leymaster, G. R., and Ward, T. G. (1948). *Am. J. Hyg.* **48,** 45.
Leymaster, G. R., and Ward, T. G. (1949). *J. Immunol.* **61,** 95.
Linscott, W. D., and Levinson, W. E. (1969). *Proc. Natl. Acad. Sci. U.S.A.* **64,** 520.
Lonberg-Holm, K., and Yin, F. H. (1973). *J. Virol.* **12,** 114.
Long, P. H., and Olitsky, P. K. (1930). *J. Exp. Med.* **51,** 209.
McCarthy, K., and Germer, W. D. (1952). *Br. J. Exp. Pathol.* **33,** 529.
McKercher, P. D., and Giordano, A. R. (1967). *Arch. Gesamte Virusforsch.* **20,** 54.
McNeill, T. A. (1968). *J. Hyg.* **66,** 541.
Madeley, C. R., Allan, W. H., and Kendal, A. P. (1971). *J. Gen. Virol.* **12,** 69.
Maess, J. (1971). *Arch. Gesamte Virusforsch.* **33,** 194 (Engl. Summ.).
Majer, M. (1972). *Curr. Top. Microbiol. Immunol.* **58,** 69.
Majer, M., and Link, F. (1970). *Clin. Exp. Immunol.* **7,** 283.
Majer, M., and Link, F. (1971). *J. Gen. Virol.* **13,** 355.
Mandel, B. (1958). *Virology* **6,** 424.
Mandel, B. (1961). *Virology* **14,** 316.
Mandel, B. (1962). *Cold Spring Harbor Symp. Quant. Biol.* **27,** 123.
Mandel, B. (1967a). *Virology* **31,** 238.
Mandel, B. (1967b). *Virology* **31,** 248.
Mandel, B. (1971a). *Methods Virol.* **5,** 375–397.
Mandel, B. (1971b). *Virology* **44,** 554.
Mandel, B. (1976). *Virology* **69,** 500.
Markenson, J. A., Daniels, C. A., Notkins, A. L., Hoofnagle, J. H., Gerety, J., and Barker, L. F. (1975). *Clin. Exp. Immunol.* **19,** 209.
Minamishima, Y., Graham, B. J., and Benyesh-Melnick, M. (1971). *Infect. Immun.* **4,** 368.
Miyamoto, K., Morgan, C., Hsu, K. C., and Hampar, B. (1971). *J. Natl. Cancer Inst.* **46,** 629.
Monod, J., Wyman, J., and Changeux, J. P. (1965). *J. Mol. Biol.* **12,** 88.
Morgan, I. M. (1945). *J. Immunol.* **50,** 359.
Mueller, J. H. (1931). *J. Immunol.* **20,** 17.
Muschel, L. H., and Toussaint, A. J. (1962). *J. Immunol.* **89,** 35.
Nagano, Y., and Mutai, M. (1954). *C. R. Seances Soc. Biol. Ses Fil.* **148,** 757.
Nagano, Y., and Oda, M. (1955). *C. R. Seances Soc. Biol. Ses Fil.* **149,** 863.
Neva, F. A., and Weller, T. H. (1964). *J. Immunol.* **93,** 466.
Notkins, A. L. (1971). *J. Exp. Med.* **134,** 41S.
Notkins, A. L., Mahar, S., Scheele, C., and Goffman, J. (1966). *J. Exp. Med.* **124,** 81.
Notkins, A. L., Mage, M., Ashe, W. K., and Mahar, S. (1968). *J. Immunol.* **100,** 314.
Notkins, A. L., Rosenthal, J., and Johnson, B. (1971). *Virology* **43,** 321.
Ogra, P. L., Karzon, D. T., Righthand, F., and MacGillivray, M. (1968). *N. Engl. J. Med.* **279,** 893.
Oldstone, M. B. A. (1975). *Prog. Med. Virol.* **19,** 84.
Oldstone, M. B. A., and Dixon, F. J. (1967). *Science* **158,** 1193.
Oldstone, M. B. A., and Dixon, F. J. (1969). *J. Exp. Med.* **129,** 483.
Oldstone, M. B. A., Larson, D. L., and Cooper, N. R. (1972). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **31,** 791.
Oldstone, M. B. A., Cooper, N. R., and Larson, D. L. (1974). *J. Exp. Med.* **140,** 549.

Olitsky, P. K. (1940). *J. Exp. Med.* **72,** 113.
Oroszlan, S., and Gilden, R. V. (1970). *Science* **168,** 1478.
Osler, A. G. (1976). "Complement—Mechanisms and Function." Prentice-Hall, Englewood Cliffs, New Jersey.
Ozaki, Y. (1968). *Arch. Gesamte Virusforsch.* **25,** 137.
