

Serology and Immunochemistry of Plant Viruses

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Preface

The purpose of this book is to review the antigenic properties of plant viruses. Their behavior as antigens is of interest for two reasons. The first one is utilitarian and is based on the fact that serology is a useful tool in the investigation of viruses. Virologists and plant pathologists have found serological techniques to be extremely useful for the identification and quantitative assay of plant viruses, for the routine diagnosis of virus diseases, and for assessing the degree of similarity between viruses. Practical aspects of plant virus serology are therefore given considerable space: three chapters are devoted to virus purification, antiserum production, and a detailed description of all the serological techniques in common use. The theoretical principles and practical execution of the various serological techniques are described in considerable detail, and two chapters are devoted to the problems of virus identification and virus classification. A list of all the plant viruses that have been studied by serology has been compiled and 1400 references have been included in the bibliography.

The second motivation for studying the antigenic properties of plant viruses is linked to a body of knowledge better described as immunochemistry rather than serology, and stems from the fact that plant viruses represent interesting models for a variety of immunological investigations. Many of them are very immunogenic and can easily be obtained in highly purified form. Since a wealth of information is available on the structural and physicochemical properties of many plant viruses, the interpretation of their behavior as antigens is greatly facilitated. Furthermore, the ease with which certain viral protein subunits associate into capsids *in vitro* makes viruses a suitable model for studying the influence of quaternary structure on protein antigenicity.

To facilitate the understanding of the molecular basis of viral antigenicity, two introductory chapters are devoted to our current knowledge of antigenic determi-

nants in proteins and to antibody structure. The immunochemistry of plant viruses is described in three later chapters on virus-antibody binding, the role of quaternary structure in antigenicity, and the structure of viral antigenic determinants. The underlying premise is that fundamental notions derived from basic immunochemistry are relevant and useful also to those interested only in applied serology.

In the past, progress in virology has often been hampered by a host-oriented approach that has led to the compartmentation of viruses into separate specialized fields. When it comes to serology, however, the *in vitro* reactions of all viral antigens are very similar, and much of general interest can be learned from the antigenic properties of each particular group of viruses, irrespective of whether they attack plants, animals, or bacteria.

This book is intended for students and research workers in plant virology, plant pathology, microbiology, and general virology. It is hoped that the dual emphasis on basic immunochemistry and applied serology will provide virologists with the incentive to innovate and adapt existing techniques to their particular needs. At the same time, it would be gratifying if this account of viral immunochemistry could further stimulate the use of plant viruses as models in basic immunological studies.

I am indebted to the many colleagues who gave their permission to reproduce published as well as unpublished material; credit has been given to them in the illustration legends. I am grateful to Gail Darroll and Michèle Schneider for preparing the art work, to Josette Vonesch for cheerful and competent secretarial help, and to my wife and parents for assisting in the unenviable task of reconstituting a reprint collection and bibliographical index destroyed by fire.

M. H. V. Van Regenmortel

List of Virus Abbreviations

ACLSV	Apple chlorotic leaf spot virus	BRLV	Black raspberry latent virus
AgMV	Agropyron mosaic virus	BSMV	Barley stripe mosaic virus
AILV	Artichoke Italian latent virus	BWYV	Beet western yellows virus
ALV	Alfalfa latent virus	BYDV	Barley yellow dwarf virus
AMCV	Artichoke mottle crinkle virus	BYMV	Bean yellow mosaic virus
AMV	Alfalfa mosaic virus	BYSV	Beet yellow stunt virus
APLV	Andean potato latent virus	BYV	Beet yellows virus
APMV	Andean potato mottle virus	CABMV	Cowpea aphid-borne mosaic virus
ApMV	Apple mosaic virus	CaMV	Cauliflower mosaic virus
ArMV	Arabis mosaic virus	CarMV	Carnation mottle virus
ASGV	Apple stem grooving virus	CasCMV	Cassava common mosaic virus
AVA	Arracacha virus A	CasLV	Cassava latent virus
BarYMV	Barley yellow mosaic virus	CCMV	Cowpea chlorotic mottle virus
BBMV	Broad bean mottle virus	CDV	Columbia datura virus
BBNV	Broad bean necrosis virus	CeMV	Celery mosaic virus
BBSSV	Blueberry shoestring virus	CERV	Carnation etched ring virus
BBSV	Broad bean stain virus	CGMMV	Cucumber green mottle mosaic virus
BBTMV	Broad bean true mosaic virus	ChYMV	Chicory yellow mottle virus
BBWV	Broad bean wilt virus	CiLRV	Citrus leaf rugose virus
BCMV	Bean common mosaic virus	CIRSV	Carnation Italian ringspot virus
BCTV	Beet curly top virus	CLRV	Cherry leaf roll virus
BelMV	Belladonna mottle virus	CLV	Carnation latent virus
BGMV	Bean golden mosaic virus	CIYMV	Clover yellow mosaic virus
BidMV	Bidens mottle virus	CIYVV	Clover yellow vein virus
BIMV	Bearded iris mosaic virus	CMMV	Cocksfoot mild mosaic virus
BICMV	Blackeye cowpea mosaic virus	CMotV	Carrot mottle virus
BMMV	Bean mild mosaic virus	CMV	Cucumber mosaic virus
BMV	Brome mosaic virus	CNFV	Carnation necrotic fleck virus
BMYV	Beet mild yellowing virus	CNV	Cacao necrosis virus .
BNYV	Broccoli necrotic yellows virus	CoMV	Cocksfoot mottle virus
BNYVV	Beet necrotic yellow vein virus	CPMMV	Cowpea mild mottle virus
BPMV	Bean pod mottle virus	CPMotV	Cowpea mottle virus
		CPMV	Cowpea mosaic virus

CPRSV	Cowpea ringspot virus	HyMV	Hypochoeris mosaic virus
CPSMV	Cowpea severe mosaic virus	HyRSV	Hydrangea ringspot virus
CRLV	Cherry rasp leaf virus	IMMV	Iris mild mosaic virus
CRMV	Cherry rugose mosaic virus	KYMV	Kennedy yellow mosaic virus
CRSV	Carnation ringspot virus	LALV	Lucerne Australian latent virus
CSMV	Chloris striate mosaic virus	LCLV	Lilac chlorotic leafspot virus
CSSV	Cacao swollen shoot virus	LiMV	Lilac mottle virus
CSV	Cocksfoot streak virus	LLV	Lonicera latent virus
CTDV	Cereal tillering disease virus	LMV	Lettuce mosaic virus
CTLV	Carrot thin leaf virus	LNYV	Lettuce necrotic yellows virus
CTV	Citrus tristeza virus	LRMV	Lilac ring mottle virus
CuNV	Cucumber necrosis virus	LRSV	Lychnis ringspot virus
CVB	Chrysanthemum virus B	LSV	Lily symptomless virus
CVMV	Carnation vein mottle virus	LTSV	Lucerne transient streak virus
CVV	Citrus variegation virus	MacMV	Maclura mosaic virus
CVX	Cactus virus X	MCDV	Maize chlorotic dwarf virus
CybMV	Cymbidium mosaic virus	MDMV	Maize dwarf mosaic virus
CybRSV	Cymbidium ringspot virus	MiMV	Mirabilis mosaic virus
CYFV	Carnation yellow fleck virus	MLRSV	Myrobalan latent ringspot virus
CYMV	Cacao yellow mosaic virus	MLV	Mulberry latent virus
CYVV	Clitoria yellow vein virus	MMV	Maize mosaic virus
DasMV	Dasheen mosaic virus	MRDV	Maize rough dwarf virus
DMV	Dahlia mosaic virus	MRFV	Maize Rayado Fino virus
DuMV	Dulcamara mottle virus	MRSV	Mulberry ringspot virus
DVX	Daphne virus X	MSV	Maize streak virus
DYMV	Desmodium yellow mottle virus	NaLV	Narcissus latent virus
ELV	Elderberry latent virus	NaMV	Narcissus mosaic virus
EMMV	Eggplant mild mottle	NeLV	Nerine latent virus
EMotV	Elm mottle virus	NeVX	Nerine virus X
EMV	Eggplant mosaic virus	NTNV	Narcissus tip necrosis virus
EryLV	Erysimum latent virus	NVMV	Nicotiana velutina mosaic virus
FDV	Fiji disease virus	NYSV	Narcissus yellow stripe virus
FMV	Frangipani mosaic virus	OBDV	Oat blue dwarf virus
FoMV	Foxtail mosaic virus	OkMV	Okra mosaic virus
GBLV	Grapevine Bulgarian latent virus	ONMV	Oat necrotic mottle virus
GCMV	Grapevine chrome mosaic virus	ORSV	Odontoglossum ringspot virus
GFLV	Grapevine fanleaf virus	OSDV	Oat sterile dwarf virus
GGMV	Guinea grass mosaic virus	OYDV	Onion yellow dwarf virus
GSMV	Gloriosa stripe mosaic virus	OYMV	Ononis yellow mosaic virus
HCRSV	Hibiscus chlorotic ringspot virus	PAMV	Potato aucuba mosaic virus
HelVS	Helenium virus S	PanMV	Parsnip mosaic virus
HeIYY	Helenium virus Y	PanSV	Pangola stunt virus
HiMV	Hippeastrum mosaic virus		
HLV	Heracleum latent virus		
HMV	Henbane mosaic virus		

PapMV	Papaya mosaic virus	RCNMV	Red clover necrotic mosaic virus
PBRSV	Potato black ringspot virus	RCVMV	Red clover vein mosaic virus
PCV	Peanut clump virus	RDV	Rice dwarf virus
PDV	Prune dwarf virus	RMV	Ribgrass mosaic virus
PeAMV	Petunia asteroid mosaic virus	RNMV	Rice necrosis mosaic virus
PeaSV	Pea streak virus	RobMV	Robinia mosaic virus
PEBV	Pea early-browning virus	RosMV	Rose mosaic virus
PeMV	Pepper mottle virus	RRSV	Rice ragged stunt virus
PEMV	Pea enation mosaic virus	RRV	Raspberry ringspot virus
PeMotV	Peanut mottle virus	RTYV	Rice transitory yellowing virus
PepMV	Pepino mosaic virus	RYMV	Rice yellow mottle virus
PFBV	Pelargonium flower-break virus	RyMV	Ryegrass mosaic virus
PhMV	Physalis mosaic virus	SBMV	Southern bean mosaic virus
PLCV	Pelargonium leaf-curl virus	SBWMV	Soil-borne wheat mosaic virus
PIMV	Plantago mottle virus	ScMV	Scrophularia mottle virus
PLPV	Plum line pattern virus	SCMV	Sugarcane mosaic virus
PLRV	Potato leafroll virus	SCV	Saguaro cactus virus
PLV	Passiflora latent virus	SDV	Satsuma dwarf virus
PMotV	Phleum mottle virus	SHMV	Sunn-hemp mosaic virus
PMTV	Potato mop-top virus	SLRSV	Strawberry latent ringspot virus
PMV	Panicum mosaic virus	SLV	Shallot latent virus
PNRSV	Prunus necrotic ringspot virus	SMV	Squash mosaic virus
PokMV	Pokeweed mosaic virus	SOV	Sammons opuntia virus
PopMV	Poplar mosaic virus	SowMV	Sowbane mosaic virus
PPV	Plum pox virus	SoyDV	Soybean dwarf virus
PRMV	Peach rosette mosaic virus	SoyMV	Soybean mosaic virus
PRSV	Papaya ringspot virus	SPMMV	Sweet potato mild mottle virus
PSbMV	Pea seed-borne mosaic virus	StaVY	Statice virus Y
PSLV	Poa semilatent virus	SubMV	Sugar beet mosaic virus
PSV	Peanut stunt virus	SV	Satellite virus
PTV	Peru tomato virus	SVBV	Strawberry vein banding virus
PV3	Parsnip virus 3	SYNV	Sonchus yellow net virus
PV5	Parsley virus 5	SYVV	Sowthistle yellow vein virus
PVA	Potato virus A	TAMV	Tulare apple mosaic virus
PVM	Potato virus M	TAV	Tomato aspermy virus
PVMV	Pepper veinal mottle virus	TBRV	Tomato black ring virus
PVS	Potato virus S	TBSV	Tomato bushy stunt virus
PVT	Potato virus T	TBV	Tulip breaking virus
PVX	Potato virus X	TCV	Turnip crinkle virus
PVY	Potato virus Y	TEV	Tobacco etch virus
PWV	Passionfruit woodiness virus	TMV	Tobacco mosaic virus
PYFV	Parsnip yellow fleck virus	TNV	Tobacco necrosis virus
PYDV	Potato yellow dwarf virus	TomMV	Tomato mosaic virus
QPMV	Quail pea mosaic virus	TomRSV	Tomato ringspot virus
RaMV	Radish mosaic virus	TRoSV	Turnip rosette virus
RBDV	Raspberry bushy dwarf virus	TRSV	Tobacco ringspot virus
RBSDV	Rice black-streaked dwarf virus	TRV	Tobacco rattle virus
RCMV	Red clover mottle virus	TSV	Tobacco streak virus

TSWV	Tomato spotted wilt virus	WLV	Wineberry latent virus
TurMV	Turnip mosaic virus	WMV	Watermelon mosaic virus
TYMV	Turnip yellow mosaic virus	WPMV	Wild potato mosaic virus
TYV	Turnip yellows virus	WSMV	Wheat streak mosaic virus
VMV	Viola mottle virus	WStMV	Wheat striate mosaic virus
WCIMV	White clover mosaic virus	WTV	Wound tumor virus
WCMV	Wild cucumber mosaic virus	WYLV	Wheat yellow leaf virus
		ZVX	Zygocactus virus X

Antigens and Antigenic Determinants

A. DEFINITIONS

An antigen is defined as any substance capable of inducing an immune response when it is introduced into an appropriate animal. The suitability of a particular animal for revealing the antigenicity of a substance depends on whether it possesses lymphoid cells endowed with receptors capable of combining specifically with the antigen. The presence of such receptors leads to a specific recognition of the antigen at the surface of the lymphoid cell, and this induces the proliferation of plasma cells that secrete antibodies directed against the antigen. Such a sequence of events corresponds to the situation known as humoral immunity.

A second type of immune response exists, which consists of the proliferation of immune lymphocytes bearing receptors specific for the antigen without any concomitant liberation of circulating antibodies. This type of response is known

as cell-mediated immunity and plays an important role in the protection of animals against virus infections (Zinkernagel, 1979; Sissons and Oldstone, 1980).

The capacity of an antigen to induce an immune response is usually referred to as *immunogenicity*. Terms such as *immunogen* or *immunogenic* are used instead of *antigenic* when the only property that is being considered is the ability of the substance to induce either a humoral or cell-mediated immunity. No particular term is in general use to refer to the second characteristic property of antigens, namely, the capacity to react specifically with an antibody or with the receptor of a lymphoid cell. Some authors use the terms *antigenicity* or *antigenic specificity* to refer to this property, but since these words have several other connotations, it would seem preferable to speak of *antigenic reactivity*.

The antigenic reactivity of a substance describes its capacity to undergo specific binding with antibodies or lymphoid cell receptors. This reactivity resides in restricted parts of the molecule known as antigenic determinants or *epitopes* (Jerne, 1960). An epitope possesses a three-dimensional structure complementary to that of the binding site of the antibody molecule. In the case of proteins, the size of an epitope is usually about 5–7 amino acid residues.

By means of enzymatic or chemical cleavage reactions, it is sometimes possible to cut out antigenic regions from a protein molecule and to show that the resulting peptides are capable of combining specifically with antibodies directed against the whole antigen. Although such isolated epitopes are too small to be able to induce an immune response on their own, they regain their immunogenicity when coupled to a large carrier molecule such as bovine serum albumin. A great many chemical substances of low molecular weight are able to acquire immunogenic properties after being coupled to carrier molecules of sufficient size. Such substances, which are called *haptens*, are structurally equivalent to isolated epitopes and are able to react with specific antibodies.

From innumerable studies on hapten-carrier conjugates, it would appear that virtually any chemical grouping is able to function as an epitope. Initially, this view seemed to lead to a paradox, since it was taken to imply that the immune system was capable of generating an infinite variety of antibodies with a relatively small amount of genetic information devoted to this task. This difficulty was resolved when it became apparent that the notion of antigenic specificity was less absolute than originally thought. It is now clear that an antibody molecule is not predestined to react with only a single antigenic determinant, but that it can, in fact, bind to several related structures (Richards *et al.*, 1975). As a result of such overlapping cross-reactivities, antibodies of sufficient affinity may be produced to any structural determinant (Goodman, 1975a).

The distinction between immunogenicity and antigenic reactivity is not purely a theoretical one. Although both properties are usually found to coexist in the same molecule, this is not always the case. Haptens are examples of nonimmunogenic substances that possess antigenic reactivity. The reverse situation

exists with molecules which induce the formation of antibodies that are unable to react with the immunogen (Mäkelä, 1965; Loor, 1971). It is possible that such nonreactive antibodies (also called heterospecific or heteroclitic antibodies) contribute to the rise in "normal" immunoglobulin concentration that usually accompanies the appearance of specific antibody during immunization.

B. IMMUNOGENICITY

The immunogenic capacity of a substance depends on its physicochemical nature, on the recipient animal, and on the method of immunization. Although several exceptions are known to exist, it can be stated that in general a minimum molecular weight of 5000 is required for a protein to be immunogenic. This is probably linked to the necessity of having at least two antigenic determinants on the same molecule.

According to the currently accepted view of the mechanism of induction of antibody synthesis, a cellular cooperation between lymphoid T and B cells is essential. This cooperation requires the presence on the same antigen of at least two epitopes that will interact with the two different cell types (Sercarz *et al.*, 1977).

Large antigens with a great number of determinants could thus be expected to be particularly well-suited for inducing this cellular cooperation. In the case of large aggregated protein structures, such as polymerized flagellin, the requirement for T cell cooperation does not exist. Such antigens are called T cell-independent (or thymus-independent) antigens. It is believed that the multiplicity of identical epitopes at the surface of the polymer is responsible for the triggering of B cells into antibody-producing plasma cells. The pattern of repeating epitopes presumably is able to mimic the effect of T cells in presenting an array of similar epitopes to the B cells.

As a general rule, an organism does not induce an immune response against its own protein constituents. However, clones of lymphocytes capable of recognizing self antigens do exist in the animal, and these may be responsible for the occurrence of harmful autoimmune phenomena. The capacity to respond to a certain antigen varies with the animal species and among the individuals within a species. These differences have a genetic basis and depend on the ability of the animal to synthesize receptor sites capable of recognizing the immunogen. By using inbred lines of mice and rats, it has been found possible to reduce this variability in response.

Another requirement for immunogenicity is that the molecule should possess a certain rigidity and constancy in its three-dimensional structure. A flexible molecule, capable of assuming a variety of conformations, is unlikely to be complementary to the unique structure of a receptor binding site. There is good

evidence that both B cell receptors and antibodies recognize specifically the three-dimensional shape of a globular protein molecule. Antibodies produced against native proteins usually react poorly or not at all with the denatured unfolded form of the molecule. Similarly, antibodies obtained by immunization with unfolded proteins mostly do not react with the native configuration of the molecule. The poor immunogenicity of a molecule such as gelatin is partly due to the flexible nature of this fibrous protein. When the rigidity of gelatin is increased by the incorporation of tyrosine residues, its immunogenicity is enhanced considerably.

Since conformation plays an important role in the definition of antigenic specificity, the main features of the three-dimensional structure of proteins will be summarized in Section C.

C. THREE-DIMENSIONAL STRUCTURE OF PROTEINS

It is convenient to distinguish four levels of protein structure. *Primary structure* is the sequence of amino acid residues in the polypeptide chain and is derived by a one-dimensional synthetic process from the coding information present in an RNA template. In the absence of any stabilizing interactions, a string of amino acid residues in continuous conformational flux would be produced. This state, called random coil, corresponds to a protein that has undergone complete denaturation.

Secondary structure refers to the regular arrangement of the backbone of the polypeptide chain into periodic hydrogen-bonded structures such as the α -helix and the β -pleated sheet.

Tertiary structure refers to the arrangement of the backbone that is not composed of regular repeating elements. This term is also used loosely to describe the overall three-dimensional structure of globular proteins.

Quaternary structure refers to the aggregated state of individual polypeptide chains that is brought about by covalent or noncovalent bonding.

The folding of a polypeptide chain is dictated by its amino acid sequence and depends on a complex network of short- and long-range noncovalent interactions. The resulting native structure is in a state of minimal conformational free energy in which most of the hydrophobic nonpolar side chains are buried inside the molecule and most of the polar chains are located at the surface. The nonpolar groups are relegated to the interior of the protein to maximize the free energy derived from fewer contacts with water. However, the belief that polar groups, internally hydrogen-bonded or not, are hydrophilic and occur mainly at the protein surface has been shown to be an oversimplification (Chothia, 1976). In fact, many polar groups also become buried when the protein folds and these can then no longer form hydrogen (H) bonds with water molecules. To compensate

for the resulting loss of enthalpy, the buried groups form internal H bonds with suitably located partners in the folded protein and in so doing, they give rise to most of the secondary structure. Such internal hydrogen bonded polar groups are no longer hydrophilic but are, in fact, hydrophobic (Chothia, 1974).

The hydrophobic nature of the interfaces between the elements of secondary structure explains the close packing achieved at the level of tertiary structure. This packing reflects the need to have as much as possible of the hydrophobic surfaces removed from contact with water.

The interaction of native globular proteins with water is of central importance to any theory of protein structure. Lee and Richards (1971) introduced the notion of *accessible surface area* to quantitatively describe the extent to which the protein surface can form contacts with water. The free energy gained from hydrophobicity when the polypeptide chain folds is proportional to the difference in accessible surface area between the unfolded and native forms of the molecule.

In lysozyme, the accessible surface area of the native structure is 6500 \AA^2 , whereas that of the unfolded molecule is $20,500 \text{ \AA}^2$. The smaller accessible surface area stabilizes the native structure by a $\Delta F = 350 \text{ kcal/mole}$, which represents the hydrophobic effect. This is slightly larger than the loss in conformational entropy of about 300 kcal/mole , which occurs when the molecule folds (Janin, 1979).

For proteins of 50–250 amino acid residues the stabilization by hydrophobicity increases with the number of residues. In a polypeptide chain shorter than 50 residues, the accessible surface area is relatively large and few residues are buried within the structure. In this case the hydrophobic effect is too small to compensate for the loss of chain entropy when the chain folds. Short peptides with an average composition will thus tend to be relatively insoluble.

It has been shown by Janin (1976) that the surface area of globular proteins of molecular weight 6000–35,000 is proportional to the two-third power of their molecular weight, i.e., $ASA = 11.1 M^{2/3}$ where ASA is the accessible surface area, in \AA^2 . The proportionately factor is exactly double that found for a solid sphere of the same mass and density. This relationship makes it possible to calculate the total surface area of an antigen that is available for binding to antibody.

In related proteins that present a great number of sequence differences, the overall three-dimensional structure is often conserved to a remarkable degree. It is known, for instance, that the cytochromes *c* from bacteria and fish possess a very similar folding pattern. Other examples are myoglobins from different species which have a very similar conformation, despite extensive changes in primary structure, and chicken egg-white and human lysozymes which show much resemblance in tertiary structure despite a 41% difference in sequence (Blake and Swan, 1971).

A large proportion of amino acid substitutions that occur in a series of homologous proteins are found in surface positions, and they do not lead to gross alterations in conformation. This suggests that there is a strong selective pressure against mutations that change the core of proteins and influence their folding.

The same conservation in folding pattern is also found among members of the tobamovirus group, some of which differ in sequence by as much as 60%. However, a small number of residues is strictly conserved and this allows the protein subunits of all tobacco mosaic virus (TMV) strains to aggregate into capsids that possess a very similar quaternary structure (Klug and Caspar, 1960; Butler and Durham, 1975).

D. ANTIGENIC DETERMINANTS

The antigenic analysis of a protein involves the elucidation of the structure of its antigenic determinants. As a first approximation, the number of antibody molecules capable of binding simultaneously to a protein defines the minimum number of epitopes present on the antigen. This number, which corresponds to the antigenic valence, is proportional to the outer surface (or accessible surface area) of the molecule. The antigenic valence of some proteins is presented in Table 1.1.

Large proteins and viruses are always made up of a number of identical subunits. The TMV capsid, for instance, consists of 2130 protein subunits. One could, therefore, have expected the antigenic valence of TMV to be a multiple of 2130. The valence of 800, which is obtained experimentally, is due to the steric hindrance that prevents a larger number of antibody molecules from binding simultaneously to the surface of the virus (Rappaport, 1961b, 1965; Van Regenmortel, 1967, 1978). In general the number of determinants present on an antigen is larger than its valence, and it cannot be determined by valence measurements.

TABLE 1.1
Antigenic Valence of Proteins and Viruses

Antigen	Molecular weight	Valence
Ribonuclease	12,700	5
Tobacco mosaic virus protein	17,500	5
Potato virus X protein	26,000	4
Ovalbumin	44,000	5
Serum albumin	67,000	6
Ferritin	465,000	26
Tomato bushy stunt virus	9.3×10^6	110
Tobacco mosaic virus	40×10^6	800

Even in the case of monomeric antigens, where each epitope is present only once in the molecule, the number of determinants is usually larger than the valence.

1. Types of Antigenic Determinants

It has been customary to distinguish two types of epitopes, the so-called sequential and conformational determinants (Sela *et al.*, 1967; Sela, 1969). Whereas a sequential determinant consists of a sequence of 5–7 residues in its unfolded random coil form, a conformational determinant is constituted by a number of residues that are kept in a particular conformation usually within the confines of a macromolecular structure. It is generally assumed that antibodies directed to conformational determinants do not react with the unfolded peptides derived from the corresponding part of the native molecule.

The distinction between conformation-dependent and independent determinants is somewhat arbitrary since any sequence of residues within a native globular protein possesses a particular conformation. In general, short peptides cleaved from a protein have a relatively flexible configuration, which differs from the more constrained folding found in the complete molecule.

Atassi and Smith (1978) have suggested that a more satisfactory distinction would be to differentiate between continuous and discontinuous determinants. These authors define a continuous determinant as a contiguous sequence of amino acid residues exposed at the surface of a native protein and possessing distinctive conformational features. A discontinuous determinant, on the other hand, consists in the juxtaposition in space of residues that are not contiguous in the primary structure. Distant residues could become contiguous through the folding of the polypeptide chain or by juxtaposing two separate peptide chains. The existence of discontinuous determinants has been conclusively demonstrated only in the case of lysozyme (Atassi and Lee, 1978).

The fact that antibodies raised by immunization with denatured proteins often do not react with the corresponding native molecule has given rise to the concept of hidden epitopes or cryptotopes (Jerne, 1960). These have been defined as determinants that become antigenically active only after breakage, depolymerization, or denaturation of the antigen. In the case of viral capsids, cryptotopes are found on the surfaces of the protein monomers that are turned inward and become buried after polymerization.

There is also evidence that polymerized proteins possess specific epitopes that are not present in the constituent protein subunits. Such epitopes, which have been called neotopes (Van Regenmortel, 1966b), are found in most virions (Neurath and Rubin, 1971). Neotopes probably owe their existence to conformational changes of the protein induced by intersubunit bonds; alternatively, they may arise from juxtaposing residues from neighboring subunits (see Chapter 7).

