CHAPTER 4

Serological Methods in the Identification and Characterization of Viruses

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1. INTRODUCTION

The purpose of this chapter is to present an integrated view of the various serological techniques that have been used in virology. The accent will be placed on the principles that govern each type of test and on the general applicability of the different serological techniques in all fields of virus research. In recent years, advances in serological techniques have sometimes been applied in only one area of virology, although they could have been equally useful to workers studying other groups of viruses. No doubt this stems from the host-oriented approach that has guided the compartmentation of virology into separate fields of specialization. When it comes to serological properties, however, the similarities between animal, insect, bacterial, and plant viruses are paramount. The same immunochemical principles govern the *in vitro* serological reactions of all viral antigens, and much of general interest can be learned from the findings obtained with each particular group of viruses. An attempt will be made here to emphasize the general validity of specific experimental procedures. A number of recent reviews restricted to the serology of particular groups of viruses are available

H. Fraenkel-Conrat et al. (eds.), *Comprehensive Virology*

(Cowan, 1973; Schmidt and Lennette, 1973; Ball, 1974; Kurstak and Morisset, 1974; Burns and Allison, 1975; Mazzone and Tignor, 1976; Mayr *et al.*, 1977; Tyrrell, 1978; Van Regenmortel, 1978; Cooper, 1979).

2. REAGENTS

2.1. Viral Antigens

It is beyond the scope of this chapter to consider the various purification methods that have been used to obtain viruses and viral soluble antigens suitable for antiserum production and serological analysis. The reader is referred to standard textbooks of virology and to some specialized reviews (Van Regenmortel, 1966; Francki, 1972; Appleyard and Zwartouw, 1978).

Methods of protein purification that are particularly helpful for purifying viruses are precipitation with polyethylene glycol (Juckes, 1971; Polson *et al.*, 1972; Venekamp, 1972) and separation by electrophoresis (Polson and Russell, 1967; Van Regenmortel, 1964, 1972). Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and crossed immunoelectrophoresis have been used successfully to obtain pure protein subunits of viral capsids. The band corresponding to the component of interest can be cut out of the gel and used as such for immunization (Vestergaard, 1975; Carroll *et al.*, 1978).

The degree of purity of the antigen that is required in individual cases is extremely variable. When used as a diagnostic reagent, it is important that the antigen should be free of contaminating viruses and nonspecific inhibitors that could interfere in the serological tests (Schmidt and Lennette, 1971; Polley, 1977). For the purpose of antigenic analysis, it is important that host-specific antigens should be absent (Van Regenmortel, 1963). These contaminants can be removed by the use of antihost serum and specific immunoadsorbents (Gold, 1961; Avrameas *et al.*, 1969; Birkbeck and Stephen, 1970; Sugiura and Nakajima, 1977; Polson *et al.*, 1978).

2.2. Antisera

Rabbits are commonly used for producing antisera against viral antigens, although goats and sheep are sometimes employed when large quantities of antiserum are needed. Ascitic fluid from immunized mice

is also a convenient source of viral antibody (Sartorelli *et al.*, 1966; Kiriyama and Ohsumi, 1973; Tzianabos *et al.*, 1976).

Little reliable information is available regarding the relative merits of different immunization procedures. In rabbits, the antiviral immune response measured in individual animals submitted to the same immunizing procedure was found to be highly variable (Van Regenmortel and Von Wechmar, 1970). As a result, comparative trials designed to demonstrate the superiority of a particular method are likely to be extremely onerous and, in fact, very few such comparative studies have been reported. One generalization that appears to be valid is that the use of adjuvants is advantageous. A variety of empirical procedures that give satisfactory results have been described in several reviews of immunization practices (Chase, 1967; Horwitz and Scharff, 1969*a*; Crowle, 1973; Herbert, 1978).

It seems that many workers do not appreciate the extent to which individual antisera obtained from a series of bleedings of different animals can vary, both quantitatively and qualitatively. It cannot be overemphasized that it is essential to immunize a number of animals and to take several bleedings from each one, whatever the purpose of the serological study may be. For the same reason, the practice of indiscriminately pooling antisera can be detrimental, since it could reduce the potency of an individual antiserum which possesses the desired characteristics.

The use of inbred strains of animals for decreasing the heterogeneity of the immune response has received little attention from virologists. On the other hand, the recently developed method of cell fusion of malignant cells and antibody-producing cells (Köhler and Milstein, 1975) is being applied successfully for producing homogeneous antiviral antibodies. This method, which is based on the production of somatic cell hybrids between mouse myeloma cells and spleen cells derived from mice immunized with viruses, holds tremendous promise for the elucidation of various aspects of antiviral immunity. Monoclonal antibodies against influenza and rabies viruses have been obtained from such hybridomas (Gerhard *et al.*, 1978; Wiktor and Koprowski, 1978). One interesting finding obtained from such studies is that monoclonal antibodies expressed by hybridomas appear to lack the usual cross-reactivity between virus strains and variants that is found with antibodies from normal mice. The absence of this cross-reactivity may be due to the fact that, in normal immunized mice, only a small population of splenic cells is able to recognize identical determinants in the two strains. If most clones of splenic cells do in fact express antibody specificities unique to the immunizing variant, it is

normal that most hybridoma antibodies do not recognize altered antigenic determinants in the heterologous variants. There is some evidence that monoclonal antibodies are not able to recognize a complementary antigenic determinant when it has been altered in a variant at a single amino acid position (Laver, personal communication). These preliminary results make it clear that hybridoma antibodies represent a powerful new tool for investigating viral antigens.

3. NEUTRALIZATION

Virus neutralization tests measure the loss of virus infectivity resulting from the binding of antibody molecules to the surface of virions. The mechanisms underlying this decrease in infectivity are complex and have not yet been fully elucidated. Clearly, the antibody interferes with an early stage of the infectious process, i.e., with adsorption to the host cell, penetration, or uncoating of the virions, but the precise step which is blocked may not be the same with all viruses. The sensitivity of the host cell used for the infectivity assay and the route of inoculation may also play a role. Different levels of residual infectivity may be observed when the same virus–antibody complex is assayed in different host cells (Philipson, 1966), and higher antibody titers are often obtained in less sensitive hosts.

Virus infectivity can be measured by any available titration method, using animals, embryonated eggs, monolayer cell cultures in tubes or microtiter plates, plaque assays, mechanical inoculation onto leaf surfaces, or inhibition of vector transmissibility. Since, with plant viruses, quantitative infectivity assays are neither sensitive nor very accurate, neutralization tests have rarely been used in plant virology.

Although neutralization tests generally are the most specific of all virus–antibody reactions, it must be realized that they do not provide information on the total antiviral antibody response. These tests obviously will not detect antibodies reacting with viral antigenic structures that are not directly involved in the expression of infectivity. Neutralization of influenza virus is produced only by antibodies directed against the hemagglutinin component of the membrane and not by those directed against the neuraminidase (Webster and Laver, 1967) or the matrix protein (Oxford and Schild, 1976). In the case of foot-and-mouth disease virus, the antibody-binding site present on the face of the icosahedral particle (Brown and Smale, 1970) is not involved in neutralization, whereas the two sites present on the vertices are (Rowlands *et al.*, 1971). In the case of vesicular stomatitis virus, which is com-

posed of five polypeptide chains and RNA, antisera to the virus contain antibodies to the nucleoprotein core antigen (Cartwright *et al.*, 1970) and to the spike glycoprotein. It was found that neutralizing activity of antiserum could be fully inhibited by the purified glycoprotein, which suggests that it is only the latter component which is involved in the neutralization reaction (Kelley *et al.*, 1972).

The distribution of different antigens over the surface of adenoviruses is also well established (Pettersson, 1971; Wadell, 1972; Willcox and Mautner, 1976*a*,*b*). Antisera were prepared against isolated hexons, pentons, and fibers, and were tested for neutralizing activity. By far the most efficient neutralization was obtained with antihexon antibodies. Antibodies against penton bases and fibers produced only negligible neutralization when used separately, although considerable neutralization occurred when both antisera were used together (Norrby and Wadell, 1972).

Another problem is that the extent of neutralization is very much dependent on the "quality" of the antibody, i.e.. on such factors as antibody affinity and valence. This means that neutralization assays may be unreliable for comparing the amounts of antibody present in different antisera (Haimovich and Sela, 1966; Blank *et al.*, 1972). In spite of these limitations, however, neutralization tests have been found invaluable for diagnostic and epidemiological studies, and they have contributed more than any other technique to our understanding of the interaction between virus and antibody. A number of reviews dealing with various aspects of virus neutralization are available (Fazekas de St. Groth, 1962; Svehag, 1968; Osterrieth, 1972; Daniels, 1975; Mandel, 1979).

3.1. Mechanism of Neutralization

The mechanism of neutralization has been investigated mainly by following the kinetics of the neutralization reaction. The procedure consists of mixing antibody with virus and removing, at regular time intervals, an aliquot of the mixture, which is then assayed for infectivity. When the percentage of surviving virus is plotted against time, an initial linear exponential decline in virus infectivity is usually observed (Dulbecco *et al.*, 1956; Granoff, 1965). Such apparent first-order kinetics are commonly attributed to the fact that a single hit results in virus neutralization (see Svehag, 1968). According to Daniels (1975), however, these data do not establish that one antibody molecule alone will neutralize an infectious particle. This author suggests that the

attachment of a single antibody may be the terminal event corresponding to the neutralization of an infectious virus–antibody complex formed in the early stages of the reaction (i.e., within a few seconds).

In many systems, deviations from a linear exponential decline, for instance, as an initial lag phase, have been observed (Lafferty, 1963). Furthermore, in the later stages of the reaction, a point is reached when no further decrease in infectivity occurs. The residual infective virions are not genetic variants, because their progeny are inactivated at the same rate as the original virus population. This residual infectivity, called the persistent fraction, has been ascribed to a variety of causes:

1. Aggregation of virus particles, which prevents virions in the center of the aggregate from coming into contact with antibody (Wallis and Melnick, 1967, 1970; Hahon, 1970).
2. Steric hindrance by neighboring antibodies, which prevents critical sites on the virion from being reached (Lafferty, 1963; Ashe *et al.*, 1969; Rappaport, 1970).
3. Dissociation of virus–antibody complexes (Fazekas de St. Groth and Webster, 1963).

Although each explanation is supported by some experimental evidence, other data again show that, in many systems, the hypothesis of aggregation (Ashe *et al.*, 1969; Hahon, 1970) or of dissociation (Mandel, 1961) is not tenable.