Ozaki, Y., and Tabeyi, K. (1967). *J. Immunol.* **98,** 1218.
Ozaki, Y., Kumagai, K., Kawanishi, M., and Seto, A. (1974). *Arch. Gesamte Virusforsch.* **45,** 7.
Parkman, P. D., Mundon, F. K., McCowan, J. M., and Buescher, E. L. (1964). *J. Immunol.* **93,** 608.
Pernis, B., Ghezzi, I., and Turri, M. (1963). *Nature (London)* **197,** 807.
Petty, R. E., and Steward, M. W. (1977). *Immunology* **32,** 49.
Philipson, L. (1966). *Virology* **28,** 35.
Philipson, L., and Bennich, H. (1966). *Virology* **29,** 330.
Philipson, L., Killander, J., and Albertsson, P. A. (1966). *Virology* **28,** 22.
Pierce, M. E., Kempf, J. E., and Soule, M. H. (1941). *Proc. Soc. Exp. Biol. Med.* **48,** 604.
Pollikoff, R., and Sigel, M. M. (1952). *Bacteriol. Proc.* p. 105.
Polyak, R. Y., Yabrov, A. A., and Smorodintsev, A. A. (1961). *Acta Virol. (Engl. Ed.)* **5,** 261.
Porter, D. D., and Larson, A. E. (1967). *Proc. Soc. Exp. Biol. Med.* **126,** 680.
Radwan, A. I., and Burger, D. (1973a). *Virology* **51,** 71.
Radwan, A. I., and Burger, D. (1973b). *Virology* **53,** 366.
Radwan, A. I., Burger, D., and Davis, W. C. (1973). *Virology* **53,** 372.
Rappaport, I. (1961). *Biochim. Biophys. Acta* **47,** 206.
Rappaport, I. (1970). *J. Gen. Virol.* **6,** 25.
Rawls, W. E., Desmyter, J., and Melnick, J. L. (1967). *Proc. Soc. Exp. Biol. Med.* **124,** 167.
Rowlands, D. T., Jr. (1967). *J. Immunol.* **98,** 958.
Rowlands, D. J., Sangar, D. V., and Brown, F. (1971). *J. Gen. Virol.* **13,** 85.
Rubin, H. (1957). *Virology* **4,** 533.
Rubin, H., and Franklin, R. M. (1957). *Virology* **3,** 84.
Sabin, A. B. (1935). *Br. J. Exp. Pathol.* **16,** 70.
Sabin, A. B. (1950). *Bacteriol. Rev.* **14,** 225.
Schmidt, N. J., Lennette, E. H., and Dennis, J. (1968). *J. Immunol.* **100,** 99.
Schrader, J. A., and Muschel, L. H. (1975). *Immunochemistry* **12,** 791.
Shinkai, K., and Yoshino, K. (1975a). *Jpn. J. Microbiol.* **19,** 25.
Shinkai, K., and Yoshino, K. (1975b). *Jpn. J. Microbiol.* **19,** 211.
Silverstein, S. C., and Marcus, P. I. (1964). *Virology* **23,** 370.
Smith, W. (1930). *J. Pathol. Bacteriol.* **33,** 273.
Smorodintsev, A. A., and Yabrov, A. A. (1963). *Acta Virol. (Engl. Ed.)* **7,** 193.
Spiegelberg, H. L. (1974). *Adv. Immunol.* **19,** 259.
Stemke, G. W. (1969). *J. Immunol.* **103,** 596.
Stemke, G. W., and Lennox, E. S. (1967). *J. Immunol.* **98,** 94.
Stevens, D. A., Pincus, T., Burroughs, M. A. K., and Hampar, B. (1968). *J. Immunol.* **101,** 979.
Stinski, M. F., and Cunningham, C. H. (1970). *J. Gen. Virol.* **8,** 173.
Strong, P. S. (1936). *J. Immunol.* **30,** 403.
Strunk, R. C., John, T. J., and Sieber, O. F. (1977). *Infect. Immun.* **15,** 165.

Styk, B. (1961). *Acta Virol. (Engl. Ed.)* **5,** 334.
Styk, B. (1962). *Acta Virol. (Engl. Ed.)* **6,** 327.
Styk, B. (1965). *Acta Virol. (Engl. Ed.)* **9,** 210.
Styk, B., and Hana, L. (1961a). *Acta Virol. (Engl. Ed.)* **5,** 194.
Styk, B., and Hana, L. (1961b). *Acta Virol. (Engl. Ed.)* **5,** 342.
Styk, B., and Hana, L. (1964). *Acta Virol. (Engl. Ed.)* **8,** 200.
Styk, B., and Hana, L. (1965a). *Acta Virol. (Engl. Ed.)* **9,** 109.