2. Methods Used in the Localization of Determinants

a. Modification of Particular Amino Acid Residues of the Protein

When mutants of the protein with single amino acid exchanges are available, it is possible to ascertain whether the substitution affects the serological properties of the molecule. This approach has been used, for instance, with mutants of hemoglobin (Reichlin, 1972), cytochrome *c* (Nisonoff *et al.*, 1970), and TMV (Sengbusch, 1965; Van Regenmortel, 1967). If the amino acid substitution leads to an antigenic change, it is concluded that the particular residue contributes to the structure of an epitope. In many well-documented cases, a single residue exchange was found to affect antigenicity only locally and not through long-distance conformational changes. However, without additional information, one cannot be sure that the mutated residue is located within the boundaries of an epitope. Instead of affecting antigenicity locally at the site of the mutation, the substitution may also influence antigenic reactivity only indirectly by altering the conformation of a distal antigenic region of the molecule.

When no mutants are available, it is also possible to chemically modify particular amino acid side chains by a variety of selective reagents (Atassi, 1977a), and then to assess the influence of the alteration on antigenic reactivity. However, this method is only of limited value for pinpointing antigenically active regions since usually more than one residue of a kind is modified by the chemical reaction. Furthermore, a change in antigenic reactivity may also result from a conformational alteration and need not imply that the modified residue participates directly in the structure of an epitope.

In order to discriminate between pairs of closely related antigens, quantitative microcomplement fixation tests (Prager and Wilson, 1971a,b) and radioimmunoassays (Milton *et al.*, 1980) are more sensitive than quantitative precipitin tests. This is probably due to the fact that the more discriminating tests are performed at molar concentrations of reactants of about 10^{-9} moles/liter. Such low concentrations favor an interaction with the most avid portion of the total antibody population and emphasize small differences in binding affinity. At the concentration of reactants used in precipitin tests, i.e., 10^{-6} – 10^{-5} moles/liter, small differences in binding affinity may go undetected (Reichlin, 1974, 1975).

b. Fragmentation of the Protein

This approach consists in purifying a series of peptides obtained by chemical or enzymatic cleavage of the protein and in screening them for antigenic reactivity. This is done by determining if the peptides are able to bind to antibodies and, in so doing, inhibit the reaction between antibodies and the intact antigen. Some shortcomings of this approach are first that most of the isolated peptides do not maintain the conformation they possess in the parent molecule, and second that

the cleavage may have occurred within the boundaries of an epitope. As a result, fragments that are antigenically inactive may actually be part of a reactive region in the intact molecule.

It is generally assumed that fragments exist in solution in a variety of random conformations in equilibrium with the native one, and that the native conformation can be generated by spontaneous and reversible folding of only a portion of the molecule. Since only conformations that approximate to the native form will bind to antibody, this may explain why very high molar ratios of peptide over intact antigen have to be used in inhibition experiments. This situation has been analyzed quantitatively in a study of staphylococcal nuclease antibodies (Sachs *et al.*, 1972). The specificity of antibodies obtained by immunization with the intact nuclease molecule was compared with that of antibodies raised against fragments corresponding to residues 1-126 and 99-149. It was assumed that antibodies elicited by the fragments were directed against epitopes of the unfolded molecule and that antibodies to the whole molecule were directed against a unique conformation of the native protein. By competitive binding between nuclease antibody fractions and either peptide 99-149 or the whole nuclease molecule, it was possible to calculate the fraction of peptide in the native conformation. It was assumed that only a conformation of the peptide similar to that in native nuclease could bind antibody. It was calculated that one in 5000 molecules of peptide was present in the native conformation (Sachs *et al.*, 1972).

As a result of the high molar excesses of peptides that are required for inhibition, an insidious pitfall of this approach is that small amounts of undetected contaminating peptides may, in fact, be responsible for the inhibition that is observed in any particular instance. Since fractionation of peptides to absolute purity is an arduous if not impossible task, a better strategy is to test the ability of radiolabeled peptides to bind to antibody. With this method, contaminating peptides will also become labeled but their contribution to binding, expressed as a percentage of the total radioactivity, will be very small. Minor contaminants will thus make only a small contribution to the total binding, and erroneous conclusions regarding the identity of the active peptide will be avoided.

Instead of testing fragments for residual antigenic activity by means of antibodies prepared against the whole protein, it is also possible to use the fragments for immunization. If antibodies raised against a peptide react with the native molecule, it is justifiable to conclude that the isolated peptide represents a determinant of the protein. Epitopes of lysozyme (Arnon, 1977) and β -galactosidase (Celada *et al.*, 1978) have been delineated in this way.

c. Use of Synthetic Peptides

Once an antigenic region has been roughly delineated, a series of short peptides corresponding to the possible sequence of the epitope can be synthesized.

This allows the exact boundaries of the determinant to be defined. This approach was found to be particularly successful in the case of the continuous antigenic determinants of myoglobin. The synthesis of peptides corresponding to continuous determinants is usually performed by solid-phase synthesis on insoluble supports. It is also possible to assess the antigenic reactivity of immobilized peptides without cleaving them from the support (Smith *et al.*, 1977). By stepwise synthesis of a peptide and subsequent radioimmunoabsorption of antibodies at each stage of the synthesis, the exploration of the structure of epitopes can be greatly simplified.

In order to link spatially adjacent residues that are apart in the sequence, Atassi *et al.* (1976a) developed a method called *surface simulation* synthesis, which utilizes synthetic peptides that mimic part of the surface topography of the protein. Glycine residues were used as spacers to obtain the correct separation between residues of the determinant that are apart in the sequence (Lee and Atassi, 1976).

This brief survey of the methods used for elucidating the structure of antigenic determinants should make it clear that no single approach is likely to succeed in defining the complete antigenic structure of a protein. The need for using complementary methods in order to elucidate antigenic structures will be further demonstrated in Sections E and F, which summarize our knowledge of two of the best-known protein antigens—myoglobin and lysozyme.

E. ANTIGENIC STRUCTURE OF MYOGLOBIN

Sperm-whale myoglobin is a protein composed of a single polypeptide chain comprising 153 residues folded in a highly helical compact structure. The antigenic properties of this molecule have been studied extensively by Atassi and collaborators and several reviews of the subject are available (Crumpton, 1974; Atassi, 1975, 1977b, 1979a). The elucidation of the antigenic structure of myoglobin was based on the following five approaches:

1. A study of the effect of conformational changes on the antigenic reactivity of the molecule
2. A study of the immunochemical properties of chemical derivatives of myoglobin specifically modified at particular amino acid locations
3. The isolation of immunochemically reactive fragments by selective cleavage reactions
4. A study of the effect of chemical modification of selected residues on the antigenic reactivity of the fragments
5. A study of synthetic peptides corresponding to several sequence overlaps of the active regions delineated by approaches 1 to 4

TABLE 1.2
Amino Acid Sequences of Five Antigenic Determinants of Sperm-Whale Myoglobin^a

Determinant	Residue positions	Sequence ^b
1	15-22	(Ala)—Lys—Val—Glu—Ala—Asp—Val—(Ala)
2	56-62	Lys—Ala—Ser—Glu—Asp—Leu—Lys
3	94-99	Ala—Thr—Lys—His—Lys—Ile
4	113-119	His—Val—Leu—His—Ser—Arg—His
5	145-151	(Lys)—Tyr—Lys—Glu—Leu—Gly—Tyr

^a From Atassi (1977b).

^b The residues in parentheses are part of the determinants only with respect to some of the antisera.

The results obtained by these different methods showed that five antigenic reactive regions are present in myoglobin. The primary structure of these five determinants is indicated in Table 1.2.

The location of the five determinants in the three-dimensional structure of myoglobin is illustrated in Fig. 1.1. All epitopes are prominently exposed at the surface of the molecule and four of them are situated on bends linking two adjacent helices. Some of the residues of determinant 4 are part of an α -helix.

According to Atassi (1975, 1979a) these five peptides account for the entire

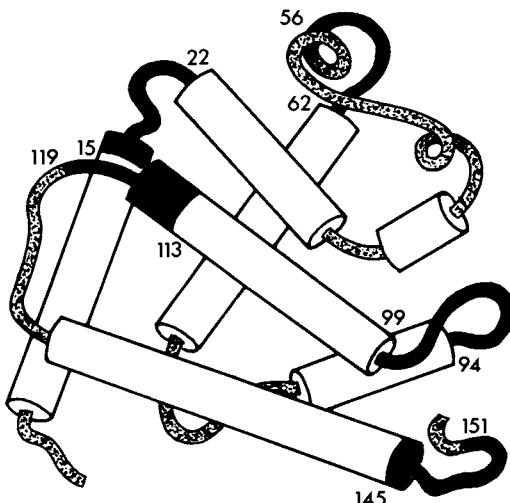


Fig. 1.1. Three-dimensional structure of myoglobin. The helices are represented as cylinders and the antigenic sites studied by Atassi (1975) are shown as black portions. The residue numbers correspond to the extremities of the different epitopes.

antigenic reactivity of the native myoglobin molecule. This conclusion was based on two types of findings: first that serial elution with the five peptides displaced about 81% of the total elutable antibody from a myoglobin-Sepharose immunoabsorbent (Atassi and Koketsu, 1975), and second that the five peptides could achieve 89–94% inhibition of the precipitin reaction between myoglobin and its antibodies (Atassi, 1977b). However, this maximum inhibition was only achieved when a large molar excess of each peptide relative to intact myoglobin was used. Two possible reasons why large excesses of peptides are required are that the peptides do not possess the appropriate native conformation, and that they carry two additional charges compared with the equivalent sequences in the intact protein. It is generally accepted that peptides are able to assume a variety of conformations and that only some of these are similar to the one found in the corresponding region of the native molecule (Crumpton and Small, 1967). When increasing concentrations of peptides are used, the probability of having some of the molecules in the correct conformation obviously also increases.

In view of the conformational restraints that the five peptides must experience within the native structure, it is truly remarkable that these hexa- and heptapeptides can account for so much of the total antigenic reactivity of myoglobin. It should be pointed out, however, that not all investigators agree that the five epitopes studied by Atassi and collaborators represent the sole antigenic regions of myoglobin (White *et al.*, 1978). Indeed, it has been shown that complete sequence homology within one of the five determinants does not guarantee that two myoglobins will cross-react serologically. In the case of sperm-whale and cattle myoglobins, which possess one identical determinant (residues 56–62) and differ in only 7 of the 35 antigenic residues (see Table 1.2.), the two molecules did not cross-react when tested by a radioimmunoassay procedure (Hurrell *et al.*, 1977; East *et al.*, 1980).

F. ANTIGENIC STRUCTURE OF LYSOZYME

Hen egg-white lysozyme consists of a single polypeptide chain made up of 129 amino acid residues and internally cross-linked by four disulfide bonds. Cleavage of the disulfide bonds leads to an unfolding of the molecule, which is then no longer able to react with antibodies directed to native lysozyme. The unfolded molecule is nevertheless immunogenic, but it induces antibodies that do not cross-react with native lysozyme in the usual *in vitro* tests. However, when tests that measure cell-mediated immunity are used, the native and unfolded forms do exhibit cross-reactivity (Arnon, 1977).

An interesting serological cross-reaction has been discovered between lysozyme and α -lactalbumin. As shown in Fig. 1.2, these two proteins are structurally related, since 49 out of 129 residues are identical and the position of

EGG - WHITE LYSOZYME

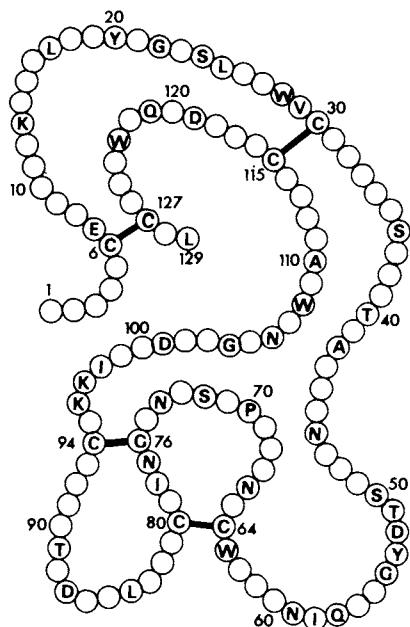
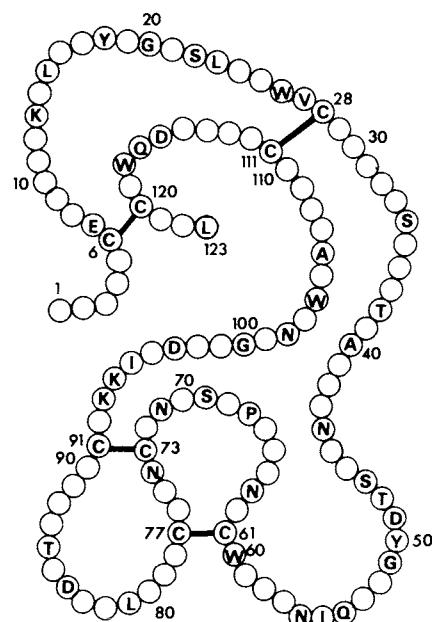
 α -LACTALBUMIN

Fig. 1.2. Structural similarities between egg-white lysozyme and α -lactalbumin. The letters correspond to homologous amino acid residues in the two molecules.

the four disulfide bridges is the same (Arnon, 1977). When the two native molecules were tested with antisera directed against each one, no cross-reactivity could be detected by various sensitive assays such as microcomplement fixation, passive hemagglutination, and phage inactivation (Arnon and Maron, 1970). However, when antisera produced against the two unfolded proteins were used in comparative tests, lysozyme and α -lactalbumin were found to be immunochemically related. Several other studies have confirmed that immunological cross-reactivity and sequence homologies between related proteins are more easily demonstrated when the unfolded molecules are compared instead of the native ones (Brawn and Dandliker, 1977).

1. Localization of Antigenic Determinants in Lysozyme

Fragments obtained by cleavage of the reduced lysozyme molecule did not possess any antigenic reactivity when tested with antibodies to native lysozyme (Young and Leung, 1970). This result may be ascribed to the fact that the preliminary rupturing of disulfide bonds required for tryptic attack destroyed the

particular conformation of the peptides required for antigenic activity. Subsequently, it was found possible, by reversible masking of amino acid groups by citraconylation, to render the lysozyme molecule susceptible to trypic digestion, without rupturing the disulfide links (Atassi *et al.*, 1973). By this approach, three disulfide-containing peptides (Fig. 1.3) were obtained that strongly inhibited the precipitation of lysozyme by its antibodies. Since the percentage inhibition amounted to 85–89%, it was claimed (Atassi and Habeeb, 1977; Atassi, 1979b) that these three peptides accounted for almost the entire antigenic reactivity of lysozyme.

Other investigators have shown that the so-called loop region, which consists of residues 64–80 (Fig. 1.3), also possesses antigenic reactivity (Arnon and Sela, 1969; Arnon *et al.*, 1971; Teicher *et al.*, 1973). It was found by several immunological techniques that the reactivity of this loop peptide was drastically reduced when the disulfide bridge joining residues 64–80 was cleaved (Arnon, 1977). Additional evidence for the presence of a determinant in this region was obtained by showing that antiloop antibodies were able to distinguish between two lysozymes that present a substitution at position 68 (Fainaru *et al.*, 1974;

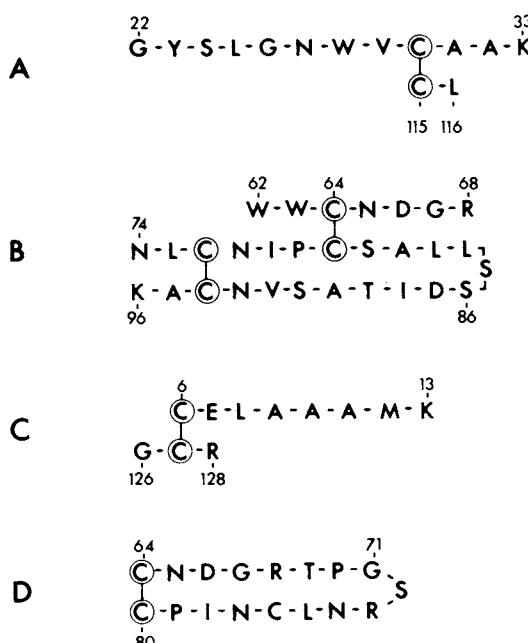


Fig. 1.3. Structure of three peptides (A, B, and C) of lysozyme obtained by trypic hydrolysis and capable of inhibiting (85–89%) the reaction of lysozyme with its specific antiserum (Atassi *et al.*, 1973). Peptide D is the antigenically active loop peptide studied by Arnon (1977) and collaborators.

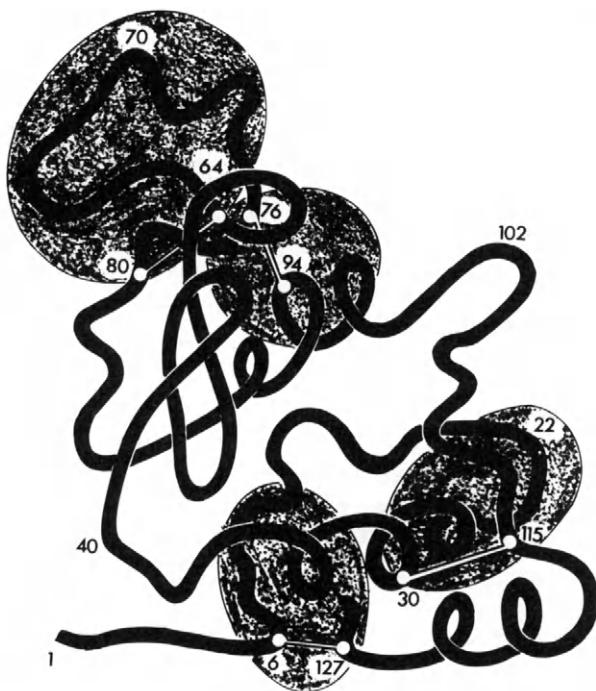


Fig. 1.4. Mode of folding of lysozyme. The four antigenic regions situated in the vicinity of the disulfide bridges are indicated by grey patches.

Ibrahim *et al.*, 1980). The approximate location of the four antigenic regions in the three-dimensional structure of lysozyme is presented in Fig. 1.4.

A more precise localization of the determinants within the three peptides isolated by Atassi *et al.* (1973) was achieved by the method of surface-simulation synthesis (Atassi *et al.*, 1976a,b). A series of discontinuous determinants were constructed by linking spacially adjacent residues that are apart in the sequence by means of glycine spacers. Several peptides that do not exist in lysozyme, but which simulate parts of the surface topography of the molecule, were obtained and their inhibitory activity was measured. These studies led to the proposal that three peptides, illustrated in Fig. 1.5, accounted for 90–95% of the antigenic reactivity of native lysozyme (Atassi and Lee, 1978). Since a large excess of these peptides was needed to achieve maximum inhibition, it was argued that the synthetic structures did not correspond exactly to the conformation of the antigenic areas of the native molecule (Atassi and Habeeb, 1977).

A word of caution should be introduced here about conclusions of a quantitative nature, which are based on the percentage inhibition observed in immunochemical reactions. The measurement of antigen–antibody interaction inevitably entails operational limitations, which make it difficult to account for the

<i>EPITOPE A</i>	116	113	114	34	33
CONSTITUANT RESIDUES	:	K — N — R — F — K			
SYNTHETIC PEPTIDE	:	K — N — R — G — F — K			
<i>EPITOPE B</i>	62	97	96	93	89
CONSTITUANT RESIDUES	:	W — K — K — N — T — D			
SYNTHETIC PEPTIDE	:	F — G — K — K — N — T — D			
<i>EPITOPE C</i>	125	5	7	14	13
CONSTITUANT RESIDUES	:	R — R — E — R — K			
SYNTHETIC PEPTIDE	:	R — G — G — R — G — E — G — G — R — K			

Fig. 1.5. Structure of the three epitopes alleged to represent the complete antigenic structure of lysozyme (Atassi and Lee, 1978). The numbers of the constituent residues correspond to their position in the primary structure. The synthetic peptides obtained by surface-simulation synthesis mimic parts of the surface topography of the lysozyme molecule.

behavior of all the different types of antibodies present in an antiserum. For instance, a method that measures preferentially the antibodies of highest affinity present in an antiserum may well overlook the existence of a majority of antibodies of low affinity. Although the precipitin test, for example, is not particularly dependent on high-affinity antibodies, it is, on the other hand, very sensitive to the ratio of serological reactants. It is conceivable that the presence of a large amount of inhibitor displaces the optimal proportion point of the homologous reaction and that the diminished amount of precipitation partly reflects this shift. In view of such difficulties it seems imperative to base quantitative conclusions on several immunochemical methods that are not subject to the same operational limitations.

In the case of the three alleged determinants of lysozyme, some of the reported contradictory findings require further clarification. By comparing the structures of the three disulfide-containing peptides obtained by cleavage (Fig. 1.3) with those of the synthetic peptides (Fig. 1.5), it is obvious that in spite of their similar total inhibitory capacities, the two sets of peptides are extremely different. In particular, when the two synthetic peptides made up of residues 5-7-13-14-125 and residues 33-34-113-114-116 are compared with the corresponding degradation peptides, it can be seen that in each case, three out of the five residues comprising the synthetic epitopes were not present in the original structures obtained by cleavage. In view of this discrepancy, it is doubtful whether much significance can be given to the similar total inhibitory capacity found with the two sets of peptides.

2. Antigenic Comparisons between Lysozymes

The existence of a large number of avian and mammalian lysozymes of known primary structure provided the opportunity to test the effect of various substitu-

tions on the antigenicity of this molecule. Immunochemical comparisons with many lysozymes as well as with several other groups of related proteins have demonstrated that there is a strong correlation between degree of sequence difference and degree of antigenic difference (Prager and Wilson, 1971a,b). From the extent of correlation, it has been inferred that about 80% of the amino acid substitutions that have accumulated during the evolution of monomeric globular proteins are antigenically detectable (White *et al.*, 1978). This leads to the paradox that the majority of substitutions are able to affect antigenicity although only about 15% of the residues of lysozyme participate directly in the structure of its epitopes (according to the data of Atassi and collaborators). This discrepancy is exemplified by the finding that several bird lysozymes that present exchanges exclusively outside the four presumed determinants (i.e., the loop peptide and the three synthetic peptides shown in Fig. 1.5) are nevertheless distinguishable antigenically (Ibrahimi *et al.*, 1979).

These findings, together with the conflicting data referred to in the Section F,1 lead to the conclusion that the entire antigenic structure of lysozyme cannot be accounted for solely by the 16 residues listed in Fig. 1.5.

As indicated by Hurrell *et al.* (1977) and Ibrahimi *et al.* (1979), it seems necessary to assume that substitutions outside antigenic determinants are able, by some kind of allosteric mechanism, to produce conformational changes that alter the antigenicity. This means that the antigenic reactivity of globular proteins is modulated by long-range interactions at the level of the secondary and tertiary structure, and that it may be impossible to describe complete antigenic structures of proteins in terms of a few short peptides. Since the conformational distortions present in cleaved or synthesized peptides are likely to be at least as important as those induced in the native determinants by distant substitutions, there is little prospect of reproducing exactly the structure of epitopes by means of short synthetic peptides.

However, in spite of these limitations, it should be emphasized that studies with short fragments of protein molecules do succeed in localizing antigenic determinants. The main lesson to be drawn from the extensive studies of myoglobin and lysozyme is that their antigenic structures cannot be fully described in terms of only 3–5 epitopes of 5–7 residues each.

Immunochemical studies with viral proteins of approximately the same size as myoglobin and lysozyme will be discussed in Chapter 10. These studies led to the conclusion that an antigen such as TMV protein also possesses a considerable number of epitopes, and that most of the accessible corners and exposed protuberances on the surface of the protein harbor antigenic activity.



Antibodies

Antibodies are proteins belonging to the group of immunoglobulins capable of binding specifically to antigens. They are found in serum and are produced by lymphoid cells, predominantly plasma cells, in response to stimulation by an antigen.

All immunoglobulins have a similar basic structure, consisting of two identical light (L) chains and two identical heavy (H) chains, which are linked together by noncovalent forces and disulfide bonds. Five classes of immunoglobulins (Ig) can be distinguished on the basis of five different types of heavy chain. These classes are IgG, IgA, IgM, IgD, and IgE and their respective H chains are called γ , α , μ , δ , and ϵ . The light chains are the same in all immunoglobulin classes.

The concentration of IgD and IgE in serum is extremely low, and these two classes play little role in the immune response of animals to plant viruses. In fact, the only antibodies to plant viruses that have been studies in any detail are IgG and IgM antibodies (Bercks *et al.*, 1974, Tremaine and Chidlow, 1974). The IgG class accounts for approximately 75% of all immunoglobulins in serum, and

most of our knowledge of antibody structure is based on studies of IgG molecules.

In the past, IgG molecules used to be called γ -globulins, a name derived from the fact that they migrate to the gamma region of the electrophoretic profile of serum. IgG molecules, like all immunoglobulins, are heterogeneous and show a broad range of electrophoretic mobilities. This heterogeneity, which is based on a multiplicity of primary structures (each one corresponding to a particular antibody specificity), can be demonstrated by the technique of isoelectric focusing. In this technique, a pH gradient is established in a gel and the immunoglobulins are allowed to migrate by electrophoresis until they reach positions in the gel that correspond to their own isoelectric point. The heterogeneity of antibody molecules is revealed by the appearance of a very large number of bands (Williamson, 1978).

Much of our detailed knowledge of immunoglobulin structure has been derived from the neoplastic disease known as multiple myeloma. This disease is caused by the uncontrolled proliferation of a single clone of antibody-producing cells and leads to the appearance, in the serum of diseased individuals, of a very high concentration of identical immunoglobulin molecules. It is the availability of such homogeneous populations of immunoglobulins that made it possible to elucidate the amino acid sequences of these proteins and to explore their three-dimensional structure by X-ray diffraction. For a detailed account of the elucidation of immunoglobulin structure, several reviews may be consulted (Porter, 1973; Edelman, 1973; Kabat, 1976; Turner, 1977; Poljak, 1978).

A. STRUCTURE OF IgG

The basic structure of the rabbit IgG molecule is shown in Fig. 2.1. It consists of two L chains (molecular weight 25,000) and two H chains (molecular weight 50,000), linked by a single disulfide bridge. Two types of L chains, called κ (kappa) and λ (lambda) chains, are found, but in any one immunoglobulin molecule, the two light chains are always of the same type.

The C-terminal half of the light chain, which is about 110 residues long and is known as the C_L region, does not vary except for certain minor allotypic variations. Allotypic differences correspond to amino acid substitutions, which occur in a limited number of positions along the chain and are found only in certain individuals.

In contrast to the constant C-terminal half of the L chain, the N-terminal half, which is known as the variable or V_L region, shows considerable sequence variability. This variability is not distributed evenly along the V_L region, but occurs in three restricted areas, totalling about 25 residues, known as hypervariable regions (Fig. 2.1). These hypervariable residues are intimately involved in

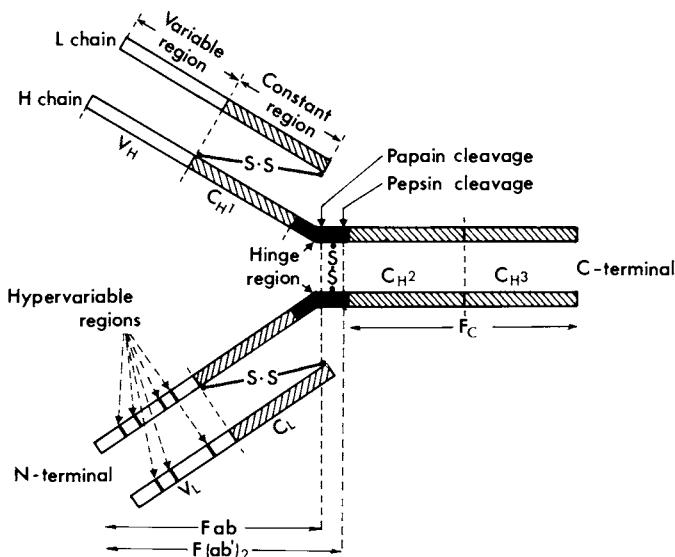


Fig. 2.1. Rabbit IgG molecule showing the interchain disulfide bridges, the location of the different domains, and the fragments obtained by papain and pepsin cleavage.

the formation of the antibody combining site and contribute to the regions of the molecule that come into contact with the antigenic determinant. The N-terminal quarter of the heavy chain is also variable and is known as the V_H region. Most of the variability of this region is concentrated in four hypervariable regions, extending over about 40 residues. The remaining C-terminal part of the H chain is subdivided into three regions, comprising about 110 residues each, known as the C_{H1} , C_{H2} , and C_{H3} regions.