Most investigators have been unable to demonstrate any significant reversibility of the virus–antibody reaction by dilution of the reaction mixture (see Svehag, 1968). This may be due to the low dissociation constant of the reaction which may necessitate intervals of several days to produce detectable dissociation. Another reason may be that, after an initial reversible reaction involving only one of the two IgG combining sites, a secondary reaction occurs whereby the second antibody site binds to a neighboring antigenic group on the virion (Lafferty and Oertelis, 1963). Such monogamous bivalent binding can occur because IgG molecules are sufficiently flexible to adjust their two identical combining sites to fit neighboring subunits on the viral surface. When such a complex is formed, the free energy change is much greater than when the antibody molecule binds by only one site (Keller, 1966; Klinman *et al.*, 1967; Hornick and Karush, 1972). With tobacco mosaic virus (TMV) and IgG antibody, it was found that no monogamous bivalent binding occurred in extreme antibody excess (Van Regenmortel and Hardie, 1976). On the other hand, when the same excess of antibody was added stepwise by a series of successive small increments, bivalent binding occurred exclusively. The more rapid

univalent binding seems to be favored when the antibody is added all at once, and this allows exactly twice as many antibody molecules to attach to the virus surface than when antibody is added more slowly (Van Regenmortel and Hardie, 1976). The energetically favored bivalent binding of antibody may thus be restricted only to conditions of antigen excess.

In the case of the tailed bacteriophages, most of the phage-neutralizing activity of antibodies appears to be directed against the tail fibers of baseplates (Franklin, 1961; King, 1968; Berget and King, 1978). It has been suggested that the binding of antibodies to the tail fibers causes some of the fibers to adopt a coiled conformation (Stemke *et al.*, 1974), and that antibody directed against the phage whiskers in the collar region immobilizes the fibers in a restricted position (Conley and Wood, 1975).

Recently, still another mechanism which applies to viruses that can undergo reversible conformational transitions has been proposed, to explain both neutralization and its apparent one-hit nature (Mandel, 1976). The capsid of poliovirus has been shown to undergo a transition between two electrophoretically recognizable states, A and B, characterized by isoelectric points of about 7 and 4.5, respectively (Mandel, 1971). When virus reacts with antibody, it becomes stabilized in state B without producing any particles of intermediate isoelectric point. It has been suggested that the virion is infectious only in state A and that the reaction of a single antibody molecule with one antigenic determinant causes the viral subunit containing this determinant to be stabilized in the B conformation. Through cooperative conformational transitions, all subunits would then become stabilized in the same state, and the altered virion would be resistant to uncoating (Mandel, 1976).

In recent years, much evidence favoring the multihit model of virus neutralization has accumulated (see Daniels, 1975; Della-Porta and Westaway, 1978). For instance, the synergistic effect of mixtures of antibody molecules directed at the different antigenic determinants of adenoviruses clearly contradicts the single-hit model of neutralization (Hierholzer and Dowdle, 1970; Norrby and Wadell, 1972). Similarly, results obtained with phenotypically mixed virus particles derived by mixed infection with two serologically distinguishable viruses show that greatly enhanced neutralization is achieved by the combined action of antibodies directed against the two sets of viral antigens (Choppin and Compans, 1970).

Further data obtained with flaviviruses and which are also incompatible with a single-hit model have been reviewed by Della-Porta and Westaway (1978). According to these authors, the "critical areas" at

the surface of virions that need to be covered by antibody to achieve neutralization are too large to allow inactivation by single immunoglobulin molecules.

On the other hand, in two recent papers (Trautman, 1976; Trautman and Harris, 1977), it has been argued that data suggesting one-hit neutralization kinetics can be reconciled with the presence of as many as five to ten critical sites on a small particle such as foot-and-mouth disease virus. Using the mass-action theory and a computer simulation approach, it was found that three of these critical sites had to be free for the virus to be infectious. Trautman (1976) derived an explicit expression for the extent of reaction between virus and antibody which allows the computer simulation of known systems. The utilization of this approach in the future should no doubt contribute to the elucidation of the mechanisms of virus neutralization.

3.2. Virus Sensitization

Virus sensitization refers to a situation where antibodies attached to the surface of virions do not neutralize the infectivity but "sensitize" the virus to subsequent inactivation by a third component such as antiglobulin, complement, or staphylococcal protein A (Notkins, 1971; Majer, 1972; Takabayashi and McIntosh, 1973; Austin and Daniels, 1974). Although virus–antibody complexes in the persistent fraction may contain sensitized virus (Majer and Link, 1970), the phenomenon of sensitization is not restricted to the non-neutralizable fraction. Antibodies directed to antigenic structures that are not involved in the expression of infectivity, such as the neuraminidase of influenza virus (Webster and Laver, 1967), are able to sensitize viruses. This means that the sensitization reaction is a useful tool for studying viral antigens that do not react with neutralizing antibody.

Neutralization of sensitized virus by antiglobulin antibodies has been found to be a sensitive method for detecting minute amounts of antiviral antibody (Goodman and Donch, 1965), for studying cross-reactions between viruses (Kjellen and Pereira, 1968), and for titrating various antiglobulin preparations (Daniels *et al.*, 1970). Certain rheumatoid factors such as IgM molecules with antiglobulin activity also bind to sensitized virus, but, in this case, neutralization is only achieved by the subsequent action of complement (Ashe *et al.*, 1971; Gipson *et al.*, 1974). It has been suggested that the inability of rheumatoid factor by itself to neutralize sensitized virus is due to the

large size of the IgM molecule which may prevent effective covering of critical sites.

Sensitization by complement components has been studied with numerous enveloped viruses, for instance, with herpesvirus (Yoshino and Taniguchi, 1965), Newcastle disease virus (Linscott and Levinson, 1969), and equine arteritis virus (Radwan and Burger, 1973). The early-acting complement components (C1 to C4) appear to neutralize infectivity through a steric hindrance phenomenon resulting from the packing of bound molecules at the surface of the virion (Radwin and Crawford, 1974). The terminal complement components (C5 to C9), on the other hand, act on the viral membrane by inducing lysis of virus particles and loss of the internal components (Oroszlan and Gilden, 1970; Daniels, 1975). The mechanism of virolysis by the action of complement is similar to the destruction of the cell membrane in immune cytolysis. Both processes result from the enzymatic breakdown of a lipoprotein membrane and can be visualized, in the electron microscope, in the form of similar lesions in the virus envelopes and cell membranes (Berry and Almeida, 1968; Oldstone, 1975). Non-membrane-containing viruses such as polyoma virus are resistant to lysis by complement even in the presence of DNase (Oldstone *et al.*, 1974).

Instead of protecting the host against unwanted infections, the interaction of complement with virus–antibody complexes *in vivo* may lead sometimes to a virus-induced immune complex disease. In persistent virus infections, the continuous antigenic challenge can lead to the formation of virus–antibody complexes that become trapped in various tissues of the host. Granular deposits of virus, host immunoglobulin, and complement in renal glomeruli and arteries have been demonstrated with numerous chronic viral infections and appear to be a common cause of immune complex disease (Oldstone, 1975).

3.3. Methods

3.3.1. Classical Neutralization Test

The classical neutralization test is performed either by incubating a series of antiserum dilutions with a constant amount of virus or, alternatively, by incubating a constant amount of antiserum with increasing dilutions of virus. The first method is more economical in the use of antiserum and is more generally used with tissue culture indicator systems. The second method is preferable when antisera have a low

antibody content. Following incubation, the infectivity of the virus can be tested in various indicator systems such as monolayer tube cultures or embryonated eggs. Detailed experimental procedures are described by Casals (1967), Habel (1969), Lennette and Schmidt (1969), Rovozzo and Burke (1973), and Mayr *et al.* (1977).

3.3.2. Microneutralization Tests

Following the development of disposable plastic microtiter plates, microneutralization tests suitable for numerous viruses have been described (Kenny *et al.*, 1970; Schmidt and Lennette, 1973). The results can be scored in terms of the suppression of a viral cytopathic effect or by utilizing the phenomenon of hemadsorption which can be brought into play by adding certain erythrocytes to the plastic cups (Sorensen, 1974; Wooley *et al.*, 1974; Greig, 1975). Results can also be evaluated colorimetrically by observing the color change of an indicator added to the growth medium (Witte, 1971). This follows from the fact that acid production which normally accompanies the growth of cells is suppressed by virus infection.

3.3.3. Plaque Reduction Test

Initially, the plaque reduction test was restricted to the study of bacteriophage neutralization, and the results were scored by the standard double agar layer method of phage titration (Adams, 1959). The assay of neutralized phage was improved by the "decision" technique of Jerne and Avegno (1956), and by the complex inactivation method, which uses an antiglobulin serum (Goodman and Donch, 1965; Krummel and Uhr, 1969). Following the development of plaque assays for animal viruses (Dulbecco and Vogt, 1954), the plaque reduction test has become increasingly used with many viruses (Fiala, 1969; Schmidt and Lennette, 1973; McVicar *et al.*, 1974). The foci of virus-infected cells (plaques) are made more easily visible by the staining of the background viable cells with vital stains.

3.3.4. Neutralization Kinetics Test

The sensitive neutralization kinetics test which measures the speed of virus neutralization by antibodies is particularly suited for determin-

ing small antigenic differences between closely related virus strains. A neutralization rate constant is determined which is normalized by expressing the results obtained in a heterologous test as a percentage of the rate constant observed in the homologous system (McBride, 1959). This method has been used in strain differentiation with herpes simplex virus (Ashe and Scherp, 1963; Wheeler *et al.*, 1969), rhinovirus (Cooney *et al.*, 1973), reovirus (Munro and Wooley, 1973), poliovirus (McBride, 1959; Thouvenot *et al.*, 1973), vaccinia virus (Dunlap and Barker, 1973), and bovine rhinotracheitis virus (Potgieter and Maré, 1974). In a recent study of foot-and-mouth disease virus strains, the method was compared with the plaque reduction and microneutralization tests and found to allow a similar degree of differentiation between strains (Rweyemamu *et al.*, 1977). In contrast, the complement fixation test was less capable of distinguishing between different strains.

3.3.5. Reactivation of Neutralized Virus

The neutralization of viruses by homologous antibody in the body fluids sometimes hampers the detection of inapparent viral infections. This is the case with Newcastle disease virus in poultry, where inapparent infections in convalescent or vaccinated birds are believed to play a role in the dissemination of the disease (Gillette *et al.*, 1975). The usual virus isolation procedures generally fail to detect these infections, and it is necessary, for efficient recovery of the virus, first to dissociate the virus–antibody complexes. Neutralized Newcastle disease virus was efficiently reactivated by proteolytic digestion with trypsin or papain (Brugh, 1977). Poliovirus and foot-and-mouth disease virus have been reactivated by treatment with fluorocarbon (Brown and Cartwright, 1960; Svehag, 1963). Other reactivation methods that have been used successfully are sonic treatment (Keller, 1965) and the use of pH extremes (Mandel, 1961; Granoff, 1965; Kjellén, 1966).