Styk, B., and Hana, L. (1965b). *Acta Virol. (Engl. Ed.)* **9,** 200.
Styk, B., Rathova, V., and Blaskovic, D. (1958). *Acta Virol. (Engl. Ed.)* **2,** 179.
Styk, B., Rathova, V., and Kociskova, D. (1961). *Acta Virol. (Engl. Ed.)* **5,** 326.
Styk, B., Hana, L., Kociskova, D., and Rathova, V. (1964). *Acta Virol. (Engl. Ed.)* **8,** 283.
Svehag, S.-E. (1965). *Arch. Gesamte Virusforsch.* **17,** 504.
Svehag, S.-E. (1966). *In* "Antibodies to Biologically Active Molecules" (B. Cinader, ed.), pp. 301–348. Pergamon, Oxford.
Svehag, S.-E. (1968). *Prog. Med. Virol.* **10,** 1.
Svehag, S.-E., and Mandel, B. (1962). *Virology* **18,** 508.
Svehag, S.-E., and Mandel, B. (1964a). *J. Exp. Med.* **119,** 1.
Svehag, S.-E., and Mandel, B. (1964b). *J. Exp. Med.* **119,** 21.
Symington, J., McCann, A. K., and Schlesinger, M. J. (1977). *Infect. Immun.* **15,** 720.
Tanaka, H. (1931). *Tokyo Iji Shinshi* **2709,** 153 (based on citation in Iwasaki and Ogura, 1968a).
Taniguchi, S., and Yoshino, K. (1965). *Virology* **26,** 54.
Taylor, R. M. (1941). *J. Immunol.* **40,** 373.
Thormar, H. (1963). *J. Immunol.* **90,** 185.
Todd, C. (1928). *Br. J. Exp. Pathol.* **9,** 244.
Tolmach, L. J. (1956). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **15,** 619.
Toolan, H. W. (1965). *Proc. Soc. Exp. Biol. Med.* **119,** 715.
Toussaint, A. J., and Muschel, L. H. (1962). *J. Immunol.* **89,** 27.
Trautman, R. (1976). *Scand. J. Immunol.* **5,** 609.
Trautman, R., and Harris, W. F. (1977). *Scand. J. Immunol.* **6,** 831.
Uhr, J. W. (1964). *Science* **145,** 457.
Uhr, J. W., and Finkelstein, M. S. (1967). *Prog. Allergy* **10,** 37.
Uhr, J. W., Finkelstein, M. S., and Baumann, J. B. (1962). *J. Exp. Med.* **115,** 655.
Van Vunakis, H. V., Barlow, J. L., and Levine, L. (1956). *Proc. Natl. Acad. Sci. U.S.A.* **42,** 391.
Vogt, A., Kopp, R., Maass, G., and Reich, L. (1964). *Science* **145,** 1447.
Wallis, C. (1971). *In* "Viruses Affecting Man and Animals" (M. Sanders and M. Schaeffer, eds.), pp. 102–123. Warren H. Green, Inc., St. Louis, Missouri.
Wallis, C., and Melnick, J. L. (1965). *Virology* **26,** 175.
Wallis, C., and Melnick, J. L. (1967). *J. Virol.* **1,** 478.
Wallis, C., and Melnick, J. L. (1970). *Virology* **42,** 128.
Wallis, C., and Melnick, J. L. (1971). *J. Immunol.* **107,** 1235.
Wallis, C., Shirley, A., and Melnick, J. L. (1973). *Intervirology* **1,** 41.
Way, H. J., and Garwes, D. J. (1970). *J. Gen. Virol.* **7,** 211.
Westaway, E. G. (1965a). *Virology* **26,** 517.
Westaway, E. G. (1965b). *Virology* **26,** 528.
Westaway, E. G. (1968). *J. Immunol.* **100,** 569.
Whitman, L. (1947). *J. Immunol.* **56,** 97.

Yoshino, K., and Isono, N. (1978). *Microbiol. Immunol.* **22** (in press).
Yoshino, K., and Kishie, T. (1973). *Jpn. J. Microbiol.* **17,** 63.
Yoshino, K., and Morishima, T. (1972). *Jpn. J. Microbiol.* **16,** 125.
Yoshino, K., and Taniguchi, S. (1964). *Virology* **22,** 193.
Yoshino, K., and Taniguchi, S. (1965a). *Virology* **26,** 44.
Yoshino, K., and Taniguchi, S. (1965b). *Virology* **26,** 61.
Yoshino, K., and Taniguchi, S. (1966). *J. Immunol.* **96,** 196.
Yoshino, K., Hashimoto, M., and Shinkai, K. (1977). *Microbiol. Immunol.* **21,** 231.