The two binding sites of IgG are found at the extremity of the two arms of the Y-shaped molecule, and are built from hypervariable residues of the V_H and V_L regions that are brought into close spatial proximity by the folding of the two polypeptide chains. The combining site is formed by a relatively shallow cleft, exposed to the solvent and composed of a number of peptide loops, the tips of which bear hypervariable residues (Richards *et al.*, 1978). The size of the cleft suggests that it could accommodate a peptide of about four to seven residues. Since the hypervariable residues are all present in loops that extend into the solution and do not influence the framework of the F_V region, different antibodies are able to display a unique antigen binding site while retaining a common three-dimensional structure.

When the amino acid sequences of the V_L , C_L , V_H , C_{H1} , C_{H2} , and C_{H3} regions are aligned, a striking degree of sequence homology is revealed. Each homology region contains one intrachain disulfide bridge in a homologous posi-

tion, spanning about 60 residues, and is folded into an independent and compact globular domain. The C_H 1 and C_H 2 domains are separated by a short region of about 15 residues called the hinge region. This region, which is rich in proline residues and is particularly sensitive to proteolytic attack, is responsible for the flexibility observed between the two Fab arms and the Fc fragment (Cathou, 1978). The angle between the two Fab arms can vary between 80 and 180° (Crothers and Metzger, 1972) and this allows an IgG molecule to adjust its binding sites to accommodate simultaneously two epitopes that are 10–20 nm apart. Not all antibody molecules, however, possess the same degree of flexibility (McGuire *et al.*, 1979).

Papain cleaves the heavy chain on the N-terminal side of the interchain disulfide bond and produces two Fab fragments (antigen binding fragments) and one Fc fragment (crystallizable fragment) per IgG molecule (Fig. 2.1). Pepsin cleaves the heavy chains at several locations on the C-terminal side of the interchain disulfide bond and produces one $F(ab') fragment. By reduction of the interchain S—S bond, two $F(ab') fragments are obtained.$$

The existence of domains which was originally inferred from sequence homologies was later confirmed by X-ray crystallography (Feinstein and Beale, 1977; Poljak, 1978). The folding of the peptide chain in the different domains was shown to be very similar and to consist of a series of antiparallel β -pleated sheets. The overall shape of the IgG molecule, as derived from the crystallographic studies of Colman *et al.* (1976) and Deisenhofer *et al.* (1976), is shown in Fig. 2.2. Most of the domains interact with each other over extended areas, except for the two C_H 2 domains, which are not in contact with each other. There is also limited longitudinal contact between V_L and C_L , and between V_H and C_H 1. The remarkable stability of the IgG molecule is mainly due to the existence of these compact hydrogen-bonded and disulfide-bonded domains. IgG molecules

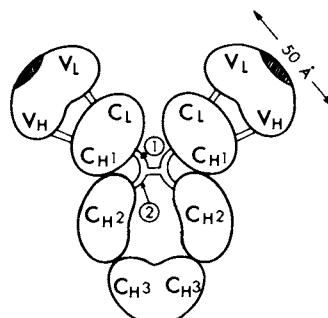


Fig. 2.2. Structure of an IgG molecule, based on the crystallographic studies of Colman *et al.* (1976) and Deisenhofer *et al.* (1976). The two antigen combining sites are located at the outer tips of the Fab regions (shaded areas). Cleavage points by papain (1) and pepsin (2) are indicated by arrows.

TABLE 2.1
Properties of the Three Main Immunological Classes of Serum

Class	Molecular weight	$s_{20,w}$	Percent carbohydrate ^a	Heavy chain	Concentration in serum (mg/ml)	Fixation of complement
IgG	150,000	7 S	3	γ	8-16	Yes
IgM	900,000	19 S	12	μ	0.5-2	Yes
IgA ^b	160,000	7 S	8	α	1-2	No

^a The carbohydrate is attached to the C_H2 region.

^b For IgA the molecular weight and sedimentation coefficient refer to the monomeric form.

are resistant to wide variations in environmental pH and ionic strength; unless the molecules have first been denatured, for instance with urea, they are also resistant to the action of reducing agents.

B. PROPERTIES OF THE MAIN IMMUNOGLOBULIN CLASSES

All five immunoglobulin classes are built on similar structural principles. The properties of the three main immunoglobulin classes found in serum are summarized in Table 2.1.

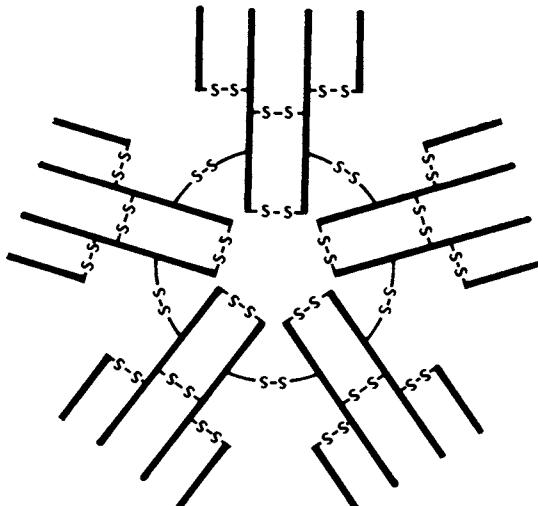


Fig. 2.3. Pentameric IgM molecule. Each monomer consists of two heavy and two light chains joined by disulfide bridges. The J chain has not been drawn. The molecule possesses 10 potential antigen combining sites.

In serum, IgM is generally found as a pentamer of five disulfide-linked subunits, each composed of two L and two H(μ) chains (Fig. 2.3). A single additional chain, called the J (joining) chain, is also present and plays a role in the polymerization of the subunits into pentamers (Koschland, 1975). IgM may be readily dissociated into 7 S subunits by reduction of the intrasubunit disulfide bridges, and differs from IgG in having four constant domains in the heavy chain, instead of three. IgM has 10 potential antigen binding sites, but these may not all be reactive simultaneously, mainly as a result of steric hindrance.

In serum, IgA is found mainly as a monomer, built of two L and two H(α) chains, but dimers and other oligomers also occur. The polymeric forms contain 1 mole of J chain per polymer (Koschland, 1975).

C. FUNCTIONAL PROPERTIES OF IMMUNOGLOBULINS

In the context of antibody interactions with plant viruses, only three functional aspects of immunoglobulins need be considered. These are binding of antigen, fixation of complement, and binding of protein A of *Staphylococcus aureus*.

1. Specificity of Antibody Combining Sites

The structural properties of the combining regions responsible for antibody specificity have been described in Section B. Although the particular amino acid sequence in the hypervariable regions of the V_L and V_H domains is directly responsible for the specificity of each antibody species, this does not mean that each antibody molecule is able to bind to only one type of antigenic determinant. In the same way that many different antibodies are able to bind a single epitope, admittedly with different degrees of affinity, the reverse situation also exists. The evidence for the concept that a single antibody molecule may be complementary to several epitopes of different structure has been summarized by Richards *et al.* (1978). Although it is difficult to assess exactly how many different epitopes are complementary to a single antibody combining site, the polyfunctional nature of combining sites is no longer in doubt. Discarding the notion of a unique one-to-one complementary relationship between antigenic determinant and antibody combining site does not mean, however, that the high degree of specificity displayed by antisera can no longer be accounted for. As pointed out by Talmage (1959) and Richards *et al.* (1975), the specificity of antisera represents a population phenomenon based on the ability of different polyfunctional antibodies to react with one and the same antigen. The fact that all the other cross-reactivities (for various antigens) present in the antiserum are different for the various antibody molecules, in effect means that they are diluted out in the antiserum. As a result, only the common reactivity directed toward the antigen used for immuni-

zation stands out, and the animal appears to have been stimulated in a highly specific manner (Richards *et al.*, 1978; Weininger and Richards, 1979).

2. Complement Binding Site

The fixation of complement is one of the most important biological properties of immunoglobulins. It amplifies the effect of antigen binding and is able to induce cell destruction and to generate physiologically active by-products. The initiation of the complement cascade reaction is brought about by the interaction of the C1q component of complement with a particular region of the C_H2 domain of IgG (Kehoe, 1978). The precise mechanism by which the binding of antigen at the extremity of the Fab region uncovers the ability of the C_H2 domain to bind C1q is still unclear. One possible mechanism would be the occurrence of an antigen-induced conformational change in the antibody leading to the exposure of a complement binding site in the Fc region. Another explanation, known as the associative model, suggests that the role of the antigen is merely to bring several antibody molecules in close proximity (Metzger, 1974). It is known that binding of C1q occurs only when at least two adjacent IgG molecules are attached to a multivalent antigen. The inability of monovalent haptens to initiate a complement fixation reaction is illustrated by the results obtained with the loop peptide of lysozyme (Arnon, 1977). Although this peptide by itself is not active in the complement fixation test, dimers prepared by coupling two loop molecules via nonamethylenediamine fix complement readily.

3. Binding of Protein A

Protein A is a molecule of 42,000 molecular weight, isolated from the cell walls of *Staphylococcus aureus*, which possesses a very high affinity for the Fc region of IgG of many animal species (Goding, 1978; Goudswaard *et al.*, 1978; Calvanico and Tomasi, 1979). This property has led to numerous applications in immununochemistry, for instance, the precipitation of antigen-antibody complexes, as well as various forms of affinity chromatography and solid phase immunoassays (Langone, 1980).

Virus Purification

A. INTRODUCTION

The various methods used for purifying plant viruses have been described in several extensive reviews (Steere, 1959; Brakke, 1967a,b; Ackers and Steere, 1967; Francki, 1972; Van Regenmortel, 1972; Venekamp, 1972; Schumaker and Rees, 1972; Noordam, 1973). In this chapter, purification methods will not be described in detail, and only general principles will be briefly summarized. The discussion will center mainly on aspects that are particularly relevant when the purification is undertaken for the purpose of studying the serological properties of plant viruses.

It is well known that immunochemical methods often detect antigenic contaminants in virus preparations that appear pure by physicochemical criteria. In fact, the ultracentrifugal and electrophoretic methods commonly used to ascertain the purity of virus preparations are not suited for establishing antigenic purity. It is of course a truism to state that immunochemical purity can only be established by immunochemical analysis.

The very fidelity with which an antibody recognizes slight conformational alterations in the antigen (see Chapter 1) sometimes leads to unexpected difficulties. This is due to the fact that many of the chemical manipulations used for isolating and purifying virus particles are able to change the conformation of the viral coat protein. It is well known that major conformational changes can be induced in proteins by extremes of pH and by the action of reagents such as detergents, organic solvents, and inorganic salts. In fact, most of the chemical treatments commonly used in virus purification are capable of inducing a certain amount of conformational reorganization in protein molecules. It is the reversible nature of these changes which in most cases allows the utilization of relatively harsh chemical procedures for virus purification. In addition, the quaternary interactions between viral subunits also render the virions more resistant to denaturation, compared with monomeric proteins. This is one of the reasons why many plant proteins can be selectively denatured by the chemical treatments that are used for clarifying crude extracts from virus-infected plants.

1. Propagation of Virus

It is important to choose a host plant in which the virus reaches a high concentration. The environmental conditions under which the inoculated plants are grown also greatly affect the virus yield. Factors such as temperature, light intensity, nutrients, age of the plants at the time of inoculation as well as time of harvesting of the infected material are known to be of considerable importance. Different parts of the infected plant may also vary in the amount of virus they contain, and although infected leaves are most commonly used, roots sometimes may be a better source of virus material (Ford, 1973).

A particular virus purification procedure can often be improved once an antiserum to the virus has been obtained. By means of quantitative serological tests, it then becomes possible to measure variations in virus concentration in various parts of the plant, and to ascertain optimal growing conditions as well as the best time of harvesting (Bartels and Völk, 1966; Schade, 1967a). The newly developed and very sensitive enzyme immunoassays, in particular, allow a very precise monitoring of the amount of virus present in infected plant tissues (Garnsey *et al.*, 1979)

2. Extraction of Virus

Infected tissue, either fresh or frozen, is usually ground in a blender in the presence of a suitable buffer. The homogenate is then strained through cheesecloth to yield the crude plant sap. After the initial grinding and juice extraction, it is often possible to obtain a further substantial quantity of virus by re-extracting the plant fiber with buffer a second time (Steere, 1959; Hollings and Stone,

1965a). Buffers that are commonly used are phosphate, citrate, borate, and EDTA (Tomlinson, 1963; Scott, 1963; Takanami and Tomaru, 1969).

With many labile viruses, it is often necessary, in order to preserve particle integrity and infectivity, to work in the cold and to add protectants as soon as the plant cells are disrupted by grinding. Substances that are commonly used include reducing agents such as sodium sulfite, mercaptoethanol, dithiothreitol, ascorbic acid, and thioglycollic acid as well as polyphenoloxidase inhibitors such as diethylidithiocarbamate (Hampton and Fulton, 1961; Harrison and Pierpoint, 1963).

3. Problems Encountered during Purification

Although the purification of viruses like TMV and turnip yellow mosaic virus (TYMV), which are stable and reach very high concentrations in their hosts, can be achieved by a variety of relatively harsh procedures, it is not possible to purify the majority of plant viruses without taking some special precautions.

a. Particle Aggregation

Particle aggregation is a serious problem encountered in the purification of many elongated viruses (Reichmann, 1959; Welsh *et al.*, 1973). This can be minimized by grinding infected tissue in high molarity buffers such as 0.5 M sodium citrate, phosphate, or borate. This approach was used for instance for purifying potyviruses such as turnip mosaic virus (TurMV) (Shepherd and Pound, 1960), lettuce mosaic virus (LMV) (Tomlinson, 1964), soybean mosaic virus (SoyMV) (Ross, 1967), potato virus Y (PVY) (Stace-Smith and Tremaine, 1970), tobacco etch virus (TEV) (Damirdagh and Shepherd, 1970), pea seed-borne mosaic virus (PSbMV) (Stevenson and Hagedorn, 1973a), maize dwarf mosaic virus (MDMV) (Gordon and Gingery, 1973), and poplar mosaic virus (PopMV) (Luisoni *et al.*, 1976). The addition of 0.5-1.0 M urea has also been found useful for dissociating aggregated potyviruses (Damirdagh and Shepherd, 1970; Makkouk and Gumpf, 1976). Detergents such as Triton X-100 and Tween-80 have been used for dispersing particles of elongated viruses that tend to aggregate and bind to plant organelles. Detergents disrupt the structure of membranes, release bound proteins and pigments, and are often able to reverse virus aggregation. This approach has been used with barley stripe mosaic virus (BSMV) (Brakke, 1959), apple chlorotic leaf spot virus (ACLSV) (Lister *et al.*, 1965), TMV (Nozu and Yamaura, 1971), plum pox virus (PPV) (Van Oosten, 1972), MDMV (Langenberg, 1973), PVY (Leiser and Richter, 1978), and the isometric cauliflower mosaic virus (CaMV) (Hull *et al.*, 1976).

b. Particle Breakage

The structural integrity of certain labile viruses requires the presence of a critical concentration of divalent cations. It was shown for instance by Lister and

Hadidi (1971) that 0.005 M MgCl₂ was needed for stabilizing the particles of ACLSV. When the MgCl was removed by dialysis or chelation, the virus particles were degraded. A beneficial side effect of such particle breakdown, however, is that it enables immunodiffusion tests to be done with long flexuous viruses, which otherwise would be unable to diffuse through agar (Lister and Hadidi, 1971). When the virus has been degraded to the level of individual protein subunits, the antigenic reactivity may be altered, and serological detection may then be less straightforward (see Chapter 7).

Virus degradation can usually be prevented by the addition of formaldehyde or glutaraldehyde in the range 0.05–2% (Hollings and Stone, 1962; Von Wechmar and Van Regenmortel, 1968; Bol and Veldstra, 1969; Van Regenmortel and Lelarge, 1973). The ability of formaldehyde to prevent the dissociation of CMV into protein subunits is illustrated in Fig. 3.1. Aldehydes act by forming cross-

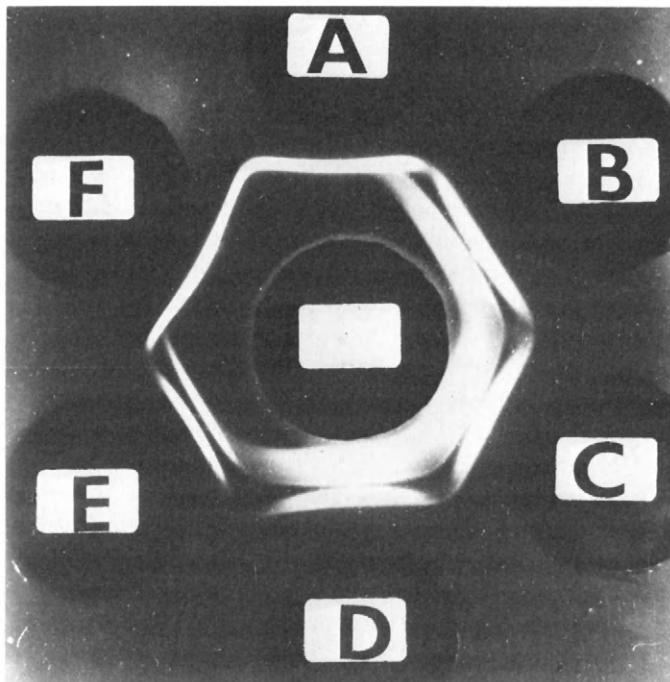


Fig. 3.1. Stabilization of cucumber mosaic virus strain S in phosphate buffer pH 7.0 containing 0.4% formaldehyde (wells A and F). Wells B and C contain CMV in phosphate buffer pH 7.0, well D contains CMV in barbital buffer pH 8.0, and well E contains CMV in acetate buffer pH 4.0. The central well contains CMV antiserum. The precipitin band nearest to the wells containing the antigen corresponds to undegraded virions. The wide internal band corresponds to protein subunits. Note the narrow subunit band produced by virus in acetate buffer, indicating partial stabilization at pH 4.0. The virus was kept for 2 days in the various buffers, prior to testing.

links mainly between the ϵ -amino groups of lysine residues (Ruschmann, 1964; Habeeb and Hiramoto, 1968) and are thus able to bind adjacent subunits together. This treatment does not seem to affect the antigenic reactivity of proteins (Habeeb, 1969; Reichlin *et al.*, 1970). Inactivated virus vaccines obtained by treating viruses with formalin have been used in animal virology for many years. With unstable plant viruses, the stabilization of capsid structure brought about by aldehyde treatment also leads to a considerable enhancement of immunogenicity (Hollings and Stone, 1962; Francki and Habil, 1972; Richter *et al.*, 1972a, 1973; Habil and Francki, 1975; Gonsalves *et al.*, 1978).

c. Proteolytic Degradation of Viral Coat Protein

The protein subunits of many potyviruses are degraded *in situ* (Hiebert and McDonald, 1973; Huttinga and Mosch, 1974; Moghal and Francki, 1976) as shown by a decrease in their molecular weights from the usual 33,000–34,000 to 31,000 and lower. A similar phenomenon has also been observed with cowpea mosaic virus (CPMV) (Niblett and Semancik, 1970), tomato bushy stunt virus (TBSV) (Ziegler *et al.*, 1974), and saguaro cactus virus (SCV) (Nelson and Tremaine, 1975). This proteolysis is mediated by plant proteases that often contaminate virus preparations.

The importance of this phenomenon for serological studies resides in the fact that proteolytically degraded viral subunits often are antigenically different from native subunits (Shepard and Secor, 1972; Koenig, 1978). In the case of potato virus X (PVX), it has been demonstrated that even when the protein subunits are still part of the intact virus, they can be degraded by a reducing agent-dependent enzyme that acts at the N-terminal end of the peptide chain (Tremaine and Agrawal, 1972; Tung and Knight, 1972; Koenig *et al.*, 1978). Another enzyme, which is a reducing agent-independent plant protease, cleaves at least another two short peptides from the C-terminus of PVX protein (Tung and Knight, 1972; Koenig *et al.*, 1978). This type of proteolytic degradation, which is enhanced when the virus is kept in prolonged contact with plant sap, is probably responsible for the differences in molecular weights of viral subunits reported by many authors (Paul, 1974). Furthermore, since the amount of proteases may vary in different plants, the extent of degradation and, therefore, the antigenic specificity of a virus may depend on the host in which the virus has been propagated (Rees and Short, 1965; Koenig, 1975). Since the addition of reducing agents to the extraction medium may lead to degradation of viral capsids, the indiscriminate use of such substances should be avoided in virus purification.

B. REMOVAL OF PLANT ANTIGENS

The presence in virus antisera of antibodies to plant antigens is undesirable for two reasons: it abolishes the specificity of the serological tests used for diagnosis

of virus infections, and it makes the interpretation of serological cross-reactivity data between viruses more difficult (Van Regenmortel, 1966a). This is due to the fact that contaminating plant antigens from different hosts often are serologically related and, thus, potentially capable of simulating a relationship between viruses (Van Regenmortel, 1963). Many workers have reported that the host antigens that contaminated their virus preparations were serologically related to plant antigens from several different species (Malkiel and Stanley, 1947; Moorhead, 1959; Tremaine, 1961; Tremaine *et al.*, 1964). Using a single antiserum prepared against fraction I protein of *Cucurbita pepo* L., it could be demonstrated that related antigens were present in 38 plant species commonly used for propagating viruses (Table 3.1).

The major plant protein that commonly contaminates virus preparations is the fraction I protein, which represents about 50% of the total proteins present in plant sap (Singer *et al.*, 1952). Fraction I protein corresponds to the enzyme

TABLE 3.1

Plant Species Shown by Immunodiffusion to Possess Serologically Related Fraction I Proteins^a

Solanaceae	Amaranthaceae
<i>Nicotiana tabacum</i> L. (tobacco)	<i>Gomphrena globosa</i> L.
<i>Solanum tuberosum</i> L. (potato)	Polygonaceae
<i>Lycopersicon esculentum</i> Mill. (tomato)	<i>Rheum raponticum</i> L. (rhubarb)
<i>Capsicum frutescens</i> L. (pepper)	Rutaceae
<i>Datura stramonium</i> L. (Jimson-weed)	<i>Citrus sinensis</i> L. (orange)
<i>Physalis floridana</i> Rydb.	<i>Citrus limon</i> L. (lemon)
<i>Petunia hybrida</i> Vilm.	Cruciferae
Leguminosae	<i>Brassica chinensis</i> L. (cabbage)
<i>Phaseolus vulgaris</i> L. (bean)	Vitaceae
<i>Vicia faba</i> L. (broad bean)	<i>Vitis vinifera</i> L. (grapevine)
<i>Vigna sinensis</i> Endl. (cowpea)	Umbelliferae
<i>Lupinus albus</i> L. (lupin)	<i>Apium graveolens</i> L. (celery)
<i>Trifolium pratense</i> L. (clover)	<i>Petroselinum crispum</i> Nym. (parsley)
<i>Medicago sativa</i> L. (alfalfa)	<i>Pastinaca sativa</i> L. (parsnip)
Rosaceae	Compositae
<i>Malus domestica</i> L. (apple)	<i>Calendula officinalis</i> L. (pot-marigold)
<i>Cydonia oblonga</i> Mill. (quince)	<i>Helianthus annuus</i> L. (sunflower)
<i>Fragaria vesca</i> L. (strawberry)	Annonaceae
<i>Prunus persica</i> L. (peach)	<i>Asimina triloba</i> L. (papaw)
Myrtaceae	Gramineae
<i>Psidium guajava</i> L. (quava)	<i>Zea mays</i> L. (corn)
Cucurbitaceae	<i>Triticum vulgare</i> L. (wheat)
<i>Cucumis sativus</i> L. (cucumber)	Liliaceae
<i>Cucurbita pepo</i> L. (marrow)	<i>Allium cepa</i> L. (onion)
Chenopodiaceae	Orchidaceae
<i>Chenopodium quinoa</i> Willd.	<i>Cymbidium</i> spp.
<i>Spinacia oleracea</i> L. (spinach)	

^a From Van Regenmortel (1966b).

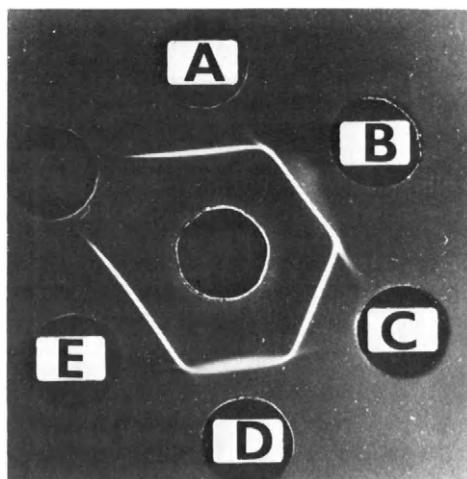


Fig. 3.2. Immunodiffusion test illustrating the serological relationship of fraction I proteins from *Chenopodium quinoa* (A); *Cucurbita pepo* (B); *Nicotiana tabacum* (C); *Zea mays* (D); and *Petunia hybridum* (E). The central well filled with antiserum to fraction I protein of *C. pepo*.

ribulose bisphosphate carboxylase, which catalyzes the photosynthetic fixation of carbon dioxide (Kawashima and Wildman, 1970). It has a sedimentation coefficient of 18 S, a molecular weight of about 550,000, and is made up of a number of large and small subunits of about 55,000 and 12,000, respectively. Fraction I proteins isolated from every green plant investigated so far have been found to possess common antigenic groups (Dorner *et al.*, 1958; Van Regenmortel, 1963; Kawashima, 1969; Kawashima and Wildman, 1971), which appear to be located on the large subunits of the molecule (Gray and Kekwick, 1974). This antigenic cross-reactivity obviously reflects the fact that the structure of the enzyme has been conserved through evolution to maintain the essential photosynthetic activity of all green plants. The serological relationship between the fraction I proteins of different plants commonly used for propagating viruses is illustrated in Fig. 3.2.

In spite of its prevalence, fraction I protein is not the only plant antigen found as a contaminant in virus preparations. Several other antigens have been identified by immunodiffusion and immunoelectrophoresis and specific antisera have been prepared against them (Marchoux, 1970; Richter *et al.*, 1975b; Schade *et al.*, 1977).