3.3.6. Serological Blocking of Aphid Transmission

This infectivity neutralization test is assayed by determining whether aphids fed on virus–antibody mixtures can transmit the virus to healthy plants (Gold and Duffus, 1967; Duffus and Gold, 1969). The method can be used to demonstrate serological relationships between plant viruses when the usual in vitro methods cannot be applied, for instance, with viruses that are not mechanically transmissible (Rochow

and Ball, 1967). This method has been of considerable help in clarifying the relationships between members of the luteovirus group (Duffus and Russell, 1975; Rochow and Duffus, 1978).

4. AGGLUTINATION

It is customary to distinguish between agglutination and precipitation reactions on the basis of the size of the reacting antigen. The term "precipitation" is used to describe the insolubilization of virus particles through the action of antibody, whereas the term "agglutination" refers to the clumping of cells or of particles of similar size. Fewer antibody molecules are needed to produce an agglutination visible to the naked eye than are required to produce a visible precipitate. This has led to the development of numerous procedures which extend the range of visual detection of serological reactions by means of reagents such as red blood cells and latex particles coated with antibody or viral antigens.

4.1. Viral Hemagglutination

Many viruses are able to bind to receptors present on the surface of red blood cells of certain animal species. This leads to the agglutination of the erythrocytes, a phenomenon called hemagglutination. Since hemagglutination does not take place when the surface of a virion is covered with antibody molecules, it is possible to use hemagglutination inhibition tests to assay for the presence of viral antibodies.

The components of the virus surface responsible for the hemagglutinating property are known as hemagglutinins and consist usually of glycoproteins or lipoproteins. In the case of adenoviruses, the hemagglutinating activity resides in the fiber component. Soluble hemagglutinins are often present in viral extracts, either because they are released from intact virions or because they are produced in the infected cell in large excess compared to the intact virions.

Soluble hemagglutinins that are monovalent with respect to their binding sites for erythrocytes cannot agglutinate cells. Although they do combine with the cell receptors, they produce agglutination only after becoming linked following the addition of antihemagglutinin antibodies (Norrby, 1968).

The best-studied hemagglutinins are those of myxoviruses, and a number of reviews describing their structural and functional properties

are available (Howe and Lee, 1972; Schulze, 1973; Laver, 1973). Much of the interest in the influenza hemagglutinin is due to the fact that it undergoes wide antigenic variation and that this phenomenon is responsible for the continual outbreaks of new influenza epidemics (Webster and Laver, 1975). Two distinct types of antigenic variation have been shown to occur. The first is antigenic drift, which involves gradual changes in the amino acid sequence of the hemagglutinin polypeptide. The second type of variation consists of major antigenic shifts which are caused by sudden, vast differences in amino acid sequence which cannot be explained by mutation. It is thought that such "new" human viruses are formed as a result of genetic recombination between "old" human viruses and animal influenza virus strains.

A phenomenon related to viral hemagglutination is hemadsorption, which occurs with those viruses that possess hemagglutinins and are released from the host cell by a process of budding. During the release of the virus at the cell surface, the infected cells are able to adsorb erythrocytes. In hemadsorption inhibition, the infected cells are treated with viral antiserum prior to the addition of red cell suspensions (Schmidt and Lennette, 1973; Norrby *et al.*, 1977).

4.2. Hemagglutination Inhibition Tests

Hemagglutination inhibition tests may be used either to identify virus isolates by means of specific antisera or to measure antibody levels by means of standard virus suspensions. They are usually somewhat less sensitive than neutralization tests for detecting viral antibodies, but they are easier to perform and take less time to complete. However, hemagglutination inhibition tests are complicated by the presence, in many sera, of various nonspecific inhibitors of agglutination. Some of these nonspecific inhibitors block agglutinating sites on the erythrocytes, while others react with the antigen itself. It is essential to remove these inhibitors, but, unfortunately, no single, universally applicable method can be recommended. Good results in eliminating the inhibitors have been obtained by treating antiserum with ether (Tauraso *et al.*, 1971), kaolin (Monath *et al.*, 1970; Inouye and Kono, 1972), dextran sulfate and $CaCl_2$ (Liebhaber, 1970; Nelson *et al.*, 1972), rivanol (Toms and Mostratos, 1973), and DEAE-Sephadex (Altemeyer *et al.*, 1970).

In addition to the problem of nonspecific inhibitors, it has also been reported (Reno and Hoffman, 1972) that certain components of

complement can enhance the hemagglutination inhibition caused by specific antibody.

In recent years, hemagglutination has been shown to occur with many more virus–erythrocyte combinations than previously known, and useful diagnostic tests are now available for most human and animal viruses, e.g., adenoviruses, enteroviruses, myxoviruses, reoviruses, and rhabdoviruses (Schmidt and Lennette, 1973). In some cases, it may be advantageous to use formalinized or glutaraldehyde-fixed erythrocytes, since they can be stored for several months; this eliminates the variability which is sometimes found in individual red blood cell preparations (Gupta and Harley, 1970; Wolff *et al.*, 1977). Freeze-dried, formalinized erythrocytes have been used in the detection of rubella antibodies (Van Weemen and Kacaki, 1976).

During the last few years, further advances have occurred in the testing of coronaviruses (Bingham *et al.*, 1975), certain herpesviruses (Klingeborn and Dinter, 1973), parvoviruses (Joo *et al.*, 1976), retroviruses (Witter *et al.*, 1973), rubella virus (Quirin *et al.*, 1972; Iwakata *et al.*, 1974), togaviruses (Della-Porta and Westaway, 1972), and papovaviruses (Favre *et al.*, 1974).

Detailed procedures for hemagglutination inhibition tests are described in Rosen (1969), Lennette and Schmidt (1969), Grist *et al.* (1974), Mayr *et al.* (1977), and Tyrrell (1978).

4.3. Passive Hemagglutination

Passive hemagglutination, also called indirect hemagglutination, utilizes erythrocytes to which virus particles have been coupled by various chemical treatments. The cells are first stabilized, for instance, with tannic acid (Stavitsky, 1977), sulfosalicyl acid (Becht, 1968), or glutaraldehyde (Becht and Malole, 1975). Bisdiazobenzidine (Arquilla, 1977) and carbodiimides were used successfully as coupling reagents. These antigen-coated red blood cells can be agglutinated by specific viral antibody, and the test is thus applicable in principle to any virus, not only to those which possess hemagglutinins. The passive hemagglutination reaction can be inhibited by prior incubation of the test serum with a suspension of the homologous virus. Such an inhibition lends itself to the measurement of very small amounts of viral antigen.

In view of the very wide applicability of direct viral hemagglutination procedures with animal viruses, passive hemagglutination tests have been used only occasionally in animal virology. Nevertheless, good

results have been reported, for instance, with herpesvirus (Bernstein and Stewart, 1971), rhinovirus (Faulk *et al.*, 1971), a murine leukemia virus (Sibal *et al.*, 1971), foot-and-mouth disease virus (Warrington and Kawakami, 1972), and the Australia antigen associated with viral hepatitis (Vyas and Shulman, 1970; Hollinger *et al.*, 1971).

In plant virology, passive hemagglutination has been used for detecting very small amounts of virus in crude and clarified plant sap (Cunningham *et al.*, 1966; Abu Salih *et al.*, 1968*a*). Red blood cells were coated with either viral antigen or antibody. Antiserum titers were determined with virus-coated erythrocytes or, in inhibition tests, with antibody-coated erythrocytes. Compared with the classical precipitin test in tubes, a thousandfold increase in serum titer can be obtained (Richter, 1967). It is also possible to detect very small amounts of virus, either with red cells sensitized with an optimal quantity of antibody or in inhibition tests using virus-coated erythrocytes. Such tests were found to be about 100–500 times more sensitive than tube precipitin tests in the detection of various elongated and isometric plant viruses (Saito and Iwata, 1964; Richter, 1971).

The sensitivity of the passive hemagglutination technique makes it well suited for studying weak cross-reactions between related viruses (Gamez *et al.*, 1967) and for demonstrating the weak serological activity of short peptides that correspond to certain antigenic determinants of a viral protein. This approach has been used in studies of the antigenic determinants of the coat protein of tobacco mosaic virus (TMV). The *C*-terminal hexapeptide of TMV protein was synthesized and coupled to erythrocytes, and passive hemagglutination tests were used to detect the presence, in TMV antisera, of antibodies reactive with the hexapeptide (Anderer and Ströbel, 1972*a*,*b*).

4.4. Latex Test

In the latex test, antigen or antibody is adsorbed onto commercially available polystyrene latex particles (Bactolatex, Difco Laboratories, Detroit, Michigan). The technique has been described in detail by Litwin (1977) and Bercks *et al.* (1972). Optimal sensitization of the latex is obtained by carefully selecting the dilution of antiserum or purified globulin preparation used for coating the particles (Bercks, 1967; Bercks and Querfurth, 1969). Different antisera should be tested since not all antisera are equally effective in this procedure.

Using antibody-coated latex, it is possible to detect 100- to 1000-

fold smaller quantities of virus than is possible with tube precipitin tests. This method has been used in the routine detection of numerous elongated and isometric plant viruses in crude extracts (Abu Salih *et al.*, 1968*b*; Maat, 1970; Schade, 1971; Fuchs, 1976; Koenig and Bode, 1978), as well as with insect and animal viruses (Fritz and Rivers, 1972; Carter, 1973).

It is possible to sensitize latex particles with viral antigens. The best results are obtained when an excess of antigen is adsorbed onto latex via an intermediate layer of previously adsorbed antibodies. This method has been used to study distant relationships within the potexvirus and tymovirus groups (Bercks and Querfurth, 1971). Homologous antiserum titers showed a maximal increase of 300-fold over the titers obtained in precipitin tests. Large increases in heterologous titers were obtained only when purified globulin fractions were used instead of unfractionated antisera.

5. PRECIPITATION

5.1. Quantitative Precipitin Tests in Tubes

Quantitative precipitin tests in fluid medium have been used mainly in plant virology because they require relatively large quantities of reactants. Purified antigen preparations have to be used since the tests cannot resolve multiple antigen–antibody systems. The tests are usually performed in tubes by mixing 0.5-ml volumes of suitable dilutions of antiserum and antigen, and determining the highest dilution of antiserum that will give a visible precipitate (i.e., the antiserum titer).