The removal of plant antigens during the various stages of a virus purification procedure can be followed easily by serological means (Oertel, 1961; Van Regenmortel, 1962, 1964a; Wetter and Paul, 1962; Wetter *et al.*, 1962; Gooding, 1963; Martelli and Hewitt, 1963; Schade, 1967b; Richter and Proll, 1970). Differences in size and electrophoretic mobility between plant and viral antigens facilitate the identification of the different precipitin lines observed in immunodiffusion and immunoelectrophoresis tests (Van Regenmortel and Engel-

brecht, 1963; Devergne and Cardin, 1967a). The concentration of plant proteins can be measured accurately by quantitative double diffusion tests (Van Regenmortel, 1964a) and rocket immunoelectrophoresis (Reichenbächer *et al.*, 1978). The relative value of different purification techniques for separating virus particles from plant antigens has been discussed by Van Regenmortel (1966b).

The most specific method of removing plant antigens from a partially purified virus preparation consists of precipitating them with an antiserum prepared against normal plant proteins (Gold, 1961; Oertel, 1961; Wetter *et al.*, 1962; Schade, 1969; Paulsen and Fulton, 1969). Instead of adding the complete antiserum prepared against plant antigens to the virus preparation, it is preferable to use only the immunoglobulin fraction (Gold, 1961; Fulton, 1967a). An even better approach is to prepare an immunoabsorbent with the plant protein antibodies (Gray and Wildman, 1976). Since the cross-reactivity between the fraction I proteins of different plants is extensive, a single immunoabsorbent can be used for purifying several viruses propagated in different hosts.

C. CLARIFICATION

After obtaining the crude sap from the infected tissues, the first step in the purification procedure consists of clarifying the extract. This is probably the most important step in the whole procedure and ideally, it should remove as much of

TABLE 3.2
Methods Used for Clarification of Plant Sap

Method	Reference
Low-speed centrifugation	Steere, 1959
Absorption to celite and charcoal	Corbett, 1961; Francki and McLean, 1968
Absorption to bentonite	Dunn and Hitchborn, 1965; De Sequeira and Lister, 1969b
Absorption to DEAE-Sephadex	Juo and Rich, 1969
Absorption to silver nitrate	Gill, 1971; Koenig <i>et al.</i> , 1978
Absorption to hydrated calcium phosphate	Fulton, 1959
Heating (40°–60°C)	Stace-Smith and Tremaine, 1970
Freezing	Pratt, 1961
Acidification to pH 4–5	Matthews, 1960; Bancroft <i>et al.</i> , 1960
Precipitation and emulsification with organic solvents (chloroform, freon, <i>n</i> -butanol, ethanol, <i>n</i> -octanol, carbon tetrachloride, diethyl ether)	Steere, 1956; Wetter, 1960; Crowley <i>et al.</i> , 1965; Basit and Francki, 1970; Tomlinson <i>et al.</i> , 1973; Polak <i>et al.</i> , 1975

the plant constituents as possible without any loss of virus. Some of the most commonly used clarification methods are listed in Table 3.2.

The most gentle clarification procedures, such as treatment with chloroform, diethylether, or carbon tetrachloride, are relatively ineffective in the removal of fraction I protein (Van Regenmortel, 1964a). A larger quantity of plant antigens is removed by adsorption to hydrated calcium phosphate (Schade, 1967b; De Sequeira, 1967) and more denaturing treatments, such as acidification to pH 3-5 and the addition of 25-30% ethanol or *n*-butanol, are even more effective. Many labile viruses, however, are degraded by such harsh procedures. The concentration of reagent used is often critical; a concentration of *n*-butanol higher than 8.5%, for instance, denatures many labile viruses (Tomlinson *et al.*, 1959) whereas a lower concentration does not clarify well. If too much charcoal or bentonite is used, much virus will also be removed (Galvez, 1964; Waterworth *et al.*, 1973). The extent of clarification is sometimes improved when the crude extract is kept at room temperature for several hours, after the addition of the organic solvent (Brunt, 1966a).

D. CONCENTRATION

When the maximum extent of clarification compatible with the stability characteristics of the virus has been achieved, it is usually necessary to concentrate the virus by one of the methods listed in Table 3.3. Some of the flexuous viruses are partly disrupted when they are submitted to large centrifugal forces and compacted into a pellet at the bottom of the centrifuge tube. In such a case, the integrity of the particles may be preserved by sedimenting the virus into a 20-55% sucrose cushion placed at the bottom of the tube (Van Oosten, 1972; Randles *et al.*, 1976). Another useful variation consists of sedimenting the virus into 30% sucrose containing 4% polyethylene glycol. This procedure concen-

TABLE 3.3
Methods Used for Concentrating Virus Particles

Method	Reference
Ultracentrifugation	Steere, 1959; Noordam, 1973
Isopycnic density gradient centrifugation	Brakke, 1967a,b
Precipitation at isoelectric point of virus	Fry <i>et al.</i> , 1960; Purcifull and Shepherd, 1964
Precipitation with salts	Steere, 1959
Precipitation with polyethylene glycol	Hebert, 1963; Leberman, 1966; Juckles, 1971; Hsu and Black, 1973; Kimura, 1976; Polson, 1977

trates the virus into a loose pellet and leaves most of the contaminating host material in solution (Kneseck *et al.*, 1974).

Precipitation with polyethylene glycol has become widely used as it is extremely simple and applicable to virtually every virus. It is important to realize that the insolubilization of the virus brought about by the polyethylene glycol is only slowly reversed when buffer is added to resuspend the precipitate. If insufficient time is allowed for solubilization, much virus will be lost during the subsequent low-speed centrifugation step.

E. REMOVAL OF RESIDUAL IMPURITIES

Following the initial sap clarification and subsequent concentration of the virus, a relatively small volume of a partially purified virus preparation is obtained, which usually needs to be further fractionated. A number of biochemical and biophysical separation procedures that are only suitable for relatively small samples can then be used to remove the residual contaminants. Some of the techniques that have been found useful for virus purification are listed in Table 3.4. An example of the purification achieved by zone electrophoresis in the case of an extract that contained three different viruses is shown in Fig. 3.3.

TABLE 3.4

Methods Used for Removing Residual Impurities from Partially Purified Virus Preparations

Method	Reference
Zonal centrifugation	Brakke, 1960; Schumaker and Rees, 1972
Isopycnic centrifugation	Brakke, 1967a,b; Miki and Oshima, 1972; Bar-Joseph <i>et al.</i> , 1974; Giri and Chessin, 1975
Zone electrophoresis	Van Regenmortel, 1964b, 1972; Murant <i>et al.</i> , 1968; Wolf and Casper, 1971; Uyemoto and Gilmer, 1971
Gel filtration	Van Regenmortel, 1962, 1964a; Steere and Ackers, 1962; Steere, 1963; Ackers and Steere, 1967; Juo and Rich, 1969
Ion-exchange chromatography	Shainoff and Lauffer, 1956; Levin, 1958; Tremaine, 1961; Venekamp, 1972
Chromatography on calcium phosphate columns	Taverne <i>et al.</i> , 1958; McLean and Francki, 1968; Murant <i>et al.</i> , 1969
Immunoadsorbents	Galvez, 1966; Avraméas <i>et al.</i> , 1969; Ladipo and De Zoeten, 1971
Immunoabsorption electrophoresis	Polson <i>et al.</i> , 1978
Chromatography on controlled pore glass beads	Barton, 1977; Brunt, 1978

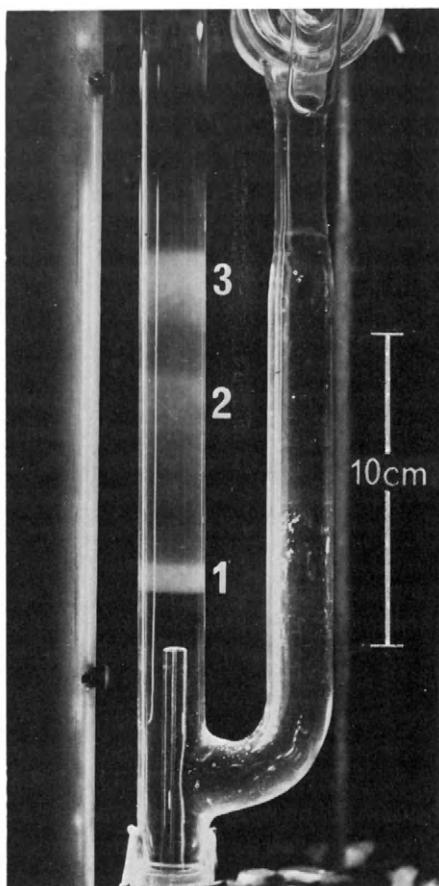


Fig. 3.3. Separation of three plant viruses by zone electrophoresis in a sugar density gradient. Zone 1 contains potato virus X, zone 2 a mixture of alfalfa mosaic virus and tobacco fraction 1 protein, and zone 3 tobacco mosaic virus. In this type of electrophoretic column, the sample is introduced by the bottom capillary and the components of the mixture are separated by ascending electrophoresis (Van Regenmortel *et al.*, 1964).

Density gradient centrifugation has been used to purify many plant viruses. The technique gives satisfactory results when aggregation of either virus or plant components is not a problem. Since zone electrophoresis in a sucrose density gradient (Polson and Russell, 1967; Van Regenmortel, 1972) and different methods of chromatography do not fractionate the components of a mixture on the basis of size, these procedures are particularly useful when the contaminants are fairly large. This is the case for instance with phytoferritin (Hyde *et al.*, 1963; Murant *et al.*, 1968) or when the plant proteins are aggregated.

F. PRESERVATION OF VIRUS MATERIAL

Once a virus has been propagated and purified, it is important to store it under conditions where its infectivity and antigenic properties remain unaltered. This will make it possible, for instance, to undertake a lengthy immunization schedule with the same antigen preparation and will do away with the need for continuous propagation in the greenhouse with its inherent danger of accidental contamination. Furthermore, efficient methods for the long-term preservation of virus material, whether in purified form or in crude extracts, are also of the utmost importance for many virological investigations. For serological diagnosis and testing of virus relationships, it is essential to dispose of a large number of reference viruses and strains. Since it would be impractical to have to propagate many viruses continuously, much effort has been spent on devising efficient methods of virus preservation. The methods that were found most successful are based on desiccation, freezing, or lyophilization of virus material (McKinney and

TABLE 3.5
Survival of Plant Viruses by Desiccation or Lyophilization^a

Virus	Virus group	Recorded survival period (years)	
		Desiccation	Lyophilization
Alfalfa mosaic virus		16	10
Arabis mosaic virus	Nepovirus		10
Barley stripe mosaic virus	Hordei	16	0
Barley yellow dwarf virus	Luteo		4
Bean pod mottle virus	Como	3	1
Bean yellow mosaic virus	Poty	2	
Broad bean mottle virus	Bromo		10
Broad bean true mosaic virus	Como		0
Brome mosaic virus	Bromo	16	
Cacao yellow mosaic virus	Tymo		1
Carnation latent virus	Carla		0
Carnation mottle virus			10
Carnation ringspot virus			6
Carnation vein mottle virus	Poty		6/12
Cherry leaf roll virus	Nepo		4
Clover yellow vein virus	Poty		5
Cucumber green mottle mosaic virus	Tobamo	10	
Cucumber mosaic virus	Cucumo	19	
Cymbidium mosaic virus	Potex		4/12
Cymbidium ringspot virus	Tombus		6

TABLE 3.5 —Continued

Virus	Virus group	Recorded survival period (years)	
		Preservation method	
		Desiccation	Lyophilization
Dahlia mosaic virus	Caulimo		0
Dulcamara mottle virus	Tymo		1
Henbane mosaic virus	Poty		10
Hydrangea ringspot virus	Potex		5/12
Lettuce mosaic virus	Poty	6/12	5/12
Narcissus mosaic virus	Potex		6/12
Narcissus yellow stripe virus	Poty		7/12
Oat mosaic virus		5	
Ononis yellow mosaic virus	Tymo		1
Pea early-browning virus	Tobra		2
Pea streak virus	Carla	2	
Pelargonium leaf curl virus	Tombus		10
Potato aucuba mosaic virus			6
Potato virus A	Poty		0
Potato virus S	Carla		10
Potato virus X	Potex	15	6
Potato virus Y	Poty	15	10
Prunus necrotic ringspot virus	Ilar	9	0
Radish mosaic virus	Como		1
Raspberry ringspot virus	Nepo		10
Southern bean mosaic virus		8	3
Sowbane mosaic virus			2
Squash mosaic virus	Como		1
Strawberry latent ringspot virus	Nepo		6
Tobacco etch virus	Poty	15	5
Tobacco mosaic virus	Tobamo		10
Tobacco necrosis virus		9	10
Tobacco rattle virus	Tobra		6
Tobacco ringspot virus	Nepo	17	10
Tobacco streak virus	Ilar	7	1
Tomato aspermy virus	Cucumo	3	10
Tomato black ring virus	Nepo		10
Tomato ringspot virus	Nepo	9	4
Tomato spotted wilt virus			6/12
Tulip breaking virus	Poty		0
Turnip crinkle virus	Tombus		7
Turnip mosaic virus	Poty	2	2
Turnip rosette virus			3
Turnip yellow mosaic virus	Tymo	5	3
Wheat streak mosaic virus		16	

^a Data from McKinney and Silber, 1968; Hollings and Stone, 1970; Rochow et al., 1976.

Silber, 1968; Hollings and Stone, 1970; Grivell *et al.*, 1971; Purcifull *et al.*, 1975b). The addition of 1% sodium azide to leaf tissue has been shown to protect viruses against microbial degradation and to preserve their antigenicity (Gooding and Tsakiridis, 1971). Many plant viruses can be preserved for many years in small pieces of infected leaf material dried and stored over calcium chloride in stoppered bottles (Table 3.5). It is preferable to store the desiccated material at 4°C since the infectivity of some viruses declines much more rapidly at room temperature (Bos, 1977; Bos and Benetti, 1979).

Freezing infected tissue or purified virus preparations at -20°C, or even better in Dry Ice or liquid nitrogen, has also been used successfully with many viruses. The labile tomato spotted wilt virus (TSWV) has been preserved for 6 years in Dry Ice (Best, 1961), whereas the stable TMV was still infective when kept for 50 years as unpreserved plant sap (Silber and Burk, 1965). Storing crude extracts or partially purified virus preparations at -20°C is often very convenient with viruses like barley yellow dwarf virus (BYDV) that are present in a very low concentration in the plant. A series of crude concentrates can be prepared over a period of time and pooled in a freezer until sufficient material has accumulated for the final purification (Rochow *et al.*, 1971). Long-term storage of purified preparations of stable viruses has been achieved by freezing the suspensions after addition of 10% glycerol. This method was found superior to freeze-drying of suspensions for preserving the antigenic activity of tymoviruses and tobamoviruses as well as several viruses infecting Gramineae (Paul and Querfurth, 1979). With several of the latter viruses, it was found that different methods of storage influenced their mobility in immunoelectrophoresis experiments. These differences in electrophoretic mobility are caused by the presence of viral degradation products with different net charges (Van Regenmortel and Lelarge, 1973).

In the case of some unstable viruses, it is not possible to freeze purified preparations and the best that can be done is to store them at 4°C. With the unstable cucumber mosaic virus (CMV), for instance, it was found that the antigenicity and particle integrity could be preserved for a period of 3 years, only if the purified preparation was fixed with 2% formaldehyde and stored at 4°C (Richter *et al.*, 1978a).

Lyophilization of infected plant sap in the presence of glucose and peptone, followed by storage under vacuum at room temperature, has also been used extensively (Hollings and Stone, 1970). Whenever comparative data on the same virus are available, it seems that simple desiccation preserves infectivity for a longer time than lyophilization (Table 3.5). As pointed out by Hollings and Stone (1970), viruses that belong to the same group do not necessarily survive the freeze-drying procedure equally well. In the potexvirus group, PVX survived 10 years and cymbidium mosaic virus (CybMV) less than 6 months. In the potyvirus group, bean yellow mosaic virus (BYMV) did not survive at all, whereas TurMV and PVY were still infective after 2 and 10 years, respectively.

Preparation of Antisera and Purification of Antibodies

A. PREPARATION OF ANTISERA

1. Immunogens

Purified virus preparations used for the immunization of animals should be free of contaminating plant antigens (see Chapter 3). This is easily ascertained by testing the virus preparation with antiserum prepared against normal plant proteins (Van Regenmortel, 1964a; Fulton, 1967a,b; Richter *et al.*, 1975b). If plant antigens are present in the virus preparation, they can be absorbed with immunoglobulins purified from the antiserum used for detecting the contamination. Residual globulins left in the preparation after cross-absorption will not elicit antibodies if the same species of animal is used for obtaining both the virus and plant protein antisera.

Most plant viruses are very good immunogens and antisera containing several milligrams of antibody per milliliter can easily be obtained. Some unstable plant

viruses are degraded into their constituent subunits in the body of the animal, and this may lead to the production of a considerable amount of antibody specific for these breakdown products. When the resulting antiserum is tested against the intact virions, it may appear as if very little specific viral antibody has been elicited. The antigenic specificity of monomeric viral subunits is often very different from that of the intact virion, and subunit antibodies may thus remain undetected if the antiserum is only tested against the complete virions (Van Regenmortel and Lelarge, 1973).

In the previous chapter (Section A3b) it was pointed out that the immunogenicity of unstable viruses can be enhanced considerably by aldehyde treatment (Hollings and Stone, 1962; Von Wechmar and Van Regenmortel, 1968; Francki and Habil, 1972).

In the case of some of the plant reoviruses such as rice dwarf virus (RDV), maize rough dwarf virus (MRDV), and Fiji disease virus (FDV), it was found that the virions were partly degraded in the body of the animal. The nucleic acid, which became exposed as a result, elicited antibodies specific for double-stranded RNA (Ikegami and Francki, 1973; Luisoni *et al.*, 1975). These antibodies, which were able to simulate a serological relationship between the intact virions of different viruses, could be removed by absorption with polyinosine: polycytidyllic acid [poly(I):poly(C)] (Francki and Jackson, 1972; Ikegami and Francki, 1973, 1974). Reoviruses are built up of double-shelled capsids and readily lose their outer protein shell. Electron microscopical observations showed that double-stranded RNA was attached externally to the inner cores that were used for immunization (Van der Lubbe *et al.*, 1979).

The role played by the quaternary structure of virions in their immunogenicity has been clearly demonstrated with TYMV and TMV. Marbrook and Matthews (1966) compared the immunogenicity of the virions and empty capsids of TYMV, and found that the empty protein shell induced a lower level of precipitating antibodies in immunized animals. Presumably, the presence of RNA in the virion confers greater stability to the capsid and prevents it from dissociating in the body of the animal. Only antibodies capable of reacting with intact virions were measured in these experiments, and it cannot be excluded that antibodies specific only for the protein subunits were present in a large quantity in the antiserum prepared against empty shells (Pratt *et al.*, 1980).

In the case of TMV, it was found that the dissociated protein subunits and the polymerized protein rods that are free of RNA produced much lower levels of viral antibody than an equivalent amount of intact virus (Marbrook and Matthews, 1966; Loor, 1967). It seems that the lymph node cells of immunized rabbits captured a larger amount of TMV than of TMV protein subunits, and also that they released the intact virus more slowly (Loor, 1967). As shown in Fig. 4.1, TMV antisera contained as much as 2–8 mg antibody/ml antiserum, compared with 0.5–1.0 mg/ml in subunit antisera. These values are in good agreement with

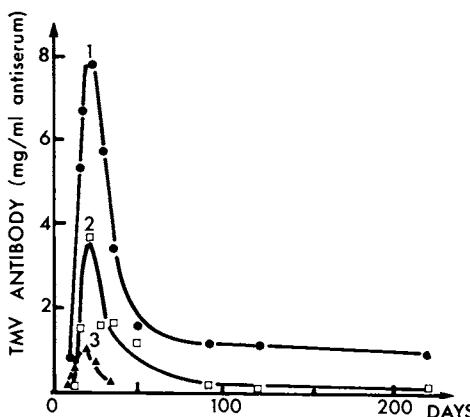


Fig. 4.1. Production of TMV antibody in rabbits immunized with TMV (curves 1 and 2 correspond to two individual rabbits) and with TMV protein (curve 3 represents average results from three rabbits). Rabbits were injected with 20 mg antigen on days 1, 3, 8, 12, and 15. No TMV antibody could be detected 1 month after the last injection of TMV protein (adapted from Loor, 1967).

earlier data of Kleczkowski (1961) who had found viral antibody levels of 1 mg/ml in TMV protein antisera and 4–6 mg/ml in TMV antisera.

The comparative immunogenicity of intact PVX and of its dissociated subunits has also been studied (Shepard and Shalla, 1970). Antisera prepared against the virus contained 3.1–3.8 mg/ml antibody reacting with the virus and 0.1–0.3 mg/ml antibody reacting with the subunits. Antisera prepared against the subunits, on the other hand, contained 1.5 mg/ml subunit antibody and 0.1 mg/ml virus antibody. These data, together with the fact that the antigenic valence of the PVX subunit was found to be 4, clearly show that the subunit antisera contain antibodies that are unable to react with the virions. The antigenic valence of 4 for the protein indicates that subunit surfaces hidden inside the capsid are able to bind antibodies, since it is impossible to accommodate so many antibody molecules on the one extremity of the molecule that is exposed in the capsid. It is clear that a reliable picture of the relative immunogenicity of dissociated subunits as compared to intact virions can only be obtained by measuring the total amount of antibody capable of reacting with all aggregation states of viral proteins.

When the immunochemical properties of viral subunits are investigated, it is important to immunize animals with protein in the monomeric state. However, concentrated preparations of viral coat protein may contain large aggregates that resemble the polymerized capsid, and this may complicate the interpretation of antigenic comparisons between virions and their subunits. Failure to control this polymerization of subunits sparked off a lengthy controversy regarding the existence of antigenic determinants specific for the monomeric state in TMV coat

protein (Starlinger, 1955; Aach, 1959; Takahashi and Gold, 1960; Kleczkowski, 1961, 1966; Van Regenmortel, 1967; Rappaport and Zaitlin, 1970; Van Regenmortel and Lelarge, 1973; Milton and Van Regenmortel, 1979). Finally, the existence of subunit-specific epitopes that are absent in the virions was clearly established.

Antisera specific for viral subunits may also be obtained by immunization with viruses that have been degraded with sodium dodecyl sulfate or other denaturing agents (Purcifull and Batchelor, 1977). When the viral subunits have been separated by polyacrylamide gel electrophoresis, it is possible to cut out the relevant bands from the gels and to immunize animals with this material after homogenization with buffer and adjuvant (Vestergaard, 1975; McMillen and Consigli, 1977; Carroll *et al.*, 1978). When the structure of the antigenic determinants of a viral protein has been elucidated, it is also possible to obtain antibodies specific for the subunit by immunizing animals with the corresponding peptide conjugated to a carrier such as serum albumin. This approach was successful with the antigenic determinants of TMV protein situated in residues 108–112 and 153–158 of the polypeptide chain (Fearney *et al.*, 1971; Anderer and Ströbel, 1972b).

2. Immunization Procedures

There is little reliable information available regarding the relative merits of different immunization procedures. Many empirical procedures give satisfactory results (Chase, 1967; Horwitz and Scharff, 1969a; Van Slogteren, 1969; Crowle, 1973; Herbert, 1978) and few workers bother to compare the effectiveness of their immunization procedure with the results produced by other methods. Admittedly, comparative trials designed to demonstrate the superiority of a particular method are likely to be laborious, mainly because the immune response measured in individual animals submitted to the same immunizing schedule can be highly variable (Wetter, 1961). Large individual differences in the reactivity of antisera from different animals (Bercks, 1963) as well as in sera taken at different stages of immunization of the same animal have been reported (Koenig and Bercks, 1968). This variability can be illustrated with the results of a study of the immune response elicited in 14 rabbits immunized with the CV4 strain of TMV (Van Regenmortel and Von Wechmar, 1970). Eight animals received a series of daily intravenous injections of 1 mg of virus over a period of 10 days, followed by two intramuscular booster injections of 5 mg of virus in Freund's incomplete adjuvant, 40 and 85 days after the start of immunization (Fig.4.2, A and B). The other six animals received four intramuscular injections of 5 mg of virus in adjuvant, the last three injections being given 15, 57, and 80 days after the first one (Fig.4.2, C and D). The development of homologous precipitin titers against CV4 and of heterologous titers against TMV (common strain) was followed by collecting antiserum from the animals at weekly intervals over a period

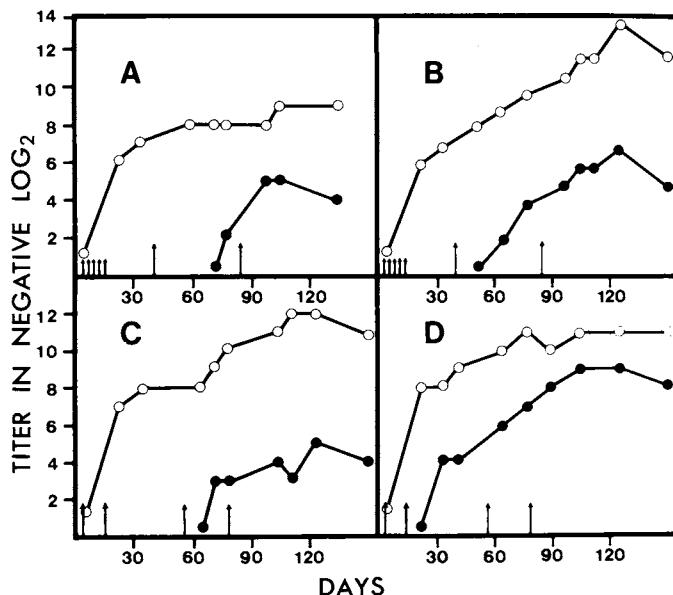


Fig. 4.2. Development of anti-CV4 homologous (○—○) and anti-TMV heterologous (●—●) precipitin titers in four rabbits immunized with the CV4 tobamovirus. Rabbits A and B received a series of intravenous injections of 1 mg of antigen (small arrows) and two intramuscular injections of 5 mg antigen in adjuvant (large arrows). Rabbits C and D received four intramuscular injections of CV4 (5mg) in adjuvant (large arrows) (Van Regenmortel and Von Wechmar, 1970).

of 5 months. It was found that the maximum homologous titers reached in animals that received the same injection schedule varied by a factor of 32; cross-reacting antibodies started to appear after 22 days in one animal, and as late as 100 days after the start of immunization in another animal. Furthermore, the number of twofold dilution steps separating homologous from heterologous titers (the so-called serological differentiation index or SDI) ranged from 1 to 7 in different rabbits (Fig. 4.2). These results clearly demonstrate that when individual animals from an outbred line of rabbits are immunized by the same procedure, they can elicit a highly variable immune response. The magnitude of these differences is such that small variations in the effectiveness of different immunization protocols are likely to be overshadowed, especially if the number of animals used for comparison is small (Van Regenmortel, 1975).

Although satisfactory antisera can be obtained by intravenous, intradermal, subcutaneous, intraperitoneal, and intramuscular injections, most workers who prepare antisera against plant viruses use either intravenous or intramuscular injections. For the latter type of injection, the antigen is emulsified in Freund's adjuvant. This type of adjuvant contains mineral oil (known under various com-

mmercial names such as Bayol F, Drakeol F) and an emulsifier such as Arlacel A (mannide monooleate) in a 9 to 1 ratio. The so-called Freund's complete adjuvant contains in addition killed mycobacteria; without the bacteria it is known as Freund's incomplete adjuvant. Both types of adjuvant are available commercially (Difco Laboratories, Detroit, Michigan), although they may also be prepared from the various ingredients (Bayol F from the Esso Company, and Arlacel from Atlas Chemical Industries, Wilmington, Delaware). The virus-adjuvant emulsion can be prepared by repeated aspirations of the mixture through a syringe (Herbert, 1978).