The various factors which influence the results of tube precipitin tests have been discussed in detail by Matthews (1967). One important point is that the size of the reacting antigen very much influences the antiserum titer. If was shown by Kleczkowski (1966) that about 10 times more antibody is required to precipitate a given amount of depolymerized tobacco mosaic virus protein than to precipitate the same amount of virus. Similarly, in the case of potato virus X and its protein subunits, Shepard and Shalla (1970) showed that the virus was a 25-times-better detector of a given amount of homologous antibody than the depolymerized protein. It is clear, therefore, that precipitin titers are not suitable for comparing antibody levels against antigens of different sizes. For the same reason, preparations of elongated viruses that have become aggregated during purification tend to give spuriously

high antiserum titers. The presence of small amounts of polyethylene glycol in the reaction mixture may also lead to higher precipitin titers (Wolf and Schmelzer, 1973).

Although it is widely believed that precipitation results from the formation of a lattice between large numbers of antigen and antibody molecules, this view appears to be too simplistic. In fact, precipitation does not occur in solutions of low ionic strength even when the reactants are present in optimal proportions and in spite of the fact that binding of antigen to antibody takes place normally in the absence of salt (Kleczkowski, 1965). Subsequent dissociation of antibody from the antigen, under acid conditions, is much facilitated when the initial binding is carried out in the absence of salt. This finding was used to develop an improved method for the isolation of specific viral antibody which possesses several advantages compared to standard procedures (Hardie and Van Regenmortel, 1977).

Tube precipitin tests have been found useful for quantifying the extent of serological cross-reaction between strains of elongated viruses, provided certain precautions are taken. It is well known that the extent of cross-reaction between two strains determined in reciprocal tests, i.e., when antisera are used against each of the two strains, can show considerable variation. Sometimes the extent of cross-reactivity is pronounced in one test and very weak or totally absent in the reciprocal test (Corbett, 1967; Van Regenmortel and Von Wechmar, 1970). However, when a sufficient number of bleedings obtained from different animals are used to compare average homologous and heterologous titers, reciprocal tests give very similar results. This allows the computation of a reliable index of serological cross-reactivity between virus strains (Van Regenmortel, 1975). The results obtained with numerous strains of tobacco mosaic virus indicate that it is possible to arrange them in a series of increasingly distantly related entities and that any sharp distinction between close and distant serological relationships is essentially arbitrary (Van Regenmortel, 1966, 1978). Similar conclusions have been drawn from serological studies of tymoviruses (Koenig and Givord, 1974; Koenig, 1976) and strains of tobacco necrosis virus (Uyemoto *et al.*, 1968).

5.2. Microprecipitin Tests in Droplets

Microprecipitin tests are performed in single drops of the mixed reactants deposited on the bottom of a petri dish. The drops are usually

covered with a layer of mineral oil to prevent drying out, and the reactions are observed by dark-field microscopy at 50–100 × magnification. Purified virus preparations as well as clarified extracts of infected tissue can be utilized for the test. The method is economical in its use of antiserum and is fairly sensitive since small precipitates are easily detected under the microscope. Microprecipitin tests have been extensively used by plant virologists for indexing plants for the presence of virus; their numerous applications have been reviewed by Van Slogteren (1969) and Ball (1974).

5.3. Immunoprecipitation

The technique of immune precipitation is a useful method for isolating intracellular proteins of virus-infected cells. During their multiplication within host cells, viruses induce the synthesis of a variety of proteins, only some of which become part of the assembled virions. Serological precipitation has been found extremely useful for following the rate of synthesis of virus-specific antigens by means of pulse-chase experiments. Usually, the labeled proteins in the extract are allowed to react with rabbit antibody specific for the antigen under study, and the resulting soluble complexes are precipitated with a second antiserum against rabbit globulins. Alternatively, the labeled antigen can be isolated by coprecipitation after addition of large amounts of the purified cold antigen and specific antibody. These procedures have been described in detail by Horwitz and Scharff (1969*b*). It is also possible to replace the second antibody directed against rabbit globulins by staphylococcal protein A. This protein binds specifically to IgG and decreases the problem of nonspecific binding observed with double antibody systems (Goding, 1978).

The immune precipitates are usually dissolved in buffers containing urea or sodium dodecyl sulfate and are analyzed by polyacrylamide gel electrophoresis (Shanmugam *et al.*, 1972). The study of virus-specific translation by immune precipitation has been especially valuable for analyzing the replication of retroviruses, since, in this case, host protein synthesis is not inhibited by virus infection and alternative methods of analysis are thus more difficult to use (Vogt and Eisenman, 1973; Van Zaane *et al.*, 1975; Naso *et al.*, 1975). Immunoprecipitation has also been used to study the radioiodinated surface antigen of Friend murine leukemia virus (Ihle *et al.*, 1975).

5.4. Double Diffusion

In double diffusion techniques, antigen and antibody diffuse toward each other in a gel which initially contained neither of them. As diffusion progresses, the two reactants meet, and precipitation occurs along a line where serological optimal proportions are reached. This line remains stationary if the ratio of the initial concentrations of the reactants corresponds to the equivalence point. In this case, the position of the line is a reflection of the Stokes' radius of the antigen. If one of the reactants is initially present in excess of the other, the precipitin line will broaden and shift toward the reservoir with the less concentrated reactant. Thus the position of the line can also be used to estimate the concentration of one or other reactant.

Another outstanding advantage of double diffusion analysis is that complex mixtures of antigens can be resolved and that the minimum number of antigen–antibody systems present can be enumerated.

Numerous parameters, in addition to size and relative concentration, can influence the diffusion process and the precipitation patterns. Agarose gels have been found superior to agar for resolving precipitin lines formed by enteroviruses (Styk and Schmidt, 1968). The electrolyte concentration in the gel (Wetter, 1967) as well as the kind of buffer used (Von Wechmar and Van Regenmortel, 1968) can markedly affect the formation of precipitin lines. The type of preservative used for keeping the gel free of microbial contamination may also be important. Mercury-containing preservatives which can degrade certain viruses are now mostly replaced by sodium azide (Cowan, 1966; Koenig, 1970).

It is generally accepted that, in a balanced immunological system, a single homogeneous antigen cannot give rise to more than one precipitin line. If artifacts arising from temperature variations or unsuspected contaminants can be excluded, the formation of double bands by apparently single systems has usually been attributed to the fact that the antigen was degraded into fragments with different serological specificities. It is unlikely, however, that this explanation applies to the two bands commonly observed with the protein subunit of tobacco mosaic virus (Kleczkowski, 1961; Van Regenmortel and Lelarge, 1973). When this subunit is isolated in the monomeric state from a single band in SDS polyacrylamide gels, it still gives rise to double precipitin lines. A possible explanation for this phenomenon is that antibody binding can induce, in the monomer, a conformational change similar to that observed during the polymerization of the subunit into capsids. Antibodies specific for each conformational state are known to be present in

most antisera (Van Regenmortel and Lelarge, 1973), and it is possible that they induce mutually exclusive surface determinants in the monomer.

Exhaustive reviews of all aspects of immunodiffusion techniques are available (Ouchterlony, 1968; Crowle, 1973; Ouchterlony and Nilsson, 1978).

5.4.1. Double Diffusion in Tubes

Double diffusion in tubes is especially valuable for measuring antigen and antibody concentration and for estimating the size of a viral antigen by means of its diffusion coefficient (Van Regenmortel, 1959). The location of a precipitin band in the tube is a linear function of the logarithm of the ratio of the concentration of the reactants and can be measured very accurately with a calibrated magnifying device (Polson, 1971). The applications of double diffusion tests in tubes for the study of viruses have been discussed by Van Regenmortel (1966).

5.4.2. Double Diffusion in Plates

Double diffusion in plates, often referred to as the Ouchterlony method, is one of the most widely used techniques in virus serology. In addition to its simplicity and economical use of reagents, its main advantage is that it is able to provide a visual demonstration of the relationships that exist between antigens. When antigens diffuse from neighboring wells in a gel toward a single antibody source, a variety of precipitation patterns can be observed. Patterns of coalescence, partial fusion, and crossing of precipitin lines reflect the presence or absence of common or related antigenic determinants on the different antigens. This type of study has been extensively used in all areas of virology for analyzing the antigenic relationship between groups of viruses (Van Regenmortel, 1966; Schmidt and Lennette, 1973; Brown *et al.*, 1973; Ball, 1974; Mazzone and Tignor, 1976; Moyer and Smith, 1977). The method has also been found invaluable for analyzing the antigenic properties of individual components of capsids, such as the hexon, penton, and fiber subunits of adenoviruses (Valentine and Pereira, 1965; Norrby, 1969) or the hemagglutinin, neuraminidase, and nucleoprotein antigens of influenza viruses (Schild and Pereira, 1969; Schild, 1970).

It is important to recognize that useful information is obtained only when two antigens are tested against a single serum. When two antisera prepared against cross-reacting antigens are tested against only one of the antigens, a pattern of coalescence of precipitin lines is always obtained (Van Regenmortel, 1966; Scott, 1973). The fusion of precipitin lines, in this case, simply reflects the fact that different antigenic regions on the surface of the virion do not diffuse independently (Grogan *et al.*, 1964). This fact is not always appreciated (Sim and Watson, 1973; Reinganum and Scotti, 1976) and, as a result, the misnomer "reaction of identity," used to describe the merging of lines, often leads authors to the erroneous conclusion that two antigens are serologically identical.

Serological comparisons between related antigens often require that antisera be cross-adsorbed with heterologous antigens. This is most conveniently done by the method of intragel adsorption (Van Regenmortel, 1967). The antigen preparation used for absorption is allowed to diffuse in the gel and will thereby establish a concentration gradient around the well. When, at a later stage, the antiserum is allowed to diffuse from the same well, the cross-reacting antibodies will be fully precipitated in the gel in the zone of equivalence. Unadsorbed antibodies will diffuse freely and will form a residual precipitin line with the homologous antigen (Von Wechmar and Van Regenmortel, 1968; Wetter and Luisoni, 1969; Granett and Shalla, 1970; Bell and Orlob, 1977). This method of serological adsorption has been used to establish that the capsids of certain viruses possess some antigenic determinants that are absent on the corresponding depolymerized capsid subunits (Van Regenmortel, 1978). These new antigenic specificities, called neotopes (Van Regenmortel, 1966), are a reflection of the quaternary structure of viral proteins and could arise either through conformational changes or through the juxtaposition of amino acid residues from neighboring subunits (Norrby and Wadell, 1972; Eppstein and Thoma, 1977).