In the author's laboratory, the routine immunization schedule consists of a series of intramuscular injections of antigen emulsified in Freund's incomplete adjuvant, given at intervals of 2 weeks. The results of a typical immunization schedule using Freund's incomplete adjuvant is illustrated in Fig. 4.3. One of the few generalizations that can be drawn from the many idiosyncrasies displayed by the procedures reported by individual workers, is that using adjuvant saves time and effort and economizes on the amount of antigen needed (Moorhead, 1959, 1961; Wetter, 1960). There appears to be no advantage in immunizing animals by the more laborious protocol of 10–15 intravenous injections given at short intervals (Van der Veken *et al.*, 1962).

The amount of antigen administered to animals in different published procedures varies widely. Most workers use a dose of 1–10 mg of virus per injection, but there has been little systematic study of the amount of antigen required to produce an adequate antibody response. With BYDV, it was found that adequate antisera could be obtained by injecting animals with a series of three immunizing doses totalling no more than 200–350 µg virus (Rochow *et al.*, 1971; Aapola and Rochow, 1971). It seems that immunization with very large doses of virus does not lead to proportionally higher antibody levels. Most workers, to be on the safe side, tend to inject a larger dose of immunogen than is required for eliciting an adequate antibody level. Unfortunately, this practice has the disadvantage that contaminants may then be present at a level where they also will induce an immune response.

The use of inbred strains of animals for decreasing the variability in immune response has also received little attention, although there is evidence that this approach could be rewarding (Sang and Sobey, 1954; Sobey, 1954; Schuster *et al.*, 1974). On the other hand, the recently developed hybridoma method for producing homogeneous monoclonal antibodies is likely to gain widespread use in the future. This method is based on production of somatic cell hybrids between mouse myeloma cells and spleen cells derived from mice immunized with viruses (Köhler and Milstein, 1975; Gerhard *et al.*, 1978; Wiktor and Koprowski, 1978; Frankel and Gerhard, 1979). The hybrid cells can be grown on a large scale in tissue culture and will produce large quantities of identical antivirus antibody molecules. Detailed descriptions of the methodology used for producing

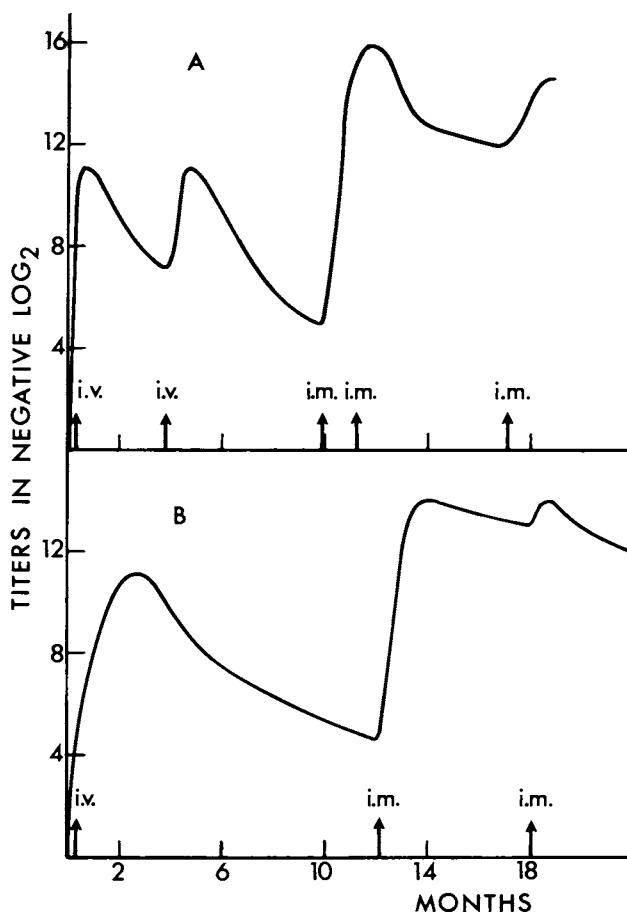


Fig. 4.3. Development of precipitin titers during immunization of rabbits with pea streak virus (A) and carnation latent virus (B). i.v., Intravenous injections; i.m. =, intramuscular injections with adjuvant (adapted from Wetter, 1960).

monoclonal antibodies are available (Köhler, 1979; Fazekas de St Groth and Scheidegger, 1980; Oi and Herzenberg, 1980).

3. Animals Used

a. Rabbits

The most commonly used animals for producing plant virus antisera are rabbits. Animals should be about 1 year old and weigh at least 3 kg. A volume of 30–50 ml of antiserum can be obtained from each animal every second week and

this is adequate for most needs. Injections are given either intravenously in a marginal ear vein, subcutaneously in an area where the skin is loose, intraperitoneally, or intramuscularly in the thigh muscle. The animals are bled by making a small incision in the marginal vein of the ear. A volume of 30 ml of blood can be drawn within 1 minute by placing the ear inside a cylindrical glass container (fitted with two outlets and held firmly against the animal's head), and applying a small negative pressure by means of a water vacuum pump.

Certain rabbit breeds have been found to be better antibody producers than others (Schuster *et al.*, 1974; Richter *et al.*, 1974), but such differences have not been studied systematically. Although quantities of at least 1 mg of antigen are usually injected each time, there is evidence that quantities of 50–100 μg may be sufficient for inducing a satisfactory immune response (Rochow *et al.*, 1971). In fact, it seems that most workers use unnecessarily large antigen doses for immunization, and, in the case of antigens that can be obtained only with difficulty, a careful study of the minimum antigen dose required for immunization may prove to be very rewarding.

b. Mice

When antigenic material is available in only very small quantities, it may be advantageous to immunize mice. Good results have been reported with mice that received an immunizing dose of virus of 2–20 μg (Marbrook and Matthews, 1966; Reddecliff and Ludwig, 1966; Francki and Habili, 1972). According to one report, as little as 0.01–0.1 μg of virus was capable of triggering a secondary immune response (Marbrook and Matthews, 1966). Adult mice weighing about 30 g can be injected intravenously in the tail (using volumes as small as 5 μl), intraperitoneally, or intramuscularly (Richter *et al.*, 1976a). Animals can be bled either from the tail, or by puncturing the retro-orbital plexus with a Pasteur pipet or by heart puncture under anesthesia. In order to prevent the blood from clotting, pipets and centrifuge tubes are treated beforehand with 2 mg/ml heparin solution (Francki and Habili, 1972). The maximum amount of blood that can be obtained from a single mouse is about 1 ml. However, by injecting mice with Ehrlich ascites tumor cells 7–14 days after the last immunizing dose of antigen, the animals develop distended abdomens from which relatively large volumes of antibody-containing fluid can be obtained. As much as 10 ml of ascitic fluid can be drained with a hypodermic needle at each successive tapping, and up to 40 ml has been collected from a single mouse (Demski and Boyle, 1969; Kiriyama and Ohsumi, 1973; Ikegami and Francki, 1974). When the Sarcoma 180/TG subline of tumor cells is used (Sartorelli *et al.*, 1966), it is possible to prolong the life of the animal and to harvest larger volumes of immune ascitic fluid.

Although it is usually stated that mice are less efficient antibody producers than rabbits, it seems that when comparisons are based on the amount of antigen

injected per kilogram of body weight, satisfactory titers of mouse antibody directed against plant viruses [e.g., TMV, CMV, PVY, BMV, southern bean mosaic virus (SBMV), cowpea chlorotic mottle virus (CCMV), MDMV, TYMV, prunus necrotic ringspot virus (PNRSV), SFDV] have been obtained by many authors (Scott *et al.*, 1969; Mink *et al.*, 1975; Moghal and Francki, 1976; Richter *et al.*, 1978b). In many cases, precipitin titers of 1:64–1:1024 have been reported.

The main advantages of using mice for immunization lie in the existence of inbred strains, which minimize genetic variations in the immune response of individual animals, and in the possibility of producing monoclonal antibody by means of hybridomas.

c. Chickens

Although chickens are convenient animals to house and are good producers of precipitating antibodies, they have been used only rarely for preparing antisera against plant viruses (Newton and Edwards, 1936; Miller and Thornberry, 1958). During the last 5 years, chickens have been used increasingly in the author's laboratory for producing antibodies against numerous plant viruses [e.g., TMV, brome mosaic virus (BMV), CCMV, TYMV, CMV, peanut stunt virus (PSV), tomato aspermy virus (TAV), TBSV, arabis mosaic virus (ArMV), grapevine fanleaf virus (GFLV)]. The animals received a series of intramuscular injections of 0.05–5 mg/ml antigen emulsified in Freund's incomplete adjuvant. Although adjuvants appear to be of little use for increasing the levels of circulating antibody in the chicken, they do allow a peak level to be maintained in the animal for a longer time (Steinberg *et al.*, 1970; French *et al.*, 1970).

The use of avian antibodies is particularly valuable in double antibody sandwich methods, which require the use of viral antibody produced in two animal species (Van Regenmortel and Burckard, 1980; Bar-Joseph and Malkinson, 1980) (see also Chapter 6, Section F,1). Since chicken antibodies do not cross-react serologically with mammalian immunoglobulins (Leslie and Clem, 1969; Esteves and Binaghi, 1972), any binding between the antivirus chicken antibody and the goat anti-rabbit globulin antibody used in indirect immunoassays is excluded (Van Regenmortel *et al.*, 1980). Another advantage of using chicken antibody is that serological reactions can be performed at high salt concentration (1.5 M NaCl) under ionic conditions that cannot be used with mammalian antibody (Benedict *et al.*, 1963; Kubo and Benedict, 1969; Benedict and Yamaga, 1976).

When laying hens are used for immunization, viral antibody can be obtained very easily from the egg yolks by precipitation with polyethylene glycol (Polson *et al.*, 1980). The passage of antibodies from the serum of hens to egg yolk is a well-known phenomenon that has been studied for many years (Brambell, 1970).

In common with the colostra of mammals, the yolks provide a supply of antibodies that confer passive immunity to the newborn chicken. Considerable quantities of immunoglobulin are present in egg yolk (Rose *et al.*, 1974), and the antibody levels in yolks are similar to those found in hen's serum (Patterson *et al.*, 1962). Some of the advantages of obtaining antibodies from eggs are (1) the fact that bleeding the animal is no longer necessary; (2) the ease of preparation of pure immunoglobulin; (3) the rapid development of suitable antibody levels in the immunized hen (Bar-Joseph and Malkinson, 1980); (4) the considerable quantities of immunoglobulin that can be obtained in a short time (Polson *et al.*, 1980).

Procedure for Purifying Immunoglobulins from the Yolks of Immunized Laying Hens. Eggs are collected daily, starting 8 days after the beginning of immunization. Immunoglobulins are extracted from lots of 10 eggs at a time. Individual yolks are separated from the albumen and washed in a jet of distilled water. Intact yolks are dropped in a large glass funnel supported on a measuring cylinder. The total volume of yolk is measured, and a volume of 0.01 M phosphate buffer pH 7.5 (containing 0.1 M NaCl and 0.01% NaN₃) equivalent to two volumes of yolk is added. Polyethylene glycol (PEG), which has been pulverized in a Waring Blender, is added to a final concentration of 3.5% by weight of polymer to volume of diluted yolk. The mixture is stirred until all the polymer is dissolved, and is then centrifuged at 10,000 rpm for 10 minutes. The supernatant is decanted into a funnel fitted with absorbent cotton wool in order to filter the yellow lipid layer. The volume of filtrate is measured and more pulverized PEG is added to bring the concentration to 12 g/100 ml. After low-speed centrifugation, the sediment is resuspended in a volume of phosphate buffer equal to that of the original diluted yolk. After a second precipitation with 12% PEG, the final pellets are compacted by a second prolonged centrifugation, and the exuded PEG solution is removed by suction. The sediment of purified immunoglobulin is dissolved in a volume of buffer equal to about one-third of the original volume of yolk. When examined in the analytical ultracentrifuge, the immunoglobulin appears homogeneous with a sedimentation coefficient of 7 S. IgM and IgA antibodies are not found in egg yolk (Rose *et al.*, 1974). From results obtained with more than 1000 eggs, the average yield of purified immunoglobulin obtained by the above procedure was 75 mg per egg. In one experiment where TMV antibody was prepared from egg immunoglobulins by the method of Hardie and Van Regenmortel (1977), the average yield of purified antibody was found to be 10 mg/egg.

d. Other Animals

When very large quantities of antiserum are needed, it may be expedient to immunize large animals such as goats, sheep, or horses (Anderer *et al.*, 1971a).

Doses of 1-10 mg/ml of immunogen have been found to give adequate responses. Plant virus antisera obtained from goats and horses have been used, for instance, in the serodiagnosis of potato virus diseases (Van Slogteren and Van Slogteren, 1957; Van der Veken, 1958; Shepard *et al.*, 1971). Other animals that have been used for antiserum production are guinea pigs (Chester, 1936; Miller and Thornberry, 1958) and frogs (Langenberg and Middleton, 1969). The use of frogs could be beneficial with antigens that are not stable at the temperature of warm-blooded animals.

4. Storage of Antisera

Blood obtained from immunized animals is allowed to clot overnight at room temperature, and the serum is carefully separated from the clot. If it is important not to waste any antiserum, a significant amount of antibodies can be extracted from blood clots (Waterworth, 1976). Since the specificity of antibodies obtained during successive bleedings of the same animal can vary considerably (Bercks, 1963; Tremaine and Wright, 1967; Allen, 1968; Koenig and Bercks, 1968; Van Regenmortel and Von Wechmar, 1970; Kassanis and Phillips, 1970) it is inadvisable to indiscriminately pool the serum from successive bleedings.

The simplest way of storing antisera is to keep them frozen at -20°C in small bottles. Some TMV antisera prepared by W. M. Stanley in the late 1930s and kept frozen since then were found by the author to have a precipitin titer of 1/512 more than 40 years later.

Waterworth *et al.* (1973) who studied the effects of various storage conditions on the activity of plant virus antisera, reported that titers were decreased after prolonged storage at 37°C but remained unchanged after storage at -70°C, -20°C, +4°C, and +26°C. Repeated freezing and thawing appeared to have no detrimental effect. Freeze-drying was found to be more effective than the addition of 50% glycerol or 0.02% sodium azide for preserving antisera at 37°C.

When antiserum is stored (at 4°C) in an equal volume of glycerol, it may be preferable for some tests to remove the glycerol by dialysis prior to use. For ring precipitin tests, the presence of glycerol is advantageous since it effectively prevents mixing when the antigen preparation is layered above the antiserum. Another simple method of preservation consists of absorbing the serum on filter paper, and storing this at room temperature in a desiccator. The serum from a 4 × 4-mm piece of paper is easily reconstituted with a drop of saline (Stapp and Bercks, 1948; Bercks, 1956). Since the presence of mercury-containing preservatives in the gel can have a detrimental effect on the formation of precipitin lines in immunodiffusion tests (Koenig, 1970), it is preferable to use sodium azide rather than merthiolate for the prevention of microbial contamination.

B. PURIFICATION OF IMMUNOGLOBULINS

For many serological tests (see Chapter 6) it is advantageous to use purified immunoglobulins instead of whole antiserum. Three simple methods for purifying immunoglobulins will be briefly described. For a detailed account of the various procedures, the reader is referred to Hudson and Hay (1980), Garvey *et al.* (1977), Nezlin (1977), Heide and Schwick (1978), and Fahey and Terry (1978).

1. Ammonium Sulfate Precipitation

Ammonium sulfate precipitation is the most commonly used procedure for preparing a crude immunoglobulin fraction from whole serum. By adjusting the salt concentration to 1/3-1/2 saturation, the globulins are precipitated, whereas the albumin and many other serum proteins will remain in solution. A high yield of IgG may be obtained by the following procedure performed at room temperature. To one volume of serum, add dropwise with constant stirring one volume of 4 M ammonium sulfate solution and adjust to pH 7.8 with 1 N NaOH. After 1 hour the suspension is centrifuged at low speed, and the precipitate is dissolved in half the volume of original serum. Further purification may be achieved by a second and third precipitation. After dissolving the final precipitate, the suspension is dialyzed against phosphate-buffered saline pH 7.8. After dialysis, the suspension is centrifuged to remove small amounts of insoluble material. The purity of the IgG will be improved if a less concentrated (3.0 M) salt solution is used for precipitation, but the yield will be lower.

2. Rivanol Precipitation

Rivanol (2-ethoxy-6,9-diaminoacridine lactate) is the soluble salt of an acridine base that is used for precipitating albumin and other serum proteins, while leaving the IgG in solution. The Rivanol itself is removed from the IgG suspension by conversion to the insoluble bromide form, and the IgG is then precipitated with ammonium sulfate. A product of very high purity is obtained (Hardie and Van Regenmortel, 1977).

The pH of the serum is adjusted to 8.0, using 0.1 N NaOH, and the total volume measured. For each milliliter of alkaline serum, 3.5 ml of 0.4% aqueous Rivanol (I.C.N. Pharmaceuticals, Plainview, New York) is added slowly with stirring. The preparation is centrifuged and the dense yellow precipitate is re-extracted with a small volume of water. This leads to an improved yield of IgG. To the combined supernatants, saturated aqueous potassium bromide is added dropwise until the suspension appears clear (Rivanol bromide forms a yellow

precipitate). The preparation is centrifuged at 15,000 rpm for 30 minutes and the supernatant is filtered to remove all traces of precipitate. The IgG is then precipitated with ammonium sulfate as described above.

3. Purification by Ion-Exchange Chromatography

In this method, an ion-exchanger such as DEAE-cellulose is equilibrated under conditions of pH and ionic strength where all serum proteins are bound except IgG. The separation may be done batchwise using 5 g wet DEAE-cellulose (in 0.01 *M* phosphate buffer, pH 8.0) per milliliter of serum (Reif, 1969). The mixture of cellulose and serum is stirred for 1 hour and the IgG is recovered from the supernatant after low-speed centrifugation.

A product of higher purity can be obtained by a column procedure using a gradient of increasing molarity for elution. The serum is applied to a 25 × 2.5 cm column that has been packed with DEAE-cellulose or DEAE-Sephadex in 0.01 *M* phosphate buffer pH 8.0. The same buffer is used to elute a first IgG fraction from the column. When a phosphate buffer of increasing molarity (up to 0.2 *M*) is passed through the column, additional IgG fractions are eluted. It has been shown by Tremaine and Chidlow (1974) that the proportion of antibodies specific for whole virions and dissociated subunits of alfalfa mosaic virus (AMV) and turnip crinkle virus (TCV) is not the same in the different IgG fractions obtained from such columns.

4. Separation of IgM and IgG

Few workers have attempted to fractionate plant virus antisera into different antibody populations possessing separate specificities (Moed and Veldstra, 1968; Tremaine and Wright, 1967; Augier de Montgremier, 1970). In order to compare the specificity of IgG (7 S) and IgM (19 S) antibodies, these two immunoglobulins have been separated by density gradient centrifugation (Allen, 1968) and gel filtration on Sephadex G-200 (Bercks *et al.*, 1974).

The 19 S and 7 S components of antiserum can be separated on 0–40% sucrose gradients in 19-ml tubes of a Beckman SW 27-1 rotor by centrifugation at 27,000 rpm for 26 hours. A volume of 0.1 ml of serum is placed on each gradient, and fractions are collected by upward displacement of the contents of the tubes by a 40% sucrose solution. Larger quantities of material can be separated on a 100 × 4.5-cm Sephadex G-200 column equilibrated with 0.05 phosphate buffer pH 7.5. Total immunoglobulins obtained by ammonium sulfate precipitation from 10 ml serum can be placed on such a column; with a flow rate of 25 ml/hour, the IgM will be eluted first after about 24 hours.

C. PURIFICATION OF ANTIBODY

All the methods used for purifying specific antibody are based on the same principle, namely, antibody is first allowed to combine with the virus, and after separating the complex from the nonreactive globulins, the antibody is dissociated from the virus at acid pH (Chester, 1936; McLaughlin *et al.*, 1980). Usually the pH has to be adjusted to 2.0 in order to achieve maximum recovery of the viral antibody (Rappaport, 1961a; Szpirer and Jeener, 1966). There is evidence, however, that a considerable loss of active antibody occurs when the IgG is submitted to such a low pH (Steward and Stanworth, 1975) and that the resulting preparations usually contain significant amounts of partially or completely denatured antibody. Furthermore, the antibodies that are extracted in a particular pH range (e.g., 2.5–3.0) are not characteristic of that pH and will not redissociate reproducibly after binding a second time to antigen (Szpirer and Jeener, 1966). These shortcomings prompted an investigation of several procedures for dissociating antibody from the virus and led to the development of an improved method in which the reactants are kept under conditions of very low ionic strength (Hardie and Van Regenmortel, 1977). In the case of some viruses that are unstable in water, it may be necessary to add small amounts of divalent cations in order to prevent particle breakdown during the experiment.

Specific IgG purified from antiserum by Rivanol precipitation is dialyzed against distilled water for 72 hours and the pH adjusted to 7.8 with 0.1 N NaOH. After centrifugation to remove the small amount of globulins that are insoluble in water, the IgG is added either to a purified virus preparation or to a virus–BSA mixed insoluble polymer. This type of polymer can be prepared by the method of Avraméas and Ternynck (1969). Combination of virus and antibody is carried out at pH 7.8, and after centrifugation of the complex, the nonreactive globulin present in the supernatant is discarded. The antigen–antibody complex is resuspended in water, brought to pH 2.9 with 0.1 N HCl, and the virus is again centrifuged immediately. The antibody–polymer complex can be sedimented at low speed within 10 minutes. This reduces the time during which the antibody is in acid solution from 60 to 15 minutes. The supernatant after the second centrifugation contains the antibody, and must be neutralized immediately to pH 7.5 with 0.1 N NaOH.

The recovery of TMV antibody from several IgG preparations by dissociation at various pH values is illustrated in Fig. 4.4. In all cases, the antibody recovery curves show a clear maximum of nearly 100% recovery at pH 2.9.

TMV antibody fractions have also been prepared by a series of successive dissociations at increasingly acid pH. After partial dissociation of the antibody at a particular pH value, the virus and residual combined antibody were centrifuged. The pellet was resuspended in water and the pH adjusted to a lower value. After centrifugation, this procedure was repeated several times. The re-

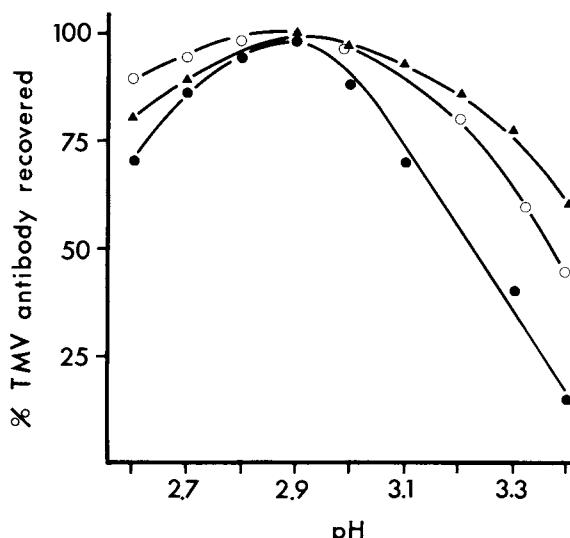


Fig. 4.4. Recovery of TMV antibody by dissociation from the virus-antibody complex at various pH values. The three curves correspond to three different rabbit IgG preparations (Hardie and Van Regenmortel, 1977).

sults presented in Table 4.1 show that the amount of antibody recovery within a pH range was highly reproducible. Moreover, if a certain antibody fraction was again recombined with antigen at pH 7.8 and then subjected to extraction at varying pH values, it was found to dissociate a second time at exactly the same pH as previously. When the relative avidity of the different antibody fractions dissociated at pH 3.3, 3.1, and 2.9 was measured in binding tests (see Chapter 5), it was found that antibodies of increasing avidity were obtained as the pH became more acid. This method may thus prove useful for the isolation of antibodies of different avidities.

Because of the ease by which virus-antibody complexes can be separated from nonspecific globulins by centrifugation, there have been few attempts to prepare specific viral antibody by means of classical immunoabsorbent columns (Parikh and Cuatrecasas, 1977; Garvey *et al.*, 1977; Nezlin, 1977). An approach of this kind, whereby the antigen is coupled to a solid matrix-like activated Sepharose could be useful, however, for isolating antibodies specific for viral subunits.

Preparation of IgG Fragments

When IgG is digested with papain at pH 6.5 under reducing conditions, monovalent Fab fragments that can be separated from the Fc fragments by CM cellulose chromatography are produced (Putnam *et al.*, 1962). If Fab with spe-

TABLE 4.1

Percentage of TMV Antibody Recovered in Successive Dissociation Steps of Increasing Acidity^a

Number of successive extractions	pH of dissociation				Total percentage of recovered antibody
	3.5	3.3	3.1	2.9	
1	—	—	—	97.6	97.6
2	—	—	77.6	19.0	96.6
3	—	57.4	19.3	19.4	96.1
4	33.6	24.0	19.5	19.9	97.0
<hr/>					
1	—	—	77.9	—	77.9
2	—	58.0	20.1	—	78.1
3	32.9	24.7	19.8	—	77.4
<hr/>					
1	—	58.3	—	—	58.3
2	33.1	24.8	—	—	57.9
<hr/>					
1	32.8				32.8

^a From Hardie and Van Regenmortel, 1977.

cific antiviral activity is required, it is preferable to first prepare Fab fragments from the total IgG fraction of an antiserum, before removing the fragments devoid of antibody activity as described in Section C (Hardie and Van Regenmortel, 1975).

When IgG is digested with pepsin at pH 4.5 in the presence of reagents that break disulfide bonds, univalent Fab' fragments are obtained that possess an additional subfragment of Fc compared with Fab. Papain and pepsin split the two heavy chains of an IgG molecule on opposite sides of the one disulfide bond that keeps the two halves of the molecule together (Fig. 2.1).

The procedure for preparing Fab' fragments is as follows (Nisonoff *et al.*, 1960). IgG (10 mg/ml) suspended in a pH 4.0 buffer containing 0.2 M sodium acetate, 0.01 M cysteine, and 0.01 M sodium azide is incubated with pepsin (1 mg enzyme per 100 mg IgG) at 37°C for 24 hours. The hydrolysis is stopped by adjusting the pH to 8.0 with 5 M NaOH and iodoacetamide is added to a final concentration of 0.06 M. After overnight dialysis at 4°C against 0.05 M phosphate-borate buffer pH 8.0, the digest is fractionated on a 40 × 1-cm Sephadex G-75 column equilibrated with the same buffer. The first eluted fraction corresponds to the Fab' fragments.

Antigen–Antibody Interaction

A. FORCES INVOLVED IN ANTIGEN–ANTIBODY INTERACTION

The noncovalent intermolecular forces that hold together antibody combining sites (or paratopes) and antigenic determinants (or epitopes) are the same as those involved in the stabilization and specific configuration of proteins. They become operative only if the paratope and epitope are able to make close contact, and the closer the contact the stronger will be the antigen–antibody bond. The degree of structural complementarity found in an epitope-paratope pair determines the combining “quality” or strength of the antibody, which is expressed quantitatively as the antibody affinity. The affinity of antibody molecules can be calculated by applying the concepts of chemical equilibria to the antigen–antibody interaction, and may be expressed either as the equilibrium constant K or as the standard free energy change ΔF . The change of free energy when 1 mole of paratope interacts with 1 mole of epitope is

$$\Delta F = - RT \ln K$$

where R is the gas constant and T the absolute temperature. The free energy

change associated with binding is composed of the two thermodynamic components, enthalpy (heat content) and entropy (order):

$$\Delta F = \Delta H - T\Delta S$$

where ΔH is the change in enthalpy and ΔS is the entropy change. Either or both of these components can be the driving force behind the formation of the complex. The contribution of ΔH is determined experimentally by measuring K and ΔF at different temperatures from

$$\Delta H = \frac{RT_1 T_2 \ln K_2 / K_1}{T_2 - T_1}$$

where K_1 and K_2 are the equilibrium constants at temperatures T_1 and T_2 . When ΔH is 0, the affinity is independent of temperature and the driving force for binding is ΔS . Such an entropy increase implies that hydrophobic bonding is the main factor responsible for the binding of antibody. On the other hand, ΔH values of -3 to -7 cal/mole would suggest the participation of hydrogen bonds or electrostatic interactions in the binding reaction (Kabat, 1976).

B. METHODS USED FOR MEASURING ANTIBODY AFFINITY

In order to calculate the equilibrium constant K , it is necessary to separate either the free epitopes or the free paratopes from the epitope-paratope complexes present at equilibrium (Steward, 1978). In the case of small haptens which diffuse through membranes that retain antibody, the bound hapten on one side of a dialysis membrane will be easily separated from free hapten. This method of equilibrium dialysis is the standard procedure used in immunochemistry for measuring antibody affinity. In the case of antigens of the size of viruses, it is the free antibody that will be more easily separated from the bound complexes. This can be done by sedimenting the virus and the complexes by ultracentrifugation and collecting the free antibody from the supernatant (Fazekas de St Groth, 1979), or by filtration of the equilibrium mixture through a sieve that will retain the large antigen and the complexes (Fazekas de St Groth, 1961, 1979; Fazekas de St Groth and Webster, 1961).

Although the virus-antibody reaction is reversible in principle, attempts at measuring the very low rate of dissociation have shown that the reaction can be regarded as irreversible for practical purposes (Bradish and Crawford, 1960; Rappaport, 1965; Krummel and Uhr, 1969).

The experimental design used for determining the affinity of TMV antibodies will be briefly outlined (Van Regenmortel and Hardie, 1976). Purified antibody was prepared by ultracentrifugation of a mixture of IgG and TMV in water at pH

TABLE 5.1

Binding Test between TMV and IgG^a

A	$d(OD)$	d	y	$f = y/A$	$f/d \times 10^{-9}$	$K_0 \times 10^{-7}$ for $n = 1$	$K_0 \times 10^{-7}$ for $n = 2$	s_r for $n = 1$	s_c for $n = 1$	$r/c \times 10^{-4}$ for $n = 1$	s_c for $n = 2$	$r/c \times 10^{-4}$ for $n = 2$
0.143	0.001	0.0050	39.995	280	48.60	10.56	24.00	0.999	71.54	1.396	31.55	6.333
0.132	0.002	0.0072	39.991	303	42.98	8.81	24.18	0.999	62.96	1.587	22.974	8.697
0.126	0.002	0.0093	39.991	318	34.19	7.47	23.95	0.999	58.28	1.714	18.298	10.919
0.118	0.003	0.0140	39.986	339	24.21	5.49	23.75	0.999	52.05	1.919	12.068	16.556
0.111	0.004	0.0158	39.984	360	22.80	5.43	38.00	0.999	46.59	2.144	6.612	30.218
0.099	0.005	0.0233	39.977	404	17.34	4.61		0.999	37.24	2.683		
0.096	0.009	0.0420	39.958	416	9.905	2.72		0.999	34.92	2.861		
0.091	0.011	0.0513	39.949	440	8.576	2.48		0.999	31.04	3.218		
0.083	0.015	0.0687	39.931	481	7.001	2.34		0.999	24.81	4.027		
0.074	0.021	0.0979	39.921	540	5.516	2.26		0.998	17.82	5.600		
0.066	0.034	0.1580	39.842	604	3.822	2.22		0.996	11.64	8.557		
0.062	0.047	0.2181	39.782	642	2.944	2.19		0.995	8.58	11.597		
0.060	0.061	0.2833	39.717	662	2.336	2.00		0.993	7.08	14.025		
0.0565	0.094	0.4375	39.563	700	1.600	2.00		0.989	4.51	21.929		
0.055	0.126	0.5893	39.411	717	1.220	2.01		0.985	3.49	28.223		
0.052	0.227	1.0582	38.942	749	0.709	2.01		0.974	1.62	60.12		

^a A, TMV concentration (moles/liter $\times 10^6$); d(OD), free IgG concentration in OD units at 280 nm; d, free IgG concentration (moles/liter $\times 10^6$); y, bound IgG concentration (moles/liter $\times 10^6$); for $n = 1$: $K_0 = f/d$ (780 - f); for $n = 2$: $K_0 = f/d$ (780 - 2f); for $n = 1$: $r = y/B_s$ and $c = A - x = 780 - A - y$ free TMV sites (moles/liter $\times 10_6$); for $n = 2$: $r = 2y/B_s$ and $c = 780 - A - 2y$. The concentration of IgG used in this experiment was $B_s = 6$ mg/ml $= 40.0 \times 10^{-6}$ moles/liter. Note that if bivalent binding of antibody is assumed to occur, no K values can be calculated for f values higher than 390, since in that case $2f > 780$ and the number of sites bound ($2y$) would become larger than the total number of sites available on the virus particle. It is clear, therefore, that in large antibody excess, the IgG molecules are bound univalently (see Fig. 5.1A for a plot of the data presented in this table).

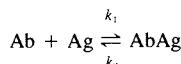
8.0, followed by dissociation of the antibody present in the pellet at pH 2.9 (Hardie and Van Regenmortel, 1977). The virus was again sedimented and specific antibody was recovered from the supernatant, which was adjusted to pH 8.

Binding tests were performed by mixing 1 ml of a series of dilutions of TMV with 1 ml of a constant concentration of specific antibody. After ultracentrifugation, the amount of free antibody present in the supernatant was determined from absorbance readings using an extinction coefficient for the antibody of $E_{280 \text{ nm}}^{0.1\%} = 1.43$. The amount of bound antibody was calculated by subtracting free from total antibody. All subsequent calculations necessary for computing K values are illustrated in Table 5.1.

C. THE LAW OF MASS ACTION

1. Graphical Representations of the Mass Action Equation

The interaction between antibody and antigen at equilibrium may be expressed as



where Ab represents free antibody, Ag free antigen, and AbAg the antibody-antigen complex. According to the law of mass action, the rate of formation of complex is proportional to the concentration of reactants. At equilibrium, the rates of association and dissociation are equal:

$$k_1[\text{Ab}][\text{Ag}] = k_2[\text{AbAg}]$$

and the equilibrium constant K may be calculated from

$$K = \frac{k_1}{k_2} = \frac{[\text{AbAg}]}{[\text{Ab}][\text{Ag}]}$$

Antibody affinity, which is expressed as K , will thus have the dimension of liters/mole. Affinity is a thermodynamic expression of the primary interaction of a single paratope with an epitope, and strictly speaking it applies only to a monovalent hapten combining with homogeneous antibody molecules. With multivalent and heterogeneous systems, the binding capacity of antibody molecules can only be expressed in terms of average values that are referred to as "avidity." Since proteins and viruses are antigenically multivalent, it is essential to include a valency term in all the equations used for avidity calculations. The

following symbols will be used: A = total antigen concentration (moles/liter); s = antigen valence; As = total antigen sites (mole sites/liter); B = total antibody concentration (moles/liter); n = antibody valence; Bn = total antibody sites (mole sites/liter); y = bound antibody concentration (mole/liter); x = bound antigen concentration (mole/liter); ny = sx = total bound sites; r = x/B = ny/sB : ratio of bound antigen to total antibody; f = y/A = sx/nA : ratio of bound antibody to total antigen; c = $A - x$: free antigen concentration; d = $B - y$: free antibody concentration. Using these symbols the equilibrium constant may be expressed as

$$K = \frac{ny}{(As - ny)(Bn - ny)} \quad (1)$$

or

$$K = \frac{sx}{(As - sx)(Bn - sx)} \quad (2)$$

These expressions are valid in the absence of interactions between epitopes, for it can be shown that the behavior of epitopes is the same, irrespective of whether they occur singly or as part of the surface of complex antigens (Day, 1972; Fazekas de St Groth, 1979).

Equations (1) and (2) are usually rearranged into forms suitable for the graphical representation of binding data. When Eq. (1) is divided by A , one obtains

$$K = \frac{y/A}{(s - ny/A)(B - y)} = \frac{f}{(s - nf)d}$$

and

$$\frac{f}{d} = K(s - nf) \quad (3)$$

This transformation of the mass action equation allows the construction of f/d versus f plots, usually referred to as Scatchard plots from which values of K (as the slope) and of the antigen valence can be derived (Rappaport, 1959; Day, 1972). It is clear that when $f/d \rightarrow 0$, then $s - nf \rightarrow 0$, and $nf \rightarrow s$.

Two such f/d versus f plots representing binding data between TMV and specific IgG and Fab, respectively, are shown in Fig. 5.1. The plots obtained with different concentrations of univalent Fab extrapolate to $s = 800$, which is the effective antigenic valence of the virus (Hardie and Van Regenmortel, 1975). With increasing Fab concentration, the curvature of the plots becomes less marked, until at a sufficiently high Fab concentration, a linear plot is obtained.

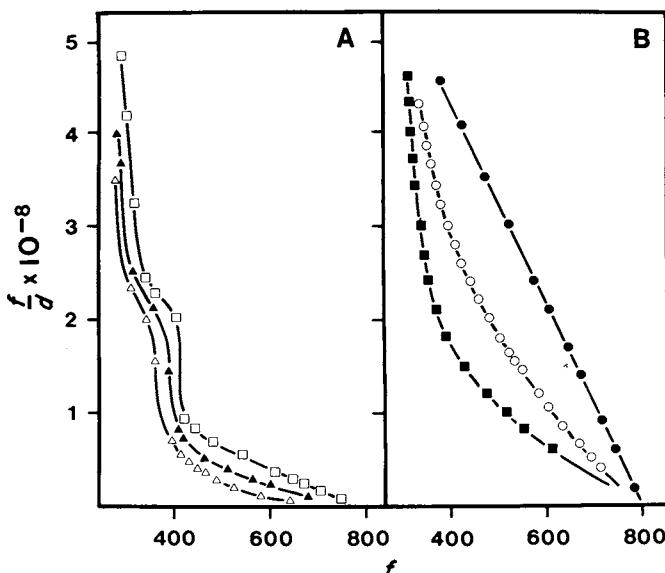


Fig. 5.1. (A) Plots of f/d versus f representing the interaction between TMV and specific IgG. □, 6 mg/ml IgG; ▲, 1.8 mg/ml IgG; △, 0.6 mg/ml IgG. The curves extrapolate to the antigenic valence of TMV, $s = 780$ (from Van Regenmortel and Hardie, 1976). (B) Plots of f/d versus f representing the interaction between TMV and specific Fab. ●, 1.9 mg/ml Fab; ○, 1.18 mg/ml Fab; ■, 0.6 mg/ml Fab. The antigenic valence of TMV is $s = 800$ (from Hardie and Van Regenmortel, 1975); see Table 5.1 for details of calculations.

The different K values corresponding to the variable slopes of these curves are presented in Fig. 5.2. In the case of curves C and D in Fig. 5.2, it is apparent that high-affinity antibodies seem to bind preferentially when the ratio of antibody to virus is lowered. On the other hand, when the antibody concentration reaches a high enough level (curves A and B), it seems that a sufficient number of high-affinity antibodies are present to bind all antigen sites preferentially. This situation then results in the appearance of homogeneous binding.

The plots obtained with IgG are biphasic and extrapolate to $s = 780$. The similarity in s values obtained with Fab and IgG plots indicates that in extreme antibody excess, the IgG molecules bind univalently. However, the biphasic nature of the curves suggests that bivalent binding of IgG molecules may occur at lower antibody-antigen ratios. This interpretation is supported by the data presented in Fig. 5.3, which show that small successive additions of antibody to a given amount of TMV result in the binding of fewer antibody molecules per virus particle than a single addition of the same total amount of antibody. The addition of a small amount of antibody to the virus preparation favors bivalent binding and

this doubles the number of antigenic sites covered without increasing the number of antibody molecules bound. In this case the antigen valence is obtained by multiplying the limiting value of f by 2, which again leads to a value of $s = 800$ (Figs. 5.1A and 5.3).

The flexibility of the two Fab arms makes it possible for antibody molecules to have both binding sites attached to neighboring epitopes on the surface of a virus particle (Lafferty and Oertelis, 1963; Almeida *et al.*, 1963; Crothers and Metzger, 1972; Karush, 1978). This type of binding is usually called monogamous bivalent binding, and has also been described as representing incestuous combinations (Fazekas de St Groth, 1979). The standard free energy change when such a complex is formed (Fig. 5.4) is much greater than when the antibody molecule binds by only one site (Greenbury *et al.*, 1965; Klinman *et al.*, 1967; Hornick and Karush, 1969, 1972; Blank *et al.*, 1972; Gopalakrishnan and Karush, 1974). This has led Day (1972) to suggest that monogamous bivalent binding is prevalent with all viruses, also under conditions of extreme antibody excess, and that in calculations of antigen valence based on antibody-antigen ratios, it is, therefore, necessary to assume $n = 2$. It seems, however, that the amount of monogamous binding varies with the relative concentration of the reactants and that no simple generalization regarding the effective antibody valence can be made (Van Regenmortel and Hardie, 1976).

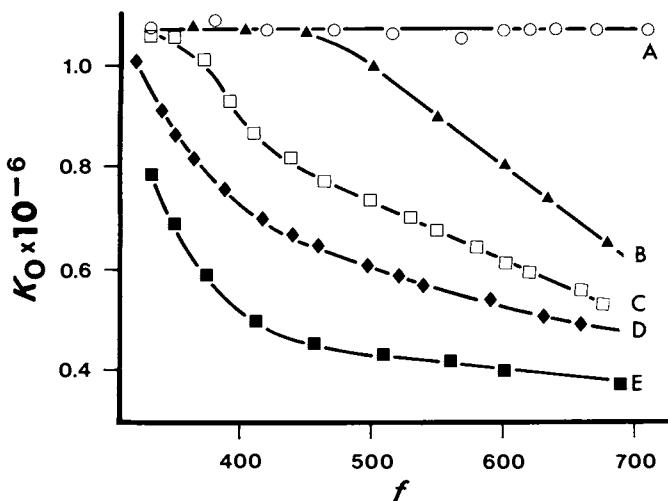


Fig. 5.2. Variation in avidity (K) of anti-TMV Fab fragments when measured at different antibody concentrations and at different f values. \circ , 1.9 mg/ml Fab; \blacktriangle , 1.6 mg/ml Fab; \square , 1.3 mg/ml Fab; \blacklozenge , 1.2 mg/ml Fab; \blacksquare , 0.9 mg/ml Fab. At the highest antibody concentration used, K is constant; this corresponds to the straight line in Fig. 5.1B.

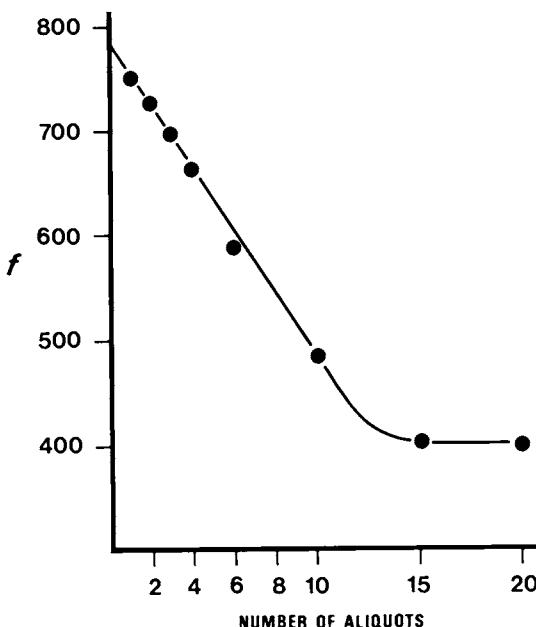


Fig. 5.3. Variation in f observed when the same total quantity of specific IgG (3.6 mg) is added to 1 mg TMV but in different aliquots. With small successive additions of IgG, the plot extrapolates to $f = 400$. Assuming monogamous bivalent binding of IgG, this corresponds to an antigenic valence of TMV of $s = 800$.

Another common transformation of the mass action equation is obtained when Eq. (2) is divided by B . This leads to

$$K = \frac{x/B}{(A - x)(n - sx/B)} = \frac{r}{c(n - sr)}$$

and

$$\frac{r}{c} = K(n - sr) \quad (4)$$

and allows the construction of Scatchard plots of the type r/c versus sr . In this case, when $r/c \rightarrow 0$, $n - sr \rightarrow 0$ and $sr \rightarrow n$.

By analogy with the f/d versus f plots, which allow the antigen valence to be determined, it would seem reasonable to expect that r/c versus sr plots will permit the effective antibody valence to be estimated. This is not the case with multivalent antigens, however, since the quantity x , cannot be obtained experimentally in such systems (the surface of virions may be only partly in the bound

state). The values of r are not obtained from experimental determinations of x/B but from ny/sB calculations derived from measurements of d (since $y = B - d$). It is thus necessary to assume a value for n in order to calculate r , and it is of course this value that will be found by extrapolation of the r/c versus sr plots.

Typical r/c versus r plots representing binding data between TMV and specific Fab or IgG, respectively, are shown in Fig. 5.5. It is remarkable that all the plots are linear (except in the immediate vicinity of $r = 1$), since linearity in this type of Scatchard plot is widely believed to imply homogeneous binding. In fact several authors (Mamet-Bratley, 1966; Anderer *et al.*, 1971b; Urbain *et al.*, 1972; Day, 1972) have concluded from the appearance of such linear plots that TMV antibodies are indeed homogeneous. That such a conclusion is invalid, is shown by the fact that the linear portions of r/c versus r plots can be transformed into smooth curves by using the f/d versus f representation (Hardie and Van Regenmortel, 1975; Van Regenmortel and Hardie, 1976). This phenomenon is illustrated in Figs. 5.1 and 5.5 since the same experimental data were used for plotting the IgG graphs of Figs. 5.1A and 5.5B and the Fab graphs of Figs. 5.1B and 5.5A, respectively. At the highest antibody concentrations used in these tests, plots of f/d versus f do become linear, and the corresponding slopes then agree with the maximum slopes observed in the r/c versus r representations of the same data. Under these conditions, it is possible that a sufficient number of high-affinity antibodies is present to bind all the antigenic sites preferentially.

When the antibody preparation used in the binding tests is partly denatured and contains nonfunctional antibody molecules, r/c versus r plots do not extrapolate to $n = 1$ or $n = 2$ but to lower values. This was observed in the TMV system by Mamet-Bratley (1966) and Anderer *et al.* (1971b). In such a case, it is possible to correct the data by calculating the concentration of active antibody B_{corr} from the relation

$$B_{\text{corr}} = B_{\text{total}} - B_{\text{total}}(n_1 - n)$$

where n_1 is the extrapolated value found in the r/c versus r plot, and n is the theoretical value (1 or 2). Once this corrected value of the concentration of active antibody has been obtained, it can be used for recalculating d , y , f , r , and K .

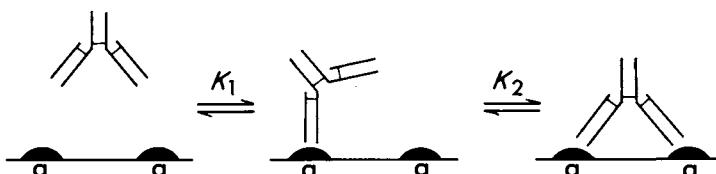


Fig. 5.4. Binding of an IgG molecule to the surface of an antigen presenting a series of repeating identical epitopes a . The value of K_1 for the single site attachment is much lower than that of K_2 for monogamous bivalent binding of IgG (see De Lisi, 1976).

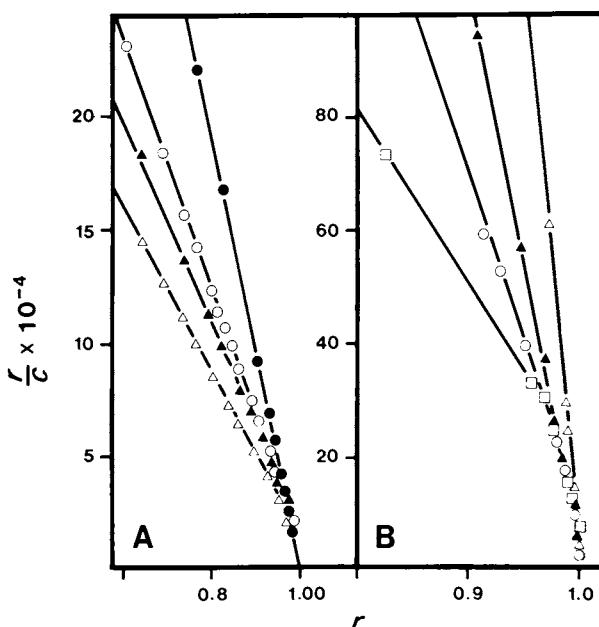


Fig. 5.5. (A) Plots of r/c versus r using the same TMV-Fab binding data represented as f/d versus f plots in Fig. 5.1B. A value of $s = 800$ was used for calculation of c . ●, 1.9 mg/ml Fab; ○, 1.3 mg/ml Fab; ▲, 1.18 mg/ml Fab; △, 0.9 mg/ml Fab. The plot obtained with 1.9 mg/ml Fab is linear and has the same slope as the straight line in Fig. 5.1B. All plots extrapolate to $r = 1$, indicating the absence of any denatured, nonfunctional molecules in the Fab preparation. (B) Plots of r/c versus r using the same TMV-IgG binding data represented as f/d versus f plots in Fig. 5.1A. A value of $s = 780$ was used for the calculation of c . △, 6.0 mg/ml IgG; ▲, 1.8 mg/ml IgG; ○, 1.2 mg/ml IgG; □, 0.6 mg/ml IgG; see Table 5.1 for details of calculations.

When the data of Anderer *et al.* (1971b) were submitted to this procedure, the f/d versus f plot of the original data, which was nonlinear and had a positive slope (Fig. 5.6A) was transformed into a linear plot that yielded an antigenic valence for TMV of about $s = 750$ (Fig. 5.6B).

In their calculations, Anderer *et al.* (1971b) had assumed that the antigenic valence of the virus was equal to the number of protein subunits, i.e., 2130. The importance of introducing the correct antigenic valence in all subsequent calculations is illustrated in Fig. 5.7. Since the data have been corrected for the amount of denatured antibody present, the r/c versus sr plots extrapolate to 1 and 2, respectively, depending on the assumed antibody valence. However, the slopes and thus the K values are considerably altered when different values of s are used.

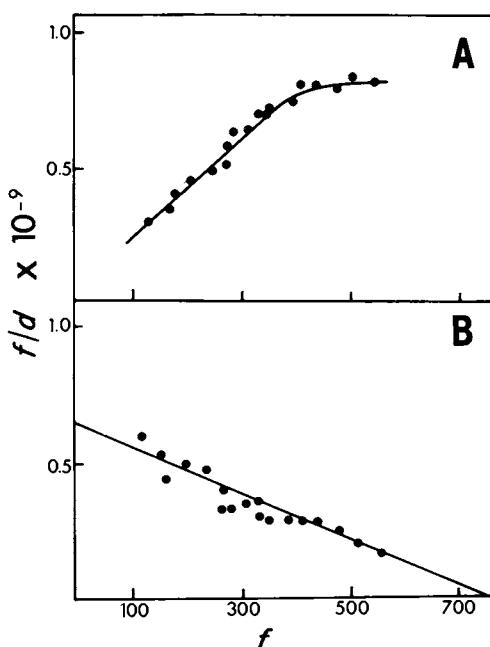


Fig. 5.6. (A) Plot of f/d versus f representing the TMV-IgG binding data published by Anderer et al. (1971b). The positive slope is indicative of the use in the calculations of an erroneous value of total antibody concentration. (B) Plot of f/d versus f for the same data of Anderer et al. (1971b), following a correction for the presence of nonfunctional molecules in the IgG preparation (see text). Extrapolation yields a value of $s = 750$ for the antigenic valence of TMV.

Other transformations of the mass action equation that are sometimes used for representing binding data take the form of the Langmuir adsorption isotherm and are derived as follows. From Eq. (3) written as

$$f = Kd (s - nf) = Kds - Kdnf$$

it follows that

$$\frac{f}{s} = Kd - Kdn \frac{f}{s}$$

Hence

$$Kd = \frac{f}{s} + \frac{f}{s} Kdn = \frac{f}{s} (1 + Kdn)$$

and

$$\begin{aligned}\frac{f}{s} &= \frac{Kd}{1 + Knd} \\ f &= \frac{sKd}{1 + Knd} \\ \frac{1}{f} &= \frac{1 + Knd}{sKd} \\ \frac{1}{f} &= \frac{1}{sKd} + \frac{n}{s}\end{aligned}\tag{5}$$

By plotting $1/f$ versus $1/d$, a line is obtained that extrapolates to $1/f = n/s$, allowing the antigen valence to be calculated. A Langmuir plot representing binding data between TMV and specific Fab is shown in Fig. 5.8. From the extrapolation at $1/f = 1.25 \times 10^{-3}$, the valence of TMV is found to be 800.

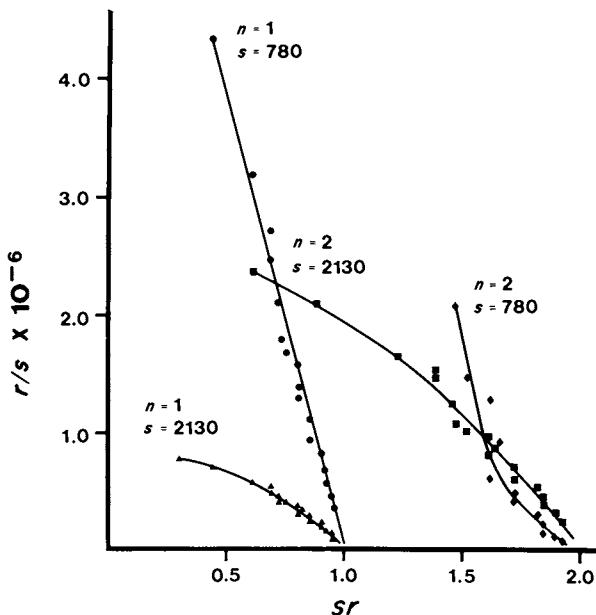


Fig. 5.7. Plots of r/c versus r representing the TMV-IgG binding data of Anderer et al. (1971b), following a correction for the presence of nonfunctional antibody in the IgG preparation. The plots extrapolate to $n = 1$ and $n = 2$ instead of to 0.59 and 1.16 in the uncorrected data. When an erroneous valence $s = 2130$ is used in the calculations, the resulting slopes and K values are considerably reduced.