Large viruses and elongated ones with a length above 500 nm diffuse poorly in 0.5–1.0% agar gel and must be degraded into smaller fragments in order to be studied by immunodiffusion (Purcifull and Gooding, 1970; Shepard, 1972; Esposito *et al*, 1977; Miller and Van der Maaten, 1977; Harrap *et al.*, 1977). Purcifull and Batchelor (1977) have listed 35 plant viruses that have been dissociated with sodium dodecylsulfate in order to permit immunodiffusion studies. Serological relationships between several potyviruses were studied with antisera prepared against their dissociated protein subunits. It was found that the subunits from different viruses were more closely related serologically

than the corresponding intact virions (Shepard *et al.*, 1974). This may be due to the fact that a larger surface area of the native viral subunit is immunochemically expressed in the monomer, compared with the capsid (Van Regenmortel and LeLarge, 1973). Another explanation is that the partial denaturation of the protein reveals internal sequence similarities that are buried in the native molecule (Arnon and Maron, 1971).

5.5. Radial Immunodiffusion

In radial immunodiffusion, one of the reactants, usually the antigen, is allowed to diffuse into a gel containing a uniform concentration of the other reactant. The antigen is deposited in a small well, cut in the gel layer, and diffuses radially into the antibody-containing agar. A ring of precipitation is formed around the charged well and will increase in diameter until such time as the amount of available antigen has been consumed (Mancini *et al.*, 1965). When the diameter of the ring no longer increases, a state reached only after several days, there is a linear relationship between antigen concentration and area of precipitate. In practice, quantitative measurements of antigen concentration are made before the maximum ring diameter is reached. This is done by a comparison with the ring diameters produced by standards of known concentration.

Because of their large size, virions diffuse only slowly in 1% agar gel. As a result, high concentrations of virus are needed to produce rings of precipitation of sufficient diameter to be useful in quantitative measurements. The method has been found to give satisfactory quantitative results with isometric plant viruses of 30 nm diameter (Richter *et al.*, 1976; Juretic and Mamula, 1978), although its ideal range of application lies with smaller antigens such as dissociated viral components.

The radial immunodiffusion procedure can also be reversed, i.e., the intact virions can be incorporated in the gel and the corresponding antiserum deposited in a well. This method was introduced by Schild *et al.*, (1972) to assay influenza virus antibodies and was also used to measure antibodies against adenovirus and vaccinia virus (Grandien and Norrby, 1975; Prakash *et al.*, 1977). In this type of test, the virions are immobilized in the gel as single particles, separated from each other by a distance equal to about 50 times their particle diameter. The ring of "precipitation" in this case is in fact a halo caused by increased light scattering of virions coated with antibody molecules (Schild *et al.*, 1972; Mostow *et al.*, 1975; Norrby *et al.*, 1977). It is also possible to determine

antibody concentrations by incorporating serial dilutions of antiserum in the gel and measuring the precipitation rings produced by standard virus preparations (Cowan and Wagner, 1970; Wagner *et al.*, 1972).

Radial immunodiffusion tests have been used extensively in plant virology to diagnose viral infections of economically important crops (Shepard and Secor, 1969). A mass testing program for potato viruses, based on the use of virions degraded by compounds such as pyrrolidine and pyridine, has been developed (Shepard, 1972).

Simplified techniques have been described which are especially adapted to large-scale screening but which still retain a sensitivity of about 1 μg antigen/ml. Slack and Shepherd (1975) eliminated tissue grinding, well cutting and chemical treatment of the antigen by embedding small pieces of infected leaf tissue in the antibody-containing agar. Van Slogteren (1976) deposited small drops of agar containing the antiserum on the bottom of a petri dish and placed droplets of plant juice containing degraded virus next to them. Precipitation inside the agar drops was observed under a dissecting microscope. Compared to radial diffusion in plates, this method achieved a twelvefold saving of antiserum.

5.6. Radial Hemolysis

Radial hemolysis combines the accuracy of radial immunodiffusion measurements with the sensitivity of hemagglutination inhibition tests. Virus-sensitized erythrocytes are incorporated in an agarose gel, and the corresponding viral antiserum, placed in wells, is allowed to diffuse radially into the gel (Russel *et al.*, 1975). In the presence of complement, a ring of hemolysis is produced around the wells. The technique has been used to assay antibodies to the hemagglutinin (Schild *et al.*, 1975) and neuraminidase antigens (Callow and Beare, 1976) of influenza virus, as well as antibodies to mumps virus (Grillner and Blomberg, 1976).

5.7. Immunoelectrophoretic Techniques

5.7.1. Immunoelectrophoresis

Immunoelectrophoresis was introduced in order to analyze complex mixtures of antigens that are not readily resolved by immunodiffusion. The antigens are first separated by electrophoresis in one dimen-

sion and are then allowed to diffuse in a second dimension toward a trough filled with antiserum (Williams, 1971). The main advantage of the method is that the antigens are defined by two independent criteria: electrophoretic mobility and antigenic specificity. The method provides mainly qualitative information, but has nevertheless been found useful in all areas of virology (Van Regenmortel, 1966; Schmidt and Lennette, 1973; Ball, 1974).

5.7.2. Counterimmunoelectrophoresis

Counterimmunoelectrophoresis is based on the fact that, in agar gel electrophoresis experiments at pH 7–8, most antigens possess a relatively high negative charge and move toward the anode, whereas antibodies that have a lower negative charge are transported toward the cathode by the electro-endosmotic flow. As a result, the two reactants, when placed in opposite wells, will migrate toward each other and will form precipitation lines very rapidly. Furthermore, much smaller quantities of antigen will be detectable than by immunodiffusion. The method, also known as immunoosmophoresis or electrosyneresis, has been applied to the detection of plant viruses (Ragetli and Weintraub, 1964; John, 1965) and Australia antigen (Gocke and Howe, 1970) and to the diagnosis of rubella and rotavirus infections (Grauballe *et al.*, 1975, 1977; Middleton *et al.*, 1976).

5.7.3. Rocket Immunoelectrophoresis

Rocket immunoelectrophoresis is a quantitative method for the measurement of antigen concentration (Laurell, 1966). Antigen samples to be compared are introduced in small wells cut side by side in a layer of antibody-containing agarose gel. When an electric current is applied, the migration of antigen into the gel leads to rocket-shaped precipitation patterns. The heights of these patterns are directly proportional to antigen concentration. The concentration of test samples can be calculated from a standard curve based on known dilutions of a reference preparation (Weeke, 1973*a*). This technique was found to be as sensitive as, but more rapid than, radial immunodiffusion for quantifying the structural proteins of influenza virus (Oxford and Schild, 1977). It has also been found useful for measuring the concentration of the major internal polypeptides of Rauscher murine leukemia virus (Robinson *et al.*, 1977). This protein has a low electrophoretic mobility but could nevertheless be measured by rocket electrophoresis, after its negative

charge had been enhanced by treatment with sodium dodecyl sulfate. In plant virology, the technique has been used to measure the concentration of the whole virions and dissociated subunits of cucumber mosaic virus (Havranek, 1978*a*,*b*).

5.7.4. Crossed Immunoelectrophoresis

Crossed immunoelectrophoresis involves first the electrophoretic separation of a mixture of antigens in agarose gel and then a second electrophoresis, at right angles to the first, into a gel containing antibodies (Weeke, 1973*b*). This method provides a higher resolution than classical immunoelectrophoresis and also allows quantitation of the individual antigenic components. It is particularly suited to the analysis of complex antigen–antibody systems, such as the large number of antigenic components of herpes simplex virus (Vestergaard *et al.*, 1977; Norrild *et al.*, 1977). Precipitates can be cut out from crossed immunoelectrophoresis gels and used to immunize rabbits. Vestergaard (1975) used this method to produce antisera suitable for typing herpes virus isolates.

6. TESTS WITH LABELED ANTIBODIES

6.1. Immunofluorescence

Antibodies labeled with fluorescent compounds such as fluorescein isothiocyanate have been used for many years in the cellular localization of viral antigens (Schmidt and Lennette, 1973). Fluorescent antibodies are able to combine with viral antigens present at the surface of infected cells, and the complex can be observed with an optical microscope using ultraviolet light. The general methodology of immunofluorescent staining has been presented in detail in several reviews (Gardner and McQuillin, 1974; Nairn, 1975; Peters and Coons, 1976; Kawamura, 1977; Faure *et al.*, 1977).

6.1.1. Direct Staining Procedure

In the direct staining procedure, the viral antibody is conjugated to the fluorescent dye and serves directly as a specific stain to detect viral antigens. Although it is not as sensitive as the indirect method, this procedure is less subject to nonspecific background staining. Viral antisera should be shown to be free of antibodies against host anti-

bodies by testing them against uninfected control cells. Unwanted antibodies can be removed by absorption of antisera with suitable preparations of host antigens (Kawamura, 1977; Nairn, 1975; Johansson *et al.*, 1976). The direct staining method is considered more reliable than other fluorescent methods for identifying many different viruses (Emmons and Riggs, 1977).

6.1.2. Indirect Staining Procedure

The indirect staining procedure consists of first allowing unlabeled viral antiserum to react with the virus. After washing the preparation to remove extraneous serum proteins, the presence of bound antibodies is revealed by a second reaction with fluorescent antiglobulin antibodies. These antibodies are prepared against the globulin of the animal species from which viral antibody was obtained. A single fluorescent antibody conjugate will thus be suitable for the detection of a large number of viruses, provided that all the viral antisera used in the primary reaction are prepared in a single animal species. Another advantage of the indirect procedure is that it is more sensitive than the direct one because the intermediate layer of viral antibody appears to increase the surface area available for the binding of the fluorescent label. On the other hand, the indirect method is more likely to produce nonspecific staining and therefore requires very stringent specificity controls (Emmons and Riggs, 1977).

6.1.3. Anticomplement Staining Procedure

The anticomplement staining procedure employs unlabeled viral antibody (IgG) that will fix complement following its interaction with antigen. An anti-C3 fluorescent antibody conjugate is used to detect the presence of bound complexes (Laing, 1974). The sensitivity of this technique is superior to that of the direct staining procedure.

6.1.4. Viral Diagnosis

In addition to its use in classical histological studies of experimental infections, the immunofluorescence procedure has been applied increasingly to the rapid diagnosis of viral infections. It has been especially valuable in the diagnosis of rabies (Lee *et al.*, 1977). Present methods of fluorescence microscopy used in clinical virology make it possible to diagnose many viral infections within a few hours of the

patient's admission to hospital (Emmons and Riggs, 1977; Gardner, 1977).

Comparative studies of the value of immunofluorescence and immunoperoxidase procedures (Kurstak *et al.*, 1977) for identifying viruses in tissue culture have shown that both methods possess similar sensitivity and specificity (Wicker, 1971; Hahon *et al.*, 1975). However, the immunoperoxidase assay is easier to perform and to interpret, and it provides a permanent record of the data (Benjamin, 1974; Benjamin and Ray, 1974; Herrmann *et al.*, 1974).

In plant virology, fluorescent antibodies have been applied successfully in the detection of different virus strains in infected protoplasts (Otsuki and Takebe, 1978) and of citrus tristeza virus in various tissues of infected citrus (Tsuchizaki *et al.*, 1978).

6.2. Immunoenzymatic Tests

Enzyme-labeled antibodies have been successfully applied for some years to the localization of antigens in tissue sections, at both the optical and the electron microscope level (Wicker and Avrameas, 1969; Faure *et al.*, 1977). When it became clear that enzyme immunoassays were also eminently suited for the quantitative determination of antigens and antibodies (Engvall and Perlmann, 1971; Van Weemen and Schuurs, 1971), the method was rapidly adopted by several laboratories for the serodiagnosis of parasitic and viral diseases (Voller *et al.*, 1974, 1976). The method has been successfully applied to the detection of measles, rubella, mumps, and Newcastle disease (Voller and Bidwell, 1977).

Numerous variations of enzyme-linked immunosorbent assays (ELISA) exist, depending on whether the antigen or antibody is being measured. Most investigators use plates of polystyrene or polyvinyl chloride that are commonly employed for hemagglutination and complement fixation tests. The indirect ELISA method used for measuring viral antibody (Voller and Bidwell, 1977) consists of the following steps: (1) coating of wells of a microtiter plate with a standard virus preparation, (2) binding of specific antibody present in a test serum to the virus, (3) binding of an enzyme-labeled antiglobulin serum to the viral antibodies, and (4) revealing the presence of bound enzyme by a colorimetric reaction with a suitable substrate. The initial coating step requires the presence of 0.05 M bicarbonate pH 9.6 buffer. Between each successive incubation with the different reagents, the wells are washed several times with phosphate-buffered saline containing 0.05% Tween. The enzyme most commonly used for preparing the conjugate is

alkaline phosphatase. Its presence in the well is detected by a yellow coloration following the addition of the substrate. In addition to visual observation, the results can be expressed quantitatively by measuring the absorbance at 405 nm. A colorimeter which measures the absorbance directly in the wells has been described (Clem and Yolken, 1978).

For the detection of plant viruses, most workers used the "double antibody sandwich" form of ELISA. In this method, the wells are first coated with antivirus globulin, and the virus in test samples is then trapped by the adsorbed antibody. The presence of trapped virus is revealed by an enzyme-labeled antivirus globulin. This method necessitates the preparation of a different antibody conjugate for each virus to be tested.

When virus antiserum prepared in two animal species is available, it is possible to combine the indirect and "double antibody sandwich" methods. This approach was followed by Scherrer and Bernard (1977) to detect the presence of rotavirus in fecal samples and of rotavirus antibodies in calf sera. The assay involved the following steps: (1) coating of wells with the globulin fraction of a rotavirus antiserum prepared in rabbits, (2) incubation with virus, (3) incubation with antirotavirus bovine antiserum, (4) incubation with purified antibovine antibodies conjugated with alkaline phosphatase, and (5) colorimetric revelation of bound enzyme. The antibodies to bovine IgG used in step 4 were prepared in rabbits and were purified by means of a polymerized bovine IgG immunoadsorbent (Avrameas and Ternynck, 1969). This method made it possible to detect rotavirus at concentrations as low as 20–30 ng/ml. Using a different method that involved antirotavirus globulin coupled to horseradish peroxidase, Ellens and De Leeuw (1977) found that 10^5–10^7 rotavirus particles/ml could be detected by ELISA. These authors concluded that the presence of rotavirus in fecal samples was detected more efficiently by ELISA than by electron microscopy and immunoelectroosmophoresis.

The extreme sensitivity of ELISA approaches that of radioimmunoassays and allows for the detection of as little as 1–10 ng/ml virus (Clark *et al.*, 1976; Kelly *et al.*, 1978; Devergne *et al.*, 1978). This has made it possible, for instance, to detect cucumber mosaic virus in single viruliferous aphids (Gera *et al.*, 1978), and various iridescent viruses in individual larvae of *Galleria mellonella* (Kelly *et al.*, 1978). ELISA has been reported to be about 100 times more sensitive than biological assays currently used for detecting potato virus Y and plum pox virus infections (Clark *et al.*, 1976; Gugerli, 1978). Enzyme immunoassays

are extremely convenient for measuring large numbers of samples and are therefore the method of choice for large-scale epidemiological screening as well as for determining the incidence of virus infection in crops by batch testing. The main limiting factors at the present time reside in the collecting, handling, and preparation of individual extracts (Thresh *et al.*, 1977; Clark and Adams, 1977). These factors limit somewhat the advantages of semiautomated apparatus that allows large numbers of samples to be assayed very rapidly.

Numerous reports indicate that ELISA is capable of discriminating between closely related virus strains. Its specificity is much greater than that of precipitin tests and equals that of neutralization tests (Mills *et al.*, 1978). A single antiserum may fail to detect different serotypes of the same virus, and a pool of antisera specific for various serotypes may be required for the detection of a broad range of strains (Barbara *et al.*, 1978; Kelly *et al.*, 1978; Lister and Rochow, 1979; Rochow and Carmichael, 1979). In some cases, when the homologous conjugated antibody is replaced by a heterologous conjugate, the detection of a cross-reaction between two strains is greatly impeded or even impossible (Koenig, 1978; Barbara *et al.*, 1978). This phenomenon is probably caused by a reduction in antibody avidity following conjugation with the enzyme. According to Koenig (1978), the specificity of the test is so great that some closely related strains (i.e., strains with a difference of two twofold dilution steps separating homologous from heterologous serum titers) cannot be shown to be related by ELISA. The low avidity of the antibody conjugate is also demonstrated by the fact that its binding to the virus is completely inhibited by an equivalent amount of simultaneously added native antibodies. This phenomenon can be put to good use in that it allows for the elimination of unwanted reactions between certain virus antisera and host antigens (Koenig, 1978). Contaminating antihost antibodies, if present in the enzyme-labeled virus antiserum, can be prevented from interfering in the reaction by adding unlabeled antibodies to normal plant proteins.

The extreme strain specificity of the double antibody sandwich method of ELISA could be an advantage when it is important to discriminate between different virus strains, but, more often, it is a drawback in diagnostic work.

When it is necessary to detect various unidentified serotypes of a virus, the indirect form of ELISA which utilizes an anti-globulin enzyme conjugate is much to be preferred. This was demonstrated in a recent comparison of the value of different ELISA procedures for detecting a wide range of tobacco mosaic virus strains (Van Regen-

mortel and Burckard, 1980). By combining the indirect and double antibody sandwich methods, it was possible to detect distantly related serotypes with antisera to one strain only (see Fig. 1). The serological distance between the various tobacco mosaic virus strains used in this study was as great as is likely to be encountered in any group of related serotypes. It is clear, therefore, that the indirect form of ELISA is the method of choice for diagnosis.

6.3. Radioimmunoassay

In radioimmunoassay procedures, either the viral antigen or the antibody is labeled with a radioisotope. The radioactivity bound in the antigen–antibody complex is measured after the unbound labeled reagent has been removed from the complex. Two main procedures can be distinguished. In solid-phase systems, one of the reactants is bound to the surface of some material, and all reactions occur at that site. In liquid phase assays, all the reactants are in a liquid suspension.

The methodology and applications of radioimmunoassays in virology have been summarized by Daugharty and Ziegler (1977). The reader is referred to that review, which covers advances up to 1976. Subsequent work has been concerned mainly with the development of additional solid-phase procedures that meet the requirements of speed, sensitivity, and large-scale operation (Middleton *et al.*, 1977; Yung *et al.*, 1977). Using an indirect radioimmunoassay with ^{125}I-labeled rabbit antibovine IgG for detecting rotavirus antibody, Babiuk *et al.* (1977) found that the assay was a thousandfold more sensitive than the counterimmunoelectrophoresis technique.

The sensitivity of direct and indirect solid-phase radioimmunoassays for detecting polyhedron protein of an insect baculovirus have been compared (Crawford *et al.*, 1977). The direct assay using ^{125}I-labeled rabbit immunoglobulin could detect 200 ng of polyhedron protein, whereas the indirect assay, using ^{125}I-labeled sheep antirabbit immunoglobulin, could detect 50 ng of polyhedron protein. Additional assays have been described for detecting viral antibody and antigen in herpes simplex and hepatitis A infections (Forghani *et al.*, 1975; Enlander *et al.*, 1976; Purcell *et al.*, 1976). A direct solid-phase radioimmunoassay, which involves binding of purified radiolabeled IgG to influenza virus hemagglutinins, has been used to study the molecular arrangement of antigenic determinants on the hemagglutinin molecule (Russell and Jackson, 1978).