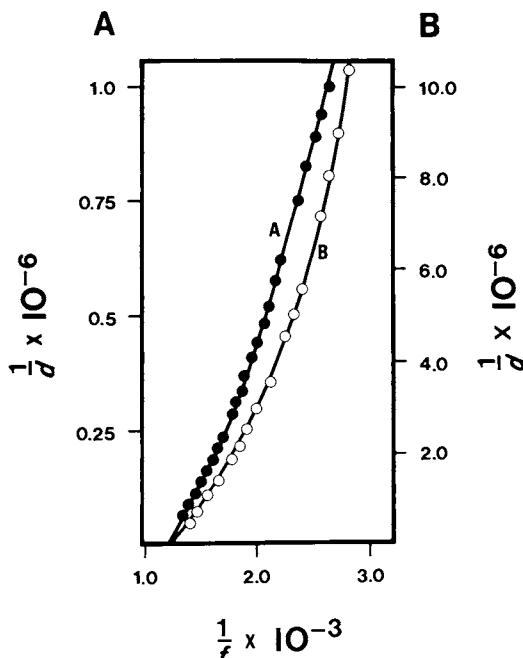


Fig. 5.8. Langmuir plot of TMV-Fab binding data. ●, 1.3 mg/ml Fab; ○, 0.23 mg/ml Fab. Both plots extrapolate to $1/f = 1/s = 1.25 \times 10^{-3}$, corresponding to an antigenic valence of TMV of $s = 800$.

In an analogous fashion, Eq. (4) can be rearranged to give

$$\frac{1}{r} = \frac{1}{nKc} + \frac{s}{n} \quad (6)$$

The utilization of Eqs. (3) – (6) for plotting binding data in the case of viruses requires a knowledge of the total antibody concentration. This is unavoidable since the amount of bound antibody (y) is calculated from the experimentally obtained value of free antibody (d) by the relation $y = B - d$. Only in the case of binding measurements with monovalent viral peptides, for instance, can the total concentration of antibody sites present in an antiserum be calculated from a transform of Eq. (1) such as

$$\frac{1}{x} = \frac{1}{KcBn} + \frac{1}{Bn} \quad (7)$$

From measurements of free and bound peptide, plots of $1/x$ versus $1/c$ can be constructed, and the concentration of total antibody sites is then obtained from

$1/x = 1/Bn$ when $1/c \rightarrow 0$. In such a case, therefore, purified antibody is not required for carrying out binding measurements.

In order to simulate the binding behavior of complex multivalent systems, computer calculations are usually necessary. Trautman (1976) showed that the following solution of the quadratic equation is useful for this purpose. From Eq. (1), an explicit expression of the extent of reaction can be derived as

$$\begin{aligned} \frac{1}{BnK} &= \frac{As}{ny}(1 - ny/Bn)(1 - ny/As) \\ \frac{ny}{AsBnK} &= 1 - ny/Bn - ny/As = \frac{n^2y^2}{BnAs} \\ \frac{n^2y^2}{BnAs} - ny \left(\frac{1}{Bn} + \frac{1}{As} + \frac{1}{AsBnK} \right) + 1 &= 0 \\ n^2y^2 - ny(Bn + As + 1/K) + AsBn &= 0 \\ ny = \frac{As + Bn + 1/K \pm [(As + Bn + 1/K)^2 - 4 AsBn]^{1/2}}{2} \end{aligned}$$

2. Evaluation of Antibody Heterogeneity

No satisfactory procedure has yet been devised for evaluating the considerable heterogeneity of antibody affinities that exists within an antibody population. It is generally assumed that the K values approximate to a normal distribution, although there is experimental evidence that the actual distribution of affinities is heavily skewed toward antibodies of low affinity (Werblin and Siskind, 1972; Werblin *et al.*, 1973; Kim *et al.*, 1974).

Many authors compute a heterogeneity index, a , from the Sips distribution

$$\frac{r}{n} = \frac{(Kc)^a}{1 + (Kc)^a} \quad (8)$$

using the logarithmic transformation

$$\log \frac{r}{n-r} = a \log K + a \log c$$

or alternatively the form

$$\log \frac{y}{B-y} = a \log K + a \log c$$

The heterogeneity index, a , is obtained from the slope of a plot of $\log r/n_r$ versus $\log c$. As the value of a approaches 1.0, the antibody population is assumed to approximate perfect homogeneity with respect to affinity constants.

The limitation of such an approach for estimating the distribution of affinities in an antiserum is illustrated by the fact that values of the heterogeneity index very close to 1 are sometimes obtained for systems known to be highly heterogeneous (Mamet-Bratley, 1966; Anderer *et al.*, 1971b; Skubitz *et al.*, 1977). Since experimental K values often depend upon the absolute quantities of interacting reactants as well as upon the relative amounts of each (Hudson, 1968; Larralde and Farber, 1972; Arend and Mannik, 1974) it has been suggested that legitimate comparisons between average affinities can only be made when the same proportion of ‘binding sites occupied’ is used in all assays (Kim *et al.*, 1975). Some attempts have been made to estimate the distribution of antibody affinities from measurements made under conditions where 50% of one of the reactants is bound (Taylor, 1975).

3. Determination of Avidity at 50% Binding of Antibody

Several authors have described methods for determining the binding parameters of antiprotein antibodies under conditions where 50% of the available antigen is bound to antibody (Celada *et al.*, 1969; Tosi and Celada, 1974; Nahm *et al.*, 1977). Celada *et al.* (1969) showed that when the log of the concentration of antiserum required to bind 50% of the available antigen is plotted against the log of total antigen concentration, a line is obtained which has a slope varying between 0 and 1. A correlation between this slope and the average affinity of the antiserum has been reported (Schirrmacher, 1972; Steward and Petty, 1972; Ahlstedt *et al.*, 1973; Devens *et al.*, 1978).

Since at 50% binding of antigen sites $As/2 = sx = ny = sc$, the appropriate equation can be derived from Eq. (1):

$$\frac{1}{K} = \frac{SC(Bn - ny)}{sx} = nd$$

$$Bn = 1/K + ny$$

$$\log Bn = \log (1/K_0 + As/2) \quad (9)$$

Two idealized plots of $\log Bn$ versus $\log As/2$ for homogeneous populations of antibody molecules with $K = 10^5$ and 10^6 are shown in Fig. 5.9 (Paul and Elfenbein (1975)). It is clear that when $As/2$ is very small (i.e., when the slopes approach 0), $\log Bn = \log 1/K$. Furthermore, the value of $\log 1/K$ can also be obtained on the abscissa at the intersection points a and a' . In the case of

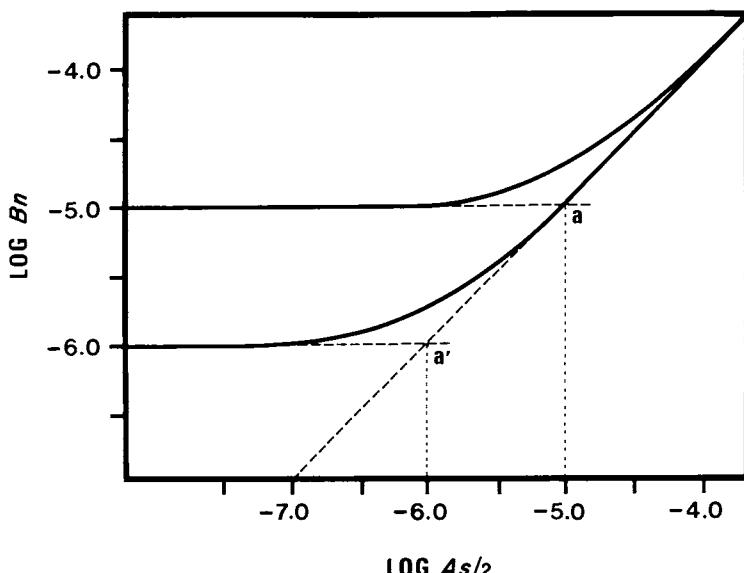


Fig. 5.9. Theoretical curves relating log conc. of total antibody sites (B_n) to log of half concentration of antigen sites ($A_s/2$) when 50% of antigen sites are bound. The curves represent the equation $\log B_n = \log(1/K + A_s/2)$ and correspond to two sets of homogeneous antibodies with affinity constants of 10^{-5} and 10^{-6} , respectively.

heterogeneous antibody populations, it has been argued (Taylor, 1975) that intermediate values of the slope are related to the distribution of antibody affinities.

When the properties of a 50% binding system were examined in the case of TMV, it was found that very low levels of free antibody were obtained at half saturation of antigen sites, and that plots of $\log B_n$ versus $\log A_s/2$ invariably were straight lines with slopes of 1 (Van Regenmortel and Hardie, 1979). On the other hand, useful plots could be obtained under conditions of 50% binding of antibody. In this case,

$$B_n/2 = ny = sx = nd$$

and the following relationships may be derived from Eq. (1):

$$\frac{1}{K} = \frac{nd(A_s - sx)}{ny} = sc$$

$$A_s = 1/K + sx$$

$$\log A_s = \log(1/K + sx) \quad (10)$$

In order to use Eq. (10) for representing binding data in the TMV system, it is necessary to first calculate the total number of antigen sites required for achieving 50% binding of antibody. This can be done by constructing plots of r/c versus sr based on the amount of free antibody found when varying amounts of virus are added to the same quantity of total antibody (Fig. 5.10). Values of r/c for $sr = 0.5$ can be obtained by extrapolation from such plots, and since $(sr)/(rc) = As - sx$, and $sx = nd = Bn/2$, it is possible to calculate the total number of antigen sites required for 50% binding of antibody from the relation $As = sc + Bn/2$.

A set of binding data representing the reaction between TMV and Fab and plotted according to Eq. (10) is shown in Fig. 5.11. The slope of the curve of $\log As$ versus $\log y$ varied between the extremes of 0 and 1, and cut the ordinate at $\log As = \log 1/K = 0.63$. The intersection a of the horizontal line and of the

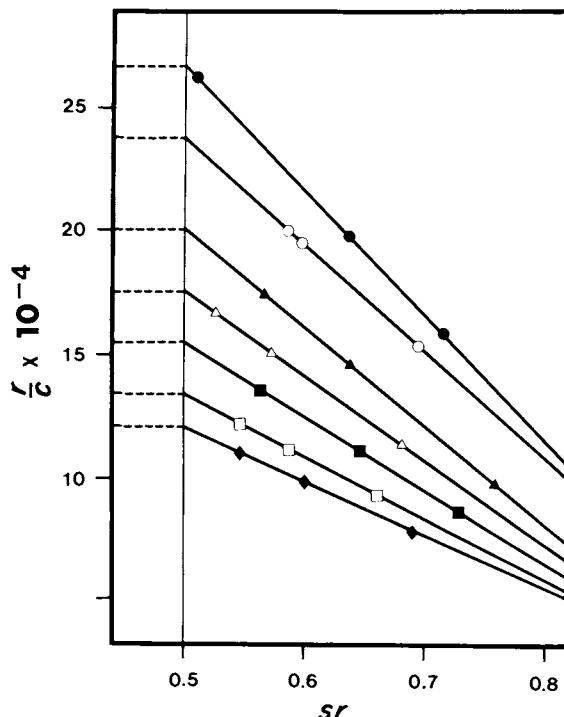


Fig. 5.10. Plots of r/c versus sr representing TMV-Fab binding data obtained by mixing three different amounts of antigen to fixed amounts of Fab. The extrapolated values of r/c when $sr = 0.5$ were used to calculate As , i.e., the concentration of antigen sites required to bring about 50% binding of antibody (from Van Regenmortel and Hardie, 1979).

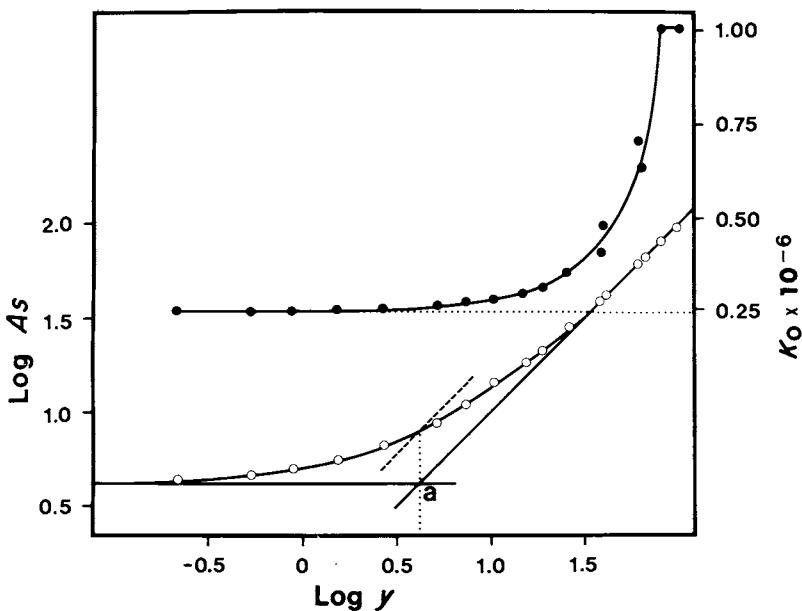


Fig. 5.11. TMV-Fab binding data obtained at 50% antibody binding. ○, Plot of $\log A_s$ versus $\log y$; ●, plot of K versus $\log y$. The K values were calculated by the mass action equation. On the ordinate, the limiting value of $\log A_s = \log 1/K = 0.63$ corresponds to a value of $K = 0.23 \times 10^{-6}$. The intersection point a lies on the abscissa at a value of $\log y = 0.63$, which corresponds to $\log A_s = 0.92$. Therefore, $A_s = B = 8.32 \times 10^{-6}$ and $sc = d = 4.16 \times 10^{-6}$, and $K = 1/sc = 1/d = 0.24 \times 10^{-6}$.

straight line of slope 1 lies on the abscissa at a value of $\log y = \log A_s = 0.63$. The same value of $1/K$ can be obtained by the intersection of the curve with a second line (broken line in Fig. 5.11) of slope 1 situated 0.3 log unit to the left of the first (Taylor, 1975).

A value of $\log A_s = 0.92$ can be read off the curve at $\log y = 0.63$ (Fig. 5.11), and at this point, the number of total antigen sites is equal to the number of total antibody sites ($A_s = B_n = 8.32 \times 10^{-6}$) and $K = 2/A_s = 2/B_n = 1/sc = 1/d = 0.24 \times 10^{-6}$. This value of K probably corresponds to antibodies of a low-affinity clonotype, whereas the maximum K value (1.03×10^6) corresponds to the antibodies of high affinity that produce straight lines in the f/d versus f plots. It seems, therefore, that measurements at 50% binding of antibody allow the calculation of average values of minimum and maximum antibody avidity, as well as an estimation of antibody heterogeneity.



Serological Techniques

In this chapter all the immunochemical techniques that have been used with plant viruses will be reviewed. The fundamental principles that govern each type of reaction will be discussed in considerable detail since a thorough understanding of the mechanism of a reaction is necessary to enable the user to adjust the parameters of any test to the requirements of his own system. Sufficient experimental details will be given to enable the reader to perform any of the described techniques. However, point by point recipes and descriptions of the numerous variations of each technique will not be presented. For additional information the following exhaustive methodological texts describing all the relevant immunochemical techniques may be consulted (Kabat and Mayer, 1961; Williams and Chase, 1967, 1971; Mayr *et al.*, 1977; Garvey *et al.*, 1977; Weir, 1978; Nowotny, 1979; Hudson and Hay, 1980). Specific reviews dealing with the serological techniques used in plant virology are also available (Matthews, 1957, 1967, 1970; Van der Veken *et al.*, 1962; Wetter, 1965; Van Regenmortel, 1966b, 1978, 1981a; Van Slogteren, 1969; Bercks *et al.*, 1972; Ball, 1964, 1974).

A. PRECIPITIN TESTS

1. Precipitation Reaction

The precipitation or precipitin reaction is the basic reaction that contributed most to the development of immunochemistry into a quantitative science (Kabat and Mayer, 1961; Maurer, 1971). The name of the reaction is derived from the fact that a visible precipitate is formed when adequate quantities of antigen and antibody are allowed to combine. It is customary to distinguish between precipitation and agglutination on the basis of the size of the reacting antigen. *Precipitation* is used to describe the insolubilization of macromolecules and virus particles, whereas *agglutination* refers to the clumping of cells or of particles of similar size. The size of the reacting antigen greatly influences the number of interacting antibody molecules required to produce an aggregation visible to the naked eye. By using red blood cells or latex particles coated with virions, it is possible to detect an agglutination with much less antibody than is required for producing a visible precipitate with the virions themselves. Large viruses or elongated viruses that have become aggregated during purification also require fewer antibody molecules to induce a precipitation than smaller virions.

The highest dilution of antiserum that will give a visible precipitate is called the antiserum precipitin titer. In view of the role played by antigen size, it is clear that precipitin titers are not suitable for comparing antibody levels against antigens of different sizes. Kleczkowski (1966) showed, for instance, that about 10 times more antibody is required to precipitate a given amount of depolymerized TMV protein than to precipitate the same amount of TMV. Similarly, in the case of PVX 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 widely believed that precipitation is caused by the formation of a lattice of antigen and antibody molecules that grows in size until it is so large that the complex becomes insoluble. According to this "lattice hypothesis" the aggregates result exclusively from the formation of bonds between epitopes and corresponding antibody combining sites. There is evidence, however, that this view is an oversimplification and that the precipitin reaction involves two different molecular mechanisms. The first stage of the reaction is immune specific and leads to the formation of relatively small antigen-antibody complexes. It has been inferred from thermodynamic considerations that very large complexes are unlikely to form as the sole product of antigen-antibody interactions (Steensgaard and Frich, 1979). The second stage of the precipitin reaction consists of the formation of a hydrophobic phase that will become insoluble under certain ionic and pH conditions (Jacobsen and Steensgaard, 1979). It has been shown with TMV and human serum albumin, for instance, that 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). Several other factors can determine whether precipitation occurs or not, for instance temperature, pH, and the type of subclass of IgG that interacts in the particular assay (Warner and Schumaker, 1970). Differences in precipitability of various immunoglobulin subclasses may be linked to the degree of flexibility of the two Fab arms in the IgG molecules (McGuire *et al.*, 1979).

At a time when the development of micromethods allows precise quantitative determinations to be made on very dilute preparations, it may be relevant to stress that the amount of serological precipitate that is obtained depends on the volume in which the reaction is carried out. This effect is clearly demonstrated in Fig. 6.1. It is obviously not possible to measure the amount of precipitate formed at a high serum dilution and then to calculate back to the quantity that would be obtained with undiluted antiserum. As pointed out by Kabat and Mayer (1961), the rather abnormal precipitin curve reported for TMV by Malkiel and Stanley (1947) may have been caused by the large dilution factors that were used in that study.

The classical quantitative analysis of the precipitin reaction was based on the measurement of nitrogen in specific precipitates, either by the micro-Kjeldahl method or by some other colorimetric procedure (Chester, 1936; Kleczkowski,

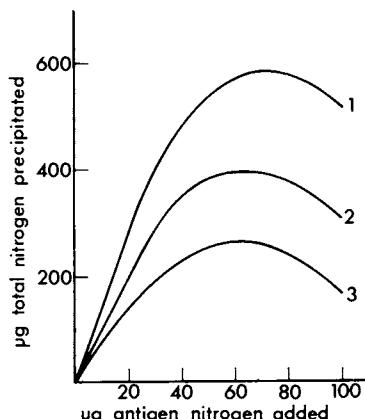


Fig. 6.1. Effect of reaction volume on amount of antigen-antibody precipitated. Curve 1 represents the amounts of precipitate formed when 1.5 ml of specific antiserum is added to the indicated quantities of egg albumin antigen. The total volume was 3 ml. Curve 2 represents the amounts precipitated when one-tenth the quantities of antigen and antiserum were allowed to react in the same volume and the values multiplied by 10. In curve 3, 1:40 dilutions of the reactants were used and the precipitate values were multiplied by 40 (from Kabat and Mayer, 1961).

1941; Kabat and Mayer, 1961). The combination of virus with antibody can also be followed by light scattering measurements (Bradish and Crawford, 1960). Usually, increasing amounts of antigen are added to a series of tubes containing a constant amount of antiserum. After incubation the tubes are centrifuged and the precipitates are analyzed for nitrogen. The supernatants can be tested by addition of antigen or antiserum to determine whether excess of antibody or antigen is present and able to produce further precipitation. When all the virus has been precipitated, it is possible to calculate the antibody content of the precipitate by subtracting the contribution of virus nitrogen.

The analysis of the supernatants normally reveals three zones: a region of antibody excess, an equivalence zone where neither antigen nor antibody is found in the supernatant, and a region of antigen excess (Kubo, 1976). With small monomeric proteins, the complete precipitin curve stretches over a fairly small range of antigen concentrations. With viruses, on the other hand, precipitation occurs over a very broad range of ratios of antibody to antigen. This is illustrated by the results of Kleczkowski (1966) shown in Fig. 6.2, in which the variation in antibody content of antisera against different antigens was eliminated by normalizing the data to the situation pertaining to 1 mg of homologous antibody. It is also apparent that the weight of antibody to weight of virus precipitated in the equivalence zone is markedly dependent on the size of the antigen. Both effects obviously reflect the large antigenic valence of virions. It is

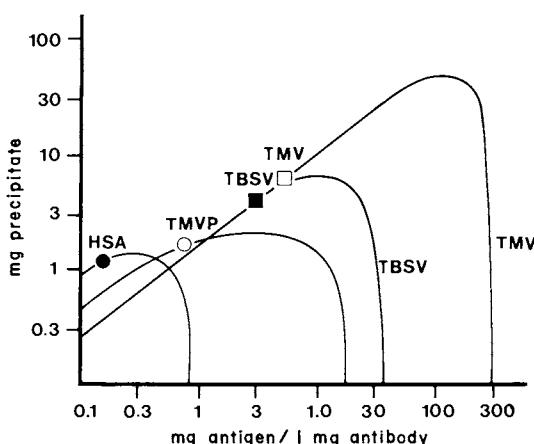


Fig. 6.2. Influence of antigen size on the precipitin curve. Amounts of precipitate formed by addition of increasing amounts of human serum albumin (HSA), TMV protein (TMVP), tomato bushy stunt virus (TBSV), and tobacco mosaic virus (TMV) to 1 mg of homologous antibody. Circles and squares on the curves correspond approximately to the ratios of equivalence (from Kleczkowski, 1966).

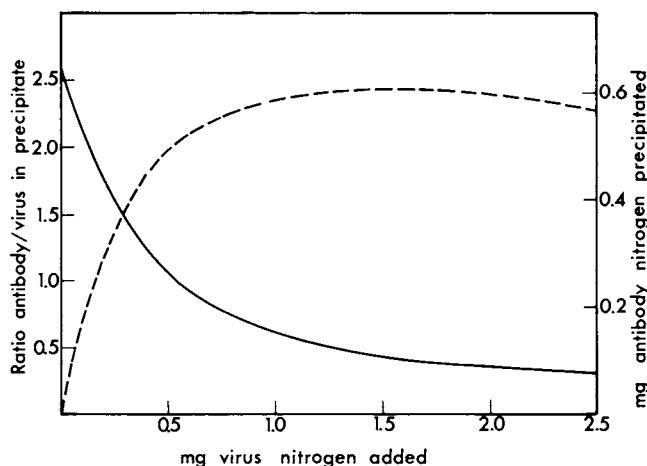


Fig. 6.3. Quantitative precipitin curve of tobacco mosaic virus, strain U2. The broken line represents the amount of antibody N precipitated, and corrected to 1.0 ml undiluted serum. The solid line represents the ratio of antibody N to antigen N in the precipitate (from Rappaport, 1957a).

interesting that the position of the TMV protein curve, compared with those of human serum albumin and TBSV (Fig. 6.2), corresponds to that of a highly aggregated protein preparation. Presumably, at the high concentration of TMV protein used (Kleczkowski, 1966), some of the material was present in disk form (Durham *et al.*, 1971). It is worth noting that the equivalence point (Fig. 6.2) always lies to the left of the zone of maximum precipitation. The reason for this characteristic feature of the precipitin reaction is unknown (Kubo, 1976). The precipitin curve of TMV was studied extensively soon after the initial work of Heidelberger and Kendall (1935) on quantitative serological precipitation (Kleczkowski, 1941; Malkiel and Stanley, 1947; Rappaport, 1957a, 1965). As shown in Fig. 6.3, the TMV precipitin curve is characterized by a large plateau region in which the maximum amount of antibody precipitated does not change appreciably. The ratio R of weight of antibody to antigen in the precipitate varies considerably and extrapolates to a value of 2.6 in extreme antibody excess. Electrophoretic and electron microscope evidence has confirmed that under these conditions the surface of the virions is completely covered with antibody molecules (Kleczkowski, 1958, 1961). Assuming that the antibody then has an effective valence of 1 (see Van Regenmortel and Hardie, 1976 and Chapter 5), the valence of TMV can be calculated to be about 700. This value is obtained by multiplying the R value by the ratio of the molecular weights of virus and antibody ($40 \times 10^6 / 15 \times 10^4 = 266$).

2. Liquid Precipitin Tests in Tubes

These tests are usually performed in small tubes of about 7 mm internal diameter with a volume of each reagent of 0.5 ml. A series of twofold dilutions of one reagent is added to a constant dilution of the other reagent and the tubes are kept in a water bath at 30°–40°C. When the tubes are placed in such a way that half the contents are immersed in the heated water, convection currents are produced that speed up precipitate formation. By recording the time for the first visible precipitation to occur in each tube of such a grid titration, a precipitation diagram such as the one shown in Fig. 6.4 can be obtained. This diagram illustrates some characteristic features of precipitin reactions:

1. The absence of precipitation in the top right area of Fig. 6.4 is due to inhibition of precipitate formation by excess antiserum.
2. The absence of precipitation in the bottom left area is due to inhibition by excess antigen.
3. For each successive row of tubes containing the same antiserum dilution, there is a constant ratio of reagents known as the α -optimum for which the precipitation reaction is fastest.
4. When a series of tubes containing the same amount of antigen but varying antiserum dilutions are compared, the fastest precipitation does not occur at the same ratio of reagents. This variable ratio defines the so-called β -optimum (Matthews, 1957).
5. The highest dilution of virus ($1.5 \mu\text{g}/\text{ml}$) that gives a visible precipitate is known as the virus end point. To find this end point, it is necessary to use diluted antiserum.
6. The highest dilution of antiserum that gives a visible precipitate is known as the antiserum titer. The determination of precipitin titers is one of the most frequently used methods for comparing different viruses serologically. To find the true antiserum titer, it is necessary to use virus concentrations in the vicinity of $10 \mu\text{g}/\text{ml}$.

The most commonly used diluents for tube precipitin tests are 0.85% NaCl or phosphate-buffered saline. The visibility of precipitates is enhanced by holding the tubes over a light box in front of a black background. Readings are usually taken after 2 hours at 30°–40°C and again after overnight incubation at 4°C.

It has been reported that the precipitin titer of antisera can be increased considerably by precipitating the antibodies with polyethylene glycol and subsequently resuspending them in saline (Wolf and Schmelzer, 1973). It is possible that this effect is caused by the presence of residual polyethylene glycol in the antibody preparation, and that this leads to a polymer-induced precipitation of soluble virus-antibody complexes (Harrington *et al.*, 1971). A similar phenomenon may explain why certain virus preparations purified by polyethylene glycol precipita-

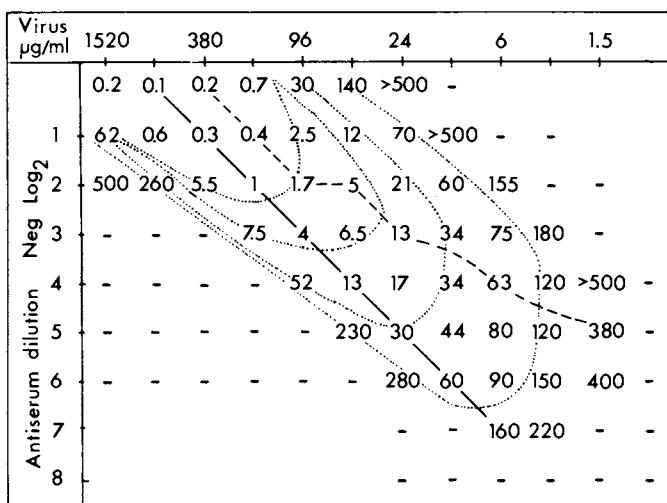


Fig. 6.4. Precipitation diagram of a grid titration of turnip yellow mosaic virus and homologous rabbit antiserum. Figures in the table are the time in minutes for visible precipitation to first appear. (-) Correspond to no precipitation after 24 hours (from Matthews, 1957).

tion sometimes give spuriously high precipitin titers. The addition of polylysine has also been found to enhance the formation of precipitates (Stahmann and Matthews, 1954).