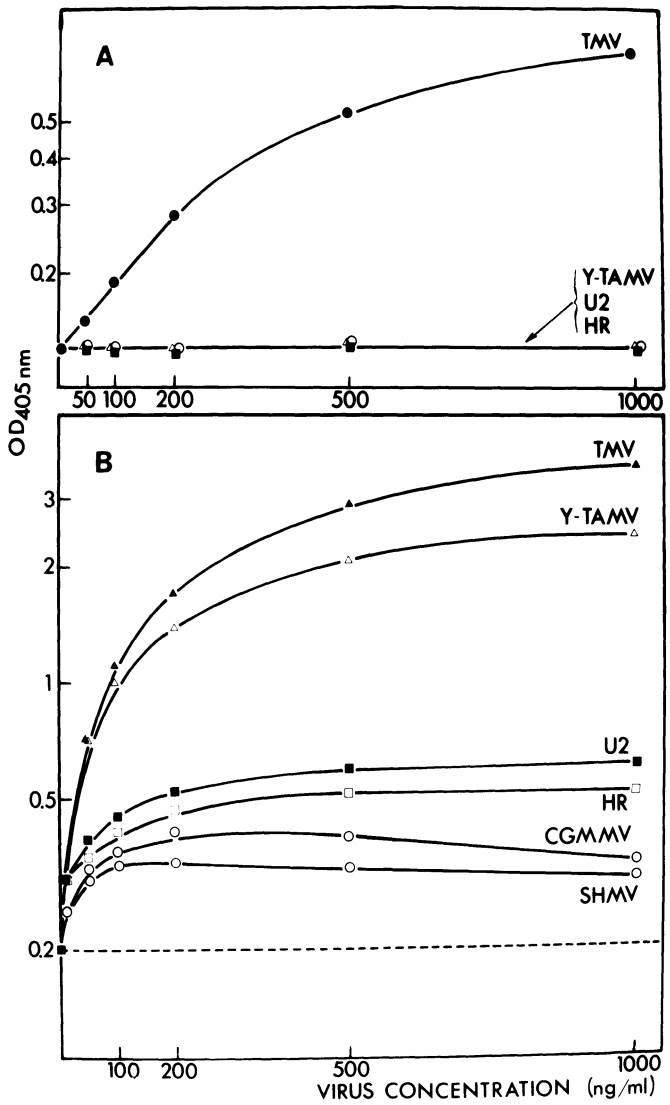

Fig. 1. Comparison of double antibody sandwich method (A) and indirect method (B) of ELISA for detecting serologically distinct strains of tobacco mosaic virus. The serological differentiation indices (SDI) between TMV and strains Y-TAMV, HR, U2, SHMV, and CGMMV are 1.2, 2.1, 2.7, 5.3, and 7.0 (Van Regenmortel, 1975). These values represent the difference between homologous and heterologous precipitin titers expressed as Neg Log_2. *Method A:* Coating of wells was done with goat anti-TMV globulin; the different viruses were incubated for 3 hr; the rabbit anti-TMV alkaline phosphatase conjugate was incubated for 2 hr. *Method B:* Same coating as in method A; after 3-hr adsorption of the viruses, rabbit anti-TMV globulin was allowed to interact for 2 hr. The enzyme conjugate (goat anti-rabbit globulin labeled with alkaline phosphatase) was incubated for 2 hr (Van Regenmortel and Burckard, 1980).

7. COMPLEMENT FIXATION

Complement fixation is extensively used in the diagnosis of a large number of virus infections of man and animals. It is based on the fact that an enzymatic system of serum proteins, known as complement, binds to antibody molecules when these have reacted with the corresponding antigen. If the antigen is associated with an erythrocyte cell surface, the fixation and concomitant activation of complement by the bound antibody leads to lysis of the red blood cell.

The complement fixation test is a two-stage procedure, involving the test system under study and an indicator system consisting of sheep erythrocytes and their corresponding antibodies. The complement present in both the test antiserum and the anti-sheep-erythrocyte serum (hemolysin) is destroyed by heating. A small amount of guinea pig complement sufficient to cause complete hemolysis of the erythrocytes present in the indicator system is added to the test antiserum.

The first part of the test consists in an overnight incubation of antigen, antibody, and complement. Depending on the amount of antigen and antibody present, different amounts of complement will be fixed. In the second part of the reaction, the sheep erythrocytes and their antibodies are introduced as an indicator. Any unused complement left in the test system will bind to the red blood cells; this will cause a certain amount of lysis. The amount of hemoglobin released from the lysed cells can be measured photometrically and is inversely proportional to the extent to which antigen and antibody have interacted and fixed complement in the test system.

For maximum sensitivity, it is important to determine, by a preliminary titration, the minimum amount of hemolysin and complement needed to lyse a predetermined number of red blood cells. The preparation of the reagents and the various titration procedures have been described in detail (Kabat and Mayer, 1961; Bradstreet and Taylor, 1962; Grist *et al.*, 1974; Mayr *et al.*, 1977).

The main difficulty encountered with this technique is the so-called anticomplementary effect. This refers to the ability of certain sera and antigen preparations to fix complement in the absence of any antigen–antibody reaction. When cell extracts or tissue culture exudates show anticomplementary activity, it is necessary to further purify the antigen. The anticomplementary activity of antisera can often be overcome by utilizing a sufficiently high serum dilution in the test. The anticomplementary activity may also be removed by a preliminary incubation with guinea pig complement, followed by heating at 60° C (Mayr *et al.*, 1977).

7.1. Microplate Technique

The microplate technique is based on the 100% hemolysis end point determined by visual inspection of the wells (Bradstreet and Taylor, 1962). The reaction can also be done in wells of cell culture trays in which virus-infected cells have been incubated. This method was used to titrate lymphocytic choriomeningitis virus in cell cultures (Gschwender and Lehmann-Grube, 1975). The microplate technique is widely used for serological diagnosis (Schmidt and Lennette, 1973) and is also suitable for strain differentiation (Forman, 1974). However, small antigenic differences between virus strains are more easily detected in tube tests, in which the degree of hemolysis is measured photometrically.

7.2. Quantitative Microcomplement Fixation Test

Various modifications of quantitative complement fixation tests in tubes have been described. In all these tests, the percentage of complement fixation is calculated from photometric measurements of the degree of hemolysis. For a given antiserum dilution, a plot of the percentage of complement fixed by increasing quantities of antigen takes the form of a bell-shaped curve (Levine and Van Vunakis, 1967). This test has been extensively used for the study of antigenic relationships (Scott, 1965; Prager and Wilson, 1971). Such studies are often done by means of inhibition of complement fixation tests, in which various quantities of inhibitor are incubated with antiserum. The residual amount of complement fixation is then measured, using the concentration of antigen which in the absence of inhibitor corresponded to the maximum of the bell-shaped curve. This method has been used, for instance, for studying the antigenic determinants of tobacco mosaic virus protein (Benjamini *et al.*, 1964). By inhibiting the complement fixation reaction by means of tryptic peptides obtained from the coat protein, it was possible to locate several antigenic regions in the capsid protein of this virus (Milton and Van Regenmortel, 1979).

8. IMMUNOELECTRON MICROSCOPY

The visualization of immunological reactions in the electron microscope is one of the most sensitive methods of antigenic analysis. Two different approaches can be distinguished, depending on whether the antigen is in suspension or is present in a cell or tissue. Histological

methods of immunocytology will not be reviewed in detail here (see Wagner, 1973; Hämmerling, 1976). These methods entail the use of labeled antibodies as a "stain," the most common labels being ferritin (Rifkind, 1976; Oshiro *et al.*, 1977) and enzymes such as horseradish peroxidase and alkaline phosphatase (Kraehenbuhl and Jamieson, 1976). Enzyme-labeled antibodies can be used for preembedding staining, in which case they are allowed to diffuse inside fixed cells and interact with antigenic sites prior to thin sectioning. Another procedure consists of applying the labeled antibody after embedding and thin sectioning of the tissue, a method which reveals only antigens that are exposed at the surface of the section. The applications of immunocytological methods for the localization of viral antigens in infected cells have been reviewed by Howe *et al.* (1969), Kurstak and Morisset (1974), and Kurstak *et al.* (1977). The methods have been described in detail by Faure *et al.* (1977) and Kurstak *et al.* (1977).

The following discussion of immunoelectron microscopy will be limited to cases where the virions are in suspension and the antigen–antibody complex is visualized directly on the electron microscope grid. A positive serological reaction can be recognized in the electron microscope by three different phenomena that may occur together or separately, i.e., clumping, decoration, and trapping on antibody-coated grids.

8.1. Clumping

The clumping method consists of mixing a suspension containing virus particles with a suitable dilution of an antiserum and incubating the mixture to allow complexes to form. The complexes can be centrifuged to a pellet in order to increase the sensitivity of detection, or they can simply be deposited on a microscope grid. An agar surface can be used to absorb impurities (Kelen *et al.* 1971). Negative staining is generally used, and aggregates can be visualized with as little as 10^6 virions present in a suspension (Almeida and Waterson, 1969). The clumping procedure is especially valuable when the virus concentration is too low to see the particles directly or when virions with a similar morphology have to be differentiated (Fauvel *et al.*, 1977).

When sufficient antibody is present in the mixture, the virions will be covered by a more or less continuous layer of antibody molecules, an effect known as decoration (Milne and Luisoni, 1977). The halo which then surrounds the virions leads to a fuzzy outline of the particles which is very characteristic (Almeida and Waterson, 1969). An excess of anti-

body may inhibit clumping in the same way as it inhibits the classical precipitation reaction.

The clumping method is extremely useful for identifying elusive human viruses that cannot be cultivated or that do not normally grow to sufficiently high titer to be identified by classical techniques, e.g., the wart viruses, coronaviruses, rhinoviruses, and the infectious hepatitis virus (Kapikian *et al.*, 1972*a*; Feinstone *et al.*, 1973; Doane and Anderson, 1977). Other uses of the method include the identification of plant viruses by the leaf-dip procedure (Ball, 1971; Langenberg, 1974), the demonstration of distinct antigens on the different morphological forms of hepatitis B particles (Neurath *et al.*, 1976; Stannard and Moodie, 1976), the establishment of antigenic relationships between certain papovaviruses (Penney and Narayan, 1973), and the localization of antigenic determinants on the tip of isolated hemagglutinin molecules of influenza virus (Wrigley *et al.*, 1977).

As in other immunoelectron microscopic procedures, the diagnostic reagent can simply be convalescent serum from patients, as in the original study of viral gastroenteritis (Kapikian *et al.*, 1972*b*). Purified globulins have also been used, as well as heat-inactivated antisera, to avoid complications due to the presence of complement (Almeida and Waterson, 1969). It is also possible to make the method more sensitive by using antiglobulin serum to further aggregate the virus–antibody complexes (Edwards *et al.*, 1975).

8.2. Decoration

The phenomenon of decoration can be visualized independently of the clumping reaction, provided that the virions are first immobilized by adsorption onto the microscope grids. When an excess of antibody is used, prior adsorption of virions to grids may be unnecessary since the particles are often completely covered with antibody molecules. In the case of elongated plant viruses that are difficult to purify, an advantage of the decoration method is that any problems resulting from contamination with antibodies to host antigens are reduced, since there is direct evidence that the virus is reacting.

The decoration method is especially valuable for the study of virions that possess a complex morphology, since it allows the site of antibody attachment to be located (Luisoni *et al.*, 1975). Seven different antigenic components on the surface of phage T4 have been distinguished in this way by means of specific antibody (Yanagida and Ahmad-Zadeh, 1970). The antibody fractions used in this work were

obtained by adsorbing anti-T4 wild-type serum with lysates of phage mutants defective in a specific gene product. After absorption, the antiserum contained only antibodies directed to the missing gene product. These antibodies were found by electron microscopy to bind to distinct areas of the phage particle which correspond to the different gene products. A similar approach was employed to locate different antigens on the surface of phage $\phi 29$ (Tosi and Anderson, 1973). In another study, polyheads of T4 phage made up of uncleaved phage head protein were shown by immunoelectron microscopy to be antigenically different from phage capsids (Yanagida, 1972).

8.3. Trapping

The trapping of plant viruses to electron microscope grids coated with specific antiserum was first described by Derrick (1973), who called the technique "serologically specific electron microscopy." The term "trapping" is used here to avoid confusion with other microscopical procedures that also rely on the visualization of virus–antibody interaction.

The method consists of the following steps. Electron microscope grids with parlodion–carbon films are allowed to float on drops of dilute virus antiserum for about 30 min. During this time, a layer of serum proteins is adsorbed to the film; excess protein is removed by washing with a buffer solution. The coated grids are then placed for 1–2 hr on drops of virus suspensions or extracts of infected tissue. Antibody molecules adsorbed to the grid specifically trap homologous virus particles. Salts and contaminants are removed by washing, and virions can be visualized after metal shadowing or negative staining.

The trapping method has been applied to the identification and quantitative analysis of numerous elongated and isometric plant viruses (Derrick and Brlansky, 1976; Paliwal, 1977; Beier and Shepherd, 1978; Milne and Lesemann, 1978; Nicolaïeff and Van Regenmortel, 1980) as well as for detecting double-stranded RNA in extracts of tobacco infected with tobacco mosaic virus (Derrick, 1978). Although mainly used by plant virologists, the trapping method will no doubt find many applications in other areas of virology as well. Recently, for instance, the method was found useful for detecting human rotaviruses in crude fecal extracts (Nicolaïeff *et al.*, 1980). Compared to standard microscopy which could detect rotavirus particles in only 20% of the specimens, the trapping procedure revealed virus particles in 71% of them.

For the quantitative assay of plant viruses in crude extracts, trapping on antiserum-coated grids is as sensitive as local lesion assays (Beier and Shepherd, 1978). Several authors have found that the log of the number of virions trapped decreases linearly with dilution and that as little as 10 ng/ml of virus can be detected (Derrick, 1973; Paliwal, 1977; Beier and Shepherd, 1978).

Optimal conditions for the test have to be determined in each case. The binding of virions to antibody-coated grids is inhibited when the antiserum used for coating is not sufficiently diluted. A similar effect is probably responsible for the fact that grids coated with normal serum adsorb practically no virions, whereas uncoated grids often adsorb a considerable number of particles. However, the number of normal serum proteins needed to prevent the particles of different viruses from adsorbing to grids is highly variable (Nicolaïeff and Van Regenmortel, 1980). This means that it is necessary to use, as controls, grids coated with normal serum diluted to the same extent as the specific antiserum.

Other parameters which may influence the extent of trapping are the temperature (Paliwal, 1977), the presence of NaCl and sucrose in the buffer (Derrick and Brlandsky, 1976; Beier and Shepherd, 1978), and the incubation time. The sensitivity of the technique can also be increased by precoating the grids with protein A from *Staphylococcus aureus* prior to coating them with specific antiserum (Shukla and Gough, 1979; Nicolaïeff *et al.*, 1980). Furthermore, it appears that maximum sensitivity and specificity can be obtained when the trapping and decoration effects are combined (Milne and Luisoni, 1977). It seems likely that both procedures will be used increasingly in the future since they offer many advantages compared to the simple clumping method.

The strain specificity of the serological trapping technique has been measured with a series of tobacco mosaic virus strains (Nicolaïeff and Van Regenmortel, 1980). Since the extent of serological cross-reactivity between these strains is known with a considerable degree of precision (Van Regenmortel, 1975), it was possible to determine how closely related serologically two strains have to be in order to be trapped on grids coated with heterologous antiserum. It was found that when two strains differed by a serological differentiation index (SDI) of about 3 (i.e., when the number of twofold dilution steps separating homologous and heterologous precipitin titers was about 3), the strains could still be detected with a single heterologous antiserum. However, when the SDI between two strains was as large as 4, the serological cross-reactivity was too low to obtain sufficient trapping on grids coated with heterologous antiserum.

9. APPLICATIONS OF SEROLOGICAL METHODS

In the following section, the different uses of serological techniques in virology will be briefly summarized.

9.1. Diagnosis

The diagnosis of viral infections represents the most widely used application of serological investigations in all fields of virology. Two main approaches can be distinguished. Virus material isolated from infected tissue may be tested for its ability to react with any one of a series of reference antisera prepared against different viruses. This is the only approach that can be used in plant virology, and its success in large-scale serological screening is well documented (Wetter, 1965; Shepard, 1972; Hollings, 1975).

In the case of viruses which infect man and vertebrates, it is also possible to diagnose an infection by demonstrating the appearance or increase of viral antibody in the blood stream of the infected host. This approach is amply illustrated in a number of extensive reviews of diagnostic procedures (Schmidt and Lennette, 1973; Bricout *et al.*, 1974; Kurstak and Kurstak, 1977; Mayr *et al.*, 1977; Hsiung, 1977).

9.2. Evaluation of Vaccines

Neutralization tests are the logical choice for evaluating the protective capacity of an antibody response induced by vaccination, since they directly measure protection against virus challenge. However, such tests are time-consuming and not very convenient for large-scale use. Other methods, such as hemagglutination inhibition, complement fixation, radial hemolysis, and mixed hemadsorption, have also been used successfully for assaying the antibody response produced by vaccination (Cox *et al.*, 1977; Grandien, 1977; Aymard, 1977).

Recently, there has been much interest in the use of subunit vaccines. Vaccination with viral structural components free of genetic material has been advocated on the grounds, first, that it avoids the injection of potentially oncogenic material (Neurath and Rubin, 1971; Rapp and Reed, 1976) and second, because it reduces the pyrogenic activity and toxic side effects of some whole-virus vaccines. Influenza subunit vaccines have been in commercial use for a number of years (Rubin and Tint, 1975). Because of the presence of toxic compounds in

rabies vaccines prepared from infected brain tissue, much effort has been spent in obtaining purified virus dissociation products that have retained their antigenicity. The spike glycoprotein of rabies virus has been purified and shown to possess an excellent immunizing capacity (Wiktor *et al.*, 1973; Cox *et al.*, 1977). With adenovirus and herpesvirus, the protective effect of nucleic-acid-free vaccines has been demonstrated in mice (Mautner and Willcox, 1974; Parks and Rapp, 1975; Kitces *et al.*, 1977).

The immunogenicity of subunit vaccines is generally much lower than that of whole-virus vaccines, and it is in this area that progress is badly needed. Several reports indicate that the efficacy of vaccines can be improved considerably by means of adjuvants (Branche and Renoux, 1972; Kreuter and Speiser, 1976; Langbeheim *et al.*, 1978).

In the case of Semlike Forest virus, protein aggregates in the form of micelles were more immunogenic than the monomeric form of the spike glycoprotein (Morein *et al.*, 1978). It is not clear whether the higher immunogenicity of aggregates is linked to the fact that multivalent antigens with repeating subunits are often thymus independent (Feldman and Basten, 1971; Ströbel, 1974) or whether it reflects the existence of new antigenic determinants induced by the quaternary structure of the proteins. The formation of such neotopes through association of monomers (Van Regenmortel, 1966, 1978) is also likely to occur in the case of glycoproteins and lipid-bound proteins (Neurath and Rubin, 1971). If neotopes contribute significantly to the overall antigenic structure of the virion, antibodies formed as a result of immunization with monomers may fail to neutralize the virus efficiently.

It should also be pointed out that, in many cases, the mechanism of immune protection is not well understood. In several virus systems, it is known that both humoral and cell-mediated immunity play a role (Burns and Allison, 1975; Cappel, 1976), while in others, there is evidence that local secretory antibody production is important (Shvartsman and Zykov, 1976).

9.3. Studies of Virus Structure

Specific antibodies are a powerful tool for localizing different antigenic structures in virions. The position of a specific gene product in the assembled virion can be visualized directly in the electron microscope by the antibody decoration technique (Yanagida and Ahmad-Zadeh, 1970), or it can be inferred from the results of hemagglutination or neu-

tralization tests (Wadell, 1972). Precipitation techniques have been extensively used for studying the various subunits found in influenza and adenoviruses (Norrby, 1969; Schild, 1970). The phenomenon of antigenic variation in influenza viruses has been shown to be related to changes in the primary structure of the hemagglutinin polypeptide (Webster and Laver, 1975). In the case of TMV strains, the degree of serological cross-reaction was found to be correlated with the extent of sequence homology in the coat proteins of the different strains (Van Regenmortel, 1975).

In recent studies, fragments of viral polypeptides obtained by proteolytic and chemical cleavage have been used to determine the precise location of antigenic sites in viral subunits. A glycopeptide which contains a major antigenic determinant of hepatitis B surface antigen particles has been isolated (Burrell *et al.*, 1976; Neurath *et al.*, 1978). Short peptides corresponding to the antigenic determinants of influenza virus hemagglutinin (Jackson *et al.*, 1978) and TMV coat protein have also been studied (Milton and Van Regenmortel, 1979).

9.4. Studies of Viral Replication

The immunoprecipitation techniques described in Section 5.3 have been extensively used for analyzing the synthesis of viral polypeptides in infected cells (Shanmugam *et al.*, 1972). In pulse-chase experiments, the radiolabeled proteins are incubated with viral antibodies and a second antiglobulin serum, and the resulting precipitate is analyzed by gel electrophoresis. In the case of retroviruses, this type of study has made it possible to identify several precursor polypeptides which appear to be processed by host enzymes (Vogt and Eisenman, 1973; Van Zaane *et al.*, 1975; Naso *et al.*, 1975).

9.5. Virus Classification

Serological cross-reactions are one of the most reliable criteria for deciding on relatedness between viruses. Indeed, viruses that are serologically related always share morphological and biochemical characteristics that place them in the same taxonomical group. Sometimes, a particular serological technique is not sufficiently sensitive to show up the presence of common antigens between two viruses. In such cases, cross-immunization with a related virus may be needed for the

heterologous antibody response to be detected (Schmidt *et al.*, 1969; Martin *et al.*, 1972).

In addition to its value for deciding on membership of a group, serology obviously also allows finer distinctions to be made. These can be based on criteria such as (1) cross-neutralization expressed as a ratio of homologous and heterologous neutralization rate constants (Honess and Watson, 1977), (2) the number of common precipitin bands observed when viruses are compared in reciprocal double diffusion tests (Honess and Watson, 1977), and (3) the ratio of homologous to heterologous titers in quantitative precipitin tests (Van Regenmortel, 1975).

Whenever a sufficient number of serologically related members of a group are examined, it is found that they can be arranged in a continuous series of increasingly distantly related entities (Koenig, 1976; Van Regenmortel, 1978). It reminds us that distinctions between close and distant serological relationships are essentially arbitrary, and that serological categories, like all categories, are constructed by the human mind and exist only by virtue of definition (Lwoff and Tournier, 1971).

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