A note of warning should be included here regarding a spurious impression of accuracy that may arise from the notation of antiserum dilutions in terms of reciprocals of large numbers, e.g., 1/4096, 1/8192, 1/16384. For many purposes it is preferable to refer to antiserum dilutions simply by the number of twofold dilution steps or negative \log_2 (Van Regenmortel and Von Wechmar, 1970). The accuracy of precipitin titer determinations is \pm one dilution step and in many cases it is likely to be even less. This means that the significance of small differences in titer should be treated with circumspection. A case in point is the change in precipitin titer observed after an antiserum has been cross-absorbed with a heterologous virus strain. It has been suggested (Kassanis, 1961; Babos and Kassanis, 1963) that when cross-absorption with a related virus strain leaves the titer against the homologous strain unchanged, the serological relationship between the two strains is "distant," whereas when the homologous titer is decreased as a result of cross-absorption, the two viruses should be considered as closely related strains. However, when the cross-absorption data reported by different authors are analyzed (see Van Regenmortel and Von Wechmar, 1970), it is clear that the inaccuracy of titer determinations does not permit such fine differentiation. When the homologous and heterologous titers of two strains differ, for instance, by 2 twofold dilution steps, only about one-fourth of the

antibodies is likely to be removed by cross-absorption, and this will probably not affect the residual homologous titer in a significant and reproducible fashion.

Although the main application of liquid precipitin tests has been the characterization and identification of viruses usually by means of antiserum titers, in recent years immunoprecipitation has also been applied to the isolation of various proteins produced during virus replication (Higgins *et al.*, 1976; Salomon *et al.*, 1978; Dougherty and Hiebert, 1980a,b,c). Rabbit antiserum or immune IgG is added to the extract containing the mixture of all the products of translation and, after an incubation period, the resulting immune complexes are precipitated with a second antiserum against rabbit globulins or with staphylococcal protein A. This protein binds specifically to IgG and produces less nonspecific binding than most double antibody systems (Goding, 1978). The immune precipitates are usually dissolved in buffers containing sodium dodecyl sulfate or urea, and the products of translation are then analyzed by polyacrylamide gel electrophoresis.

3. Precipitin Ring Test

This test is based on the appearance of a ring of precipitation at the interface between superimposed layers of antigen and antibody preparations. Antiserum, diluted in 10–30% glycerin in saline, is placed in the bottom of a small tube (3–6 mm in diameter and 5 cm in length); the antigen preparation is carefully layered onto the surface to form a sharp interface. Depending on the size of the tube used, as little as 0.1–0.2 ml of the reagents can be used. A positive reaction takes the form of a precipitin ring at the interface. When the time of appearance of the ring is recorded, and serial dilutions of the reagents are examined, the accuracy of the test can be improved considerably (Whitcomb and Black, 1961a,b; Sinha and Thottappilly, 1974). Precipitin rings can be visualized more easily when the antibodies have been labeled with fluorescein isothiocyanate (Sinha and Reddy, 1964).

The minimum virus concentration that can be detected in precipitin ring tests is about 1–10 $\mu\text{g}/\text{ml}$ (Wright and Stace-Smith, 1966; Sinha and Thottappilly, 1974).

This test has been used successfully with all virus groups (Bancroft *et al.*, 1960; Sinha, 1968; Mink *et al.*, 1969; Niblett and Semancik, 1969; Luisoni *et al.*, 1973). Compared to tube precipitin tests, the main advantage of the ring test is that smaller quantities of reactants are required and that the relative concentrations of the reactants are not critical.

4. Microprecipitin Tests

These tests are performed in single drops of the mixed reactants deposited on the bottom of a petri dish. If glass dishes are used they should be rendered

hydrophobic by a coat of silicone or 0.1% Formvar dissolved in chloroform (Desjardin, 1968; Noordam, 1973). Plastic dishes do not require such treatment. 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 10–100 \times magnification (Van Slogteren, 1955). Drops may also be observed in a specially constructed frame of glass or plastic after placing a cover slide above them (Noordam, 1973). Purified virus preparations as well as clarified extracts of infected tissue can be used for the test, and a complete grid titration can be performed in a single petri dish. Figure 6.5 shows the results of a microprecipitin test with TNV in which less than 1 $\mu\text{g}/\text{ml}$ of virus could be detected. Zones of inhibition of the reaction by excess of one or other of the reactants are much less pronounced than in grid titrations performed by tube precipitin tests. This is probably due to the fact that increased turbidity and the appearance of "clouds" that do not settle to the bottom of the tube are scored as a negative reaction in tube tests, whereas they are recognized as precipitates when examined by microscopy.

The microprecipitin test is economical in its use of antiserum and is fairly sensitive since small precipitates are easily detected under the microscope. The method has been used extensively in the diagnosis of virus diseases and for large scale indexing of vegetative propagation material (Van der Veken *et al.*, 1962; Sampson and Taylor, 1968; Van Slogteren, 1969, 1972; Ball, 1974).

A variation of the microprecipitin test in which chloroplasts and cell fragments present in crude plant sap are agglutinated upon addition of virus antiserum is often referred to as the chloroplast agglutination test (Bradley, 1953; Munro, 1954; Storms and Streets, 1962; Van der Veken *et al.*, 1962).

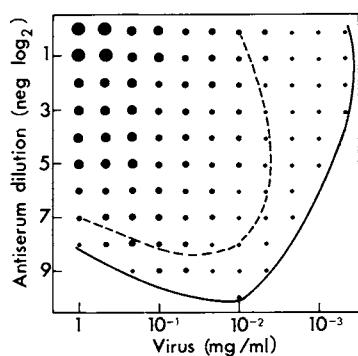


Fig. 6.5. Microprecipitin test with TNV. Dot sizes indicate relative amounts of precipitate. The broken line and solid line indicate end points after 6 hours and 66 hours, respectively (from Noordam, 1973).

B. IMMUNODIFFUSION

Immunodiffusion tests are serological precipitin tests that are carried out in gels instead of free liquid. The great advantage of this type of test is that mixtures of antigens and their corresponding antibodies may become physically separated because of different rates of diffusion in the gel. As a result immunodiffusion tests are able to provide information on the homogeneity and purity of the reactants as well as on the size of and relationships among antigens.

Two main groups of immunodiffusion tests can be distinguished: simple diffusion techniques in which one of the reactants diffuses into a gel containing the other reactant, and double diffusion techniques in which both reactants diffuse into a gel initially free of them. Depending on whether the reactions are carried out in tubes or in plates, the diffusion process is said to occur in one or in two dimensions.

Many plant viruses are sufficiently small to be able to diffuse in 0.7–1.5% agar gels (Ackers and Steere, 1962). Satisfactory results have been reported with certain potexviruses that have a length of 470 – 580 nm, e.g., clover yellow mosaic virus (C1YMV) (Ford, 1964), papaya mosaic virus (PapMV) (De Bokx, 1965), PVX (Van Regenmortel, 1966b; McCrum *et al.*, 1971), narcissus mosaic virus (NaMV) (Brunt, 1966b), and petunia asteroid mosaic virus (PAMV) (Juo and Rich, 1969). Using 0.5% agar gels and a low electrolyte concentration, Wetter (1967b) showed that it is also possible to obtain satisfactory immunodiffusion lines with carlavirus with a normal length of about 650 nm. Using this method, he confirmed the existence of cross-reactions between potato virus M (PVM), red clover vein mosaic virus (RCVMV), PSV, and carnation latent virus (CLV).

Larger viruses such as the poty- and rhabdoviruses cannot diffuse into agar unless they have been degraded chemically or by some other means (Purcifull and Shepherd, 1964; Tomlinson *et al.*, 1965; McLean *et al.*, 1971; Lin and Campbell, 1972; Thottappilly and Sinha, 1973). Sonic treatment of the filamentous particles of TurMV, for instance, produced short fragments that formed prominent precipitin lines in gels (Tomlinson and Walkey, 1967b). Depolymerization of many large filamentous viruses into small serologically active subunits has been achieved by chemical degradation with reagents such as ethanolamine (Purcifull and Gooding, 1970), pyridine (Shepard, 1970b), pyrrolidine (Shepard *et al.*, 1971), and detergents (Hamilton, 1964; Hamilton and Ball, 1966; Purcifull and Batchelor, 1977). The use of degraded virus particles has made immunodiffusion tests generally applicable to the closteroviruses (Bar-Joseph and Smookler, 1976; Gonsalves *et al.*, 1978), rhabdoviruses (Jackson and Christie, 1977), potyviruses (Gooding and Bing, 1970), and carlavirus (Shepard, 1972).

For many viruses, the ionic environment in the gel is not critical for precipitin

line formation, and a variety of buffers suitable for preserving the structural integrity of the virions can be used (Von Wechmar and Van Regenmortel, 1968). With elongated viruses, the electrolyte concentration in the gel has been found to play a considerable role in the formation of precipitin lines (Wetter, 1967a). The diffusion of TMV at high salt concentration (0.8–2% NaCl) is slowed down, presumably because of the aggregation of virus particles. In double immunodiffusion plate tests the curvature of the virus precipitin line is small in 0.01 M phosphate buffer and much more pronounced in buffers of higher molarity. Aggregation and precipitation of TMV particles are thought to occur as a result of spatial exclusion between the virus and agar molecules. A similar phenomenon also occurs with gelatin (Dudman, 1966; Van Regenmortel *et al.*, 1971).

The presence of mercury-containing preservatives such as merthiolate (sodium ethylmercurithiosalicylate) or cialit (sodium 2-ethylmercurimercaptobenzoxazole-5-carboxylate) can adversely affect precipitin reactions in gels (Bancroft, 1962; Cowan, 1966; Koenig and Jankulowa, 1968). With some viruses, these compounds appear to inhibit the diffusion process, whereas with others, they alter the electrophoretic migration of the virions (Koenig, 1969b, 1970). Merthiolate can also lead to nonspecific precipitation of viruses in the gel (Tremaine and Willison, 1962a). Since sodium azide has no such effects, the use of this preservative is recommended for keeping agar plates free of microbial contamination (Gooding and Tsakiridis, 1971).

Extensive reviews of all aspects of immunodiffusion techniques have been published (Ouchterlony, 1968; Crowle, 1973; Ouchterlony and Nilsson, 1978).

1. Single Diffusion in Tubes

This technique, also known as the Oudin method, requires that the external reactant, usually in a liquid phase, migrates into a gel containing the other reactant. This means that the diffusing reactant must be present in considerable excess compared with the internal reactant. The position of the leading edge of the precipitin band in the tube is proportional to the square root of time (Oudin, 1952):

$$h = kt^{1/2}$$

where h is the distance migrated by the leading edge of the band, t is the time, and k a proportionality constant. Figure 6.6A illustrates this relationship in a test with TMV as the external reactant. Straight line relationships also exist between k and the logarithm of virus concentration, if constant antibody is used as the internal reactant (Oudin, 1952), and between k and the logarithm of antibody concentration if constant virus is the internal reactant (Commoner and Rodenberg, 1955; Wetter, 1967a; Wetter and Luisoni, 1969). These relations are dem-

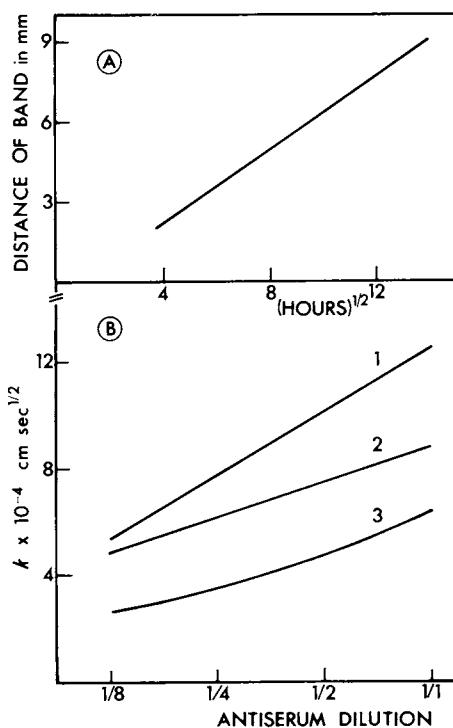


Fig. 6.6. (A) Relation between distance of precipitin band from meniscus and square root of time in single diffusion test with TMV (15 mg/ml). The internal reactant was antiserum diluted 1:100. (B) Relation between log antibody concentration and k in single diffusion tests with TMV. Line 1 corresponds to the diffusion of antibodies (undiluted antiserum) into agar containing 0.1 mg/ml TMV. Lines 2 and 3 correspond to the diffusion of TMV (15 mg/ml) into agar containing antiserum dilution 1:100 in 0.01 M phosphate buffer and in 0.01 M phosphate buffer + 0.8% NaCl, respectively (modified from Wetter, 1967a).

onstrated in Fig. 6.6B. It is also apparent that the diffusion of TMV, when used as the external reactant, is slowed down when 0.8% NaCl is added to 0.01 M phosphate buffer in the gel. Koenig (1969b) showed that the diffusion of BelMV into antiserum-containing agar was inhibited in the presence of organic mercury compounds.

The Oudin procedure is usually performed in 3–7 × 80 mm tubes, which are first coated with a layer of 0.1–0.5% agar. Agar, with the internal reactant incorporated into it, is then added to the tubes. When the gel has solidified, about 0.5 ml of the external reactant is layered above it, and the tubes are sealed. A series of relative concentrations of the reactants have to be tested empirically to ensure adequate migration of the precipitin band in the gel. The advancing edge of the band is measured at regular intervals over a period of about 2 weeks.

2. Radial Immunodiffusion

This technique, which is based on the principle of single diffusion in two dimensions, is usually performed in petri dishes filled with a layer of antibody-containing agar. Virus is placed in small wells that are cut in the gel and diffuses radially outward into the medium. The reverse placement of reactants can, of course, also be used (Schild *et al.*, 1972; Grandien and Norrby, 1975). The immune precipitate takes the form of a halo or ring of precipitation around the well and increases in diameter until such time as the total amount of available antigen has been consumed (Mancini *et al.*, 1965). When the diameter of the ring no longer increases, a situation reached only after several days, there is a linear relationship between the concentration of the external reactant (usually the antigen) and the area of precipitate. In practice, quantitative measurements of antigen concentration are made before the maximum ring diameter is reached. This is done by comparison with the ring diameters produced by standards of known concentration.

With isometric viruses such as CMV, BMV, TYMV, belladonna mottle virus (BelMV), radish mosaic virus (RaMV), and sowbane mosaic virus (SowMV) (Richter *et al.*, 1976b; Juretic and Mamula, 1978) adequate rings of precipitation were obtained with virus concentrations in the range 0.1–5 mg/ml when the agar contained antiserum diluted 1/20–1/100. Using straight line calibration plots of ring area versus virus concentration, the amount of virus in crude plant extracts could be determined fairly accurately. As shown in studies with foot-and-mouth disease virus, it is also possible to determine antibody concentrations by incorporating serial dilutions of antiserum in the gel and measuring the precipitin rings produced by standard virus preparations (Cowan and Wagner, 1970; Wagner *et al.*, 1972).

The main area of application of radial immunodiffusion in plant virology lies in the rapid diagnosis of virus infections by means of chemically dissociated capsid proteins. A mass indexing program for the presence of viruses in potato seed-stock has been developed, based on the use of virions degraded by pyrrolidine and pyridine (Shepard, 1969, 1972; Shepard and Secor, 1969; Shepard *et al.*, 1971). These organic compounds were found to be preferable to detergents as degrading agents, mainly because they produced fewer nonspecific reactions (Shepard, 1970a). Although radial immunodiffusion tests appear to be somewhat more sensitive than double diffusion procedures, they suffer from the disadvantage of requiring larger quantities of antiserum (Shepard, 1972; Uyemoto *et al.*, 1972).

Procedure

Radial immunodiffusion tests can be performed as follows. Suitable dilutions of antiserum are mixed with equal volumes of buffered 2–3% agar containing

sodium azide (at 50°C) and 10 ml of the mixture are poured into 100-mm plastic petri dishes. A template can be placed in the dish beforehand. After solidification of the agar, the template is removed leaving a large number of wells that can be used as antigen depots. The wells are then filled with the antigen preparations. In tests with chemically degraded viral subunits in 2.5% pyrrolidine, precipitin rings become visible in less than 1 hour. As many as 84 samples have been tested in a single dish containing 10 ml of antibody-agar mixture (Shepard and Secor, 1969). A large number of samples from infected plant material could be obtained in a single operation by means of an hydraulic press. This allowed a team of six people to process about 6000 samples per day (Shepard, 1972).

Simplified techniques have been described that are especially adapted to large-scale screening of plant material. Tissue grinding, well cutting, and chemical treatment of the antigen could be eliminated by embedding small pieces of infected plant tissue in agar containing the antibody and the virus dissociating agent (Slack and Shepherd, 1975; Richter and Polak, 1975). In another procedure, small drops of agar containing the antiserum were placed on the bottom of the petri dish, and droplets of plant juice containing degraded virus were placed next to them (Van Slooteren, 1976). Precipitation inside the agar drops could be observed under a dissecting microscope. Compared to radial immunodiffusion in plates, this procedure achieved a 12-fold saving of antiserum.

3. Double Diffusion in Tubes

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. According to Einstein's (1905) statistical treatment of Brownian movement and diffusion of particles in a liquid, the square root of the mean of the squares of the individual displacements of a given particle in one direction is

$$x = (2Dt)^{1/2}$$

where D is the diffusion coefficient of the particle and t is the time. For antigen and antibody molecules diffusing toward each other from opposite ends of an agar column, $x_g = (2D_g t_g)^{1/2}$ and $x_b = (2D_b t_b)^{1/2}$ where x_g and x_b are the distances moved from the menisci by antigen and antibody. Where the reactants first meet, $t_g = t_b$ and $x_g^2/x_b^2 = D_b/D_g$.

Since the value of D_b for IgG antibody is known ($4.6\text{--}4.8 \times 10^{-7} \text{ cm}^2 \text{ sec}^{-1}$; see Polson, 1971) the above relationship allows the calculation of the diffusion coefficient of antigens (Fig. 6.9). This, in turn, allows the size of the antigen to be calculated from the Stokes-Einstein equation: $r = kT/6\pi\eta D$ where r is the

radius of spherical particle, k is the Boltzmann constant, T is the absolute temperature, and η is the viscosity of the medium.

Since the rate at which a substance diffuses into the gel increases with the initial concentration of that substance, the relation $x_g^2/x_b^2 = D_b/D_g$ applies only in a statistical sense to single particles. However, if the ratio of the initial concentrations of the two reactants corresponds to the equivalence point, the precipitin band position is concentration- and time-independent and the band will thus remain stationary. Only in this case is the position of the band a reflection of the Stokes' radius of the antigen (Preer, 1956). 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. The position of the line can thus also be used to estimate the concentration of any one of the reactants.

Procedure

Double diffusion tests in tubes can be performed as follows. A suitable dilution of antiserum is mixed with an equal volume of molten buffered 1% agar, and 0.5 ml of the mixture is poured into a series of $5-10 \times 80$ mm tubes. When the agar has solidified, 1.0 ml of molten buffered 0.5% agar is poured above it. When the second layer of agar has solidified, serial dilutions of the antigen preparation are added to the top of the agar columns. The tubes should be sealed to prevent evaporation.

An improved procedure utilizes a plastic apparatus designed by Polson (1958), which allows the formation of flat menisci at both ends of the central agar column. This apparatus consists of three rectangular perspex bars $15 \times 1 \times 1$ cm, through which 10 holes, 4 mm in diameter, have been drilled (Fig. 6.7). The agar solution is introduced in the middle section which is then cut off from the adjacent section by lateral sliding of the two parts. The antiserum and antigen preparation are introduced into the holes on either side of the middle section, and these are then sealed by sliding two additional bars over them. When the agar has solidified, the three sections are moved into apposition and form a series of tubes with an agar column of constant length in the middle (Polson, 1958, 1971). By means of a microcomparator, the position of precipitin bands can be measured with great precision (Fig. 6.8). A miniaturized version of the same apparatus has also been described (Polson, 1976).

It has been found (Preer, 1956; Polson, 1958) that band position is a linear function of the logarithm of the ratio of antigen to antibody concentrations (Fig. 6.9). This means that antigen concentrations can be estimated by comparing the position of precipitin bands with a calibration curve obtained by plotting the positions of precipitin bands formed by serial twofold dilutions of antigen against constant antibody. This method of serological titration has a standard error of about 5% and has been used for quantitating many plant viruses as well as host plant antigens (Polson and Van Regenmortel, 1961; Van Regenmortel and En-

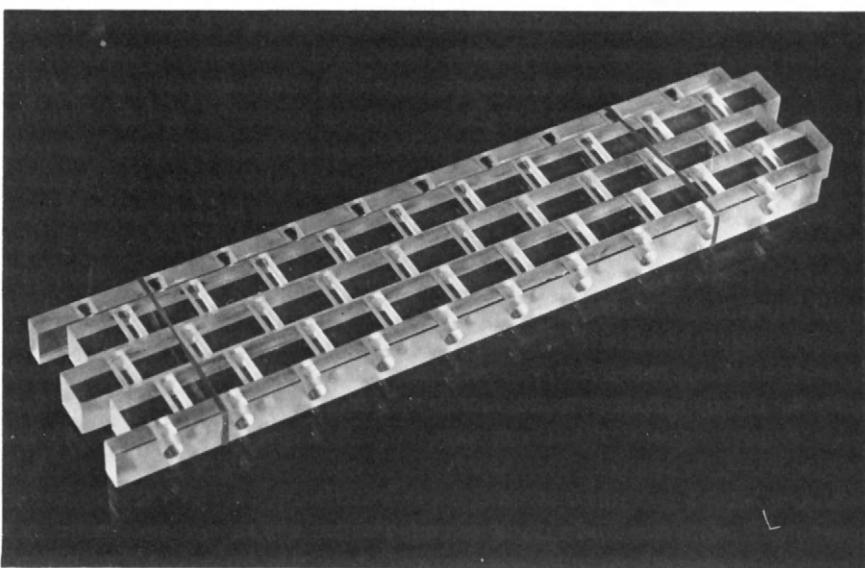
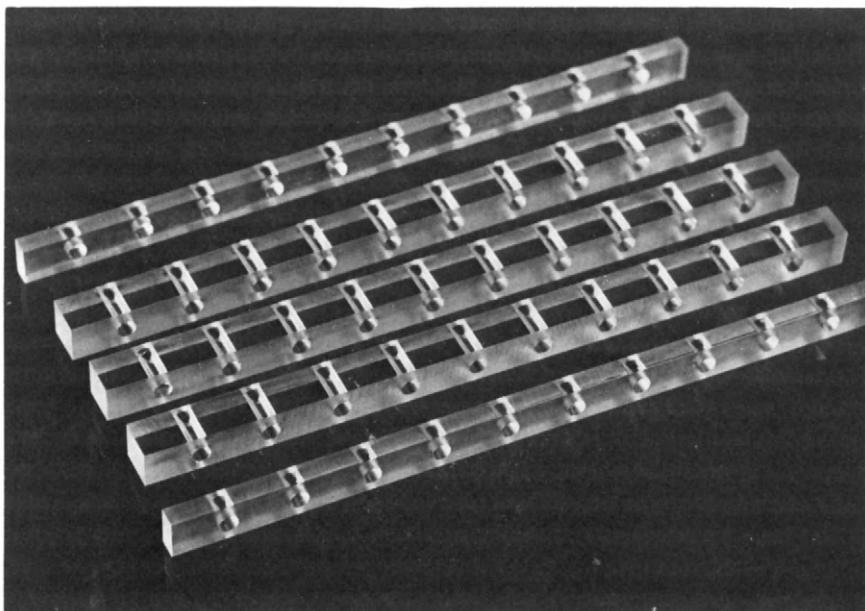


Fig. 6.7. Plastic apparatus for quantitative double diffusion tests. Developed by Polson (1958). Agar is introduced in the middle section. By lateral sliding of the various parts, agar columns of constant length are produced. Flat menisci are essential for precise determination of precipitin band position (Van Regenmortel, 1966b).

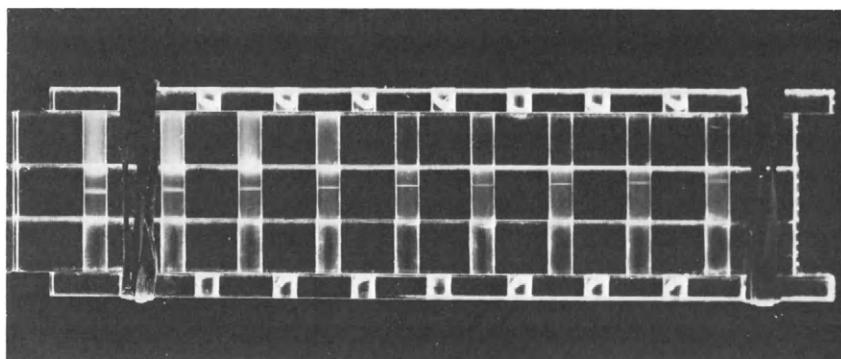


Fig. 6.8. Quantitative double diffusion test with the apparatus shown in Fig. 6.7. The holes in the bottom section were filled with 1:16 dilution of WCMV antiserum; holes in the top section were filled (from left to right) with a series of twofold dilutions of crude sap from plants infected with WCMV (Van Regenmortel, 1966b).

gelbrecht, 1963; Van Regenmortel, 1964a). The size of viral antigens can also be estimated from the diffusion coefficients measured by this technique, as shown in Fig. 6.9 (Van Regenmortel, 1959; Tremaine and Willison, 1961; Van Regenmortel and Engelbrecht, 1963; Allen and Tremaine, 1965).

4. Double Diffusion in Plates

This procedure, often referred to as the Ouchterlony method, is one of the most widely used techniques in plant virus serology. In addition to its simplicity and economical use of reagents, its main advantage is that it can provide a visible demonstration of the relationships that exist between antigens.

a. Procedure

The test can be performed either in petri dishes or on microscope slides. The gel is usually 0.7–1.5% agar or agarose in any suitable buffer. Gelatin gels have been found to be unsuitable for work with TMV (Dudman, 1965). Wells can be formed in the agar layer by positioning templates on the plate before pouring the agar, or by using gel cutters after the agar has set. Agar plugs can be removed by suction. Different well patterns suitable for various types of analysis have been described (Ouchterlony, 1968; Crowle, 1973). A pattern commonly used has a central well of 4 mm diameter, surrounded by 8 peripheral wells of 4 mm diameter at a distance 3 mm from the edge of the central one. When glass petri dishes are used, the solutions put into the wells tend to ooze under the agar, but this can be prevented by making the glass nonwettable with a silicone coat. This difficulty does not arise when plastic petri dishes are used. During the development of precipitin lines, evaporation of the solutions should be prevented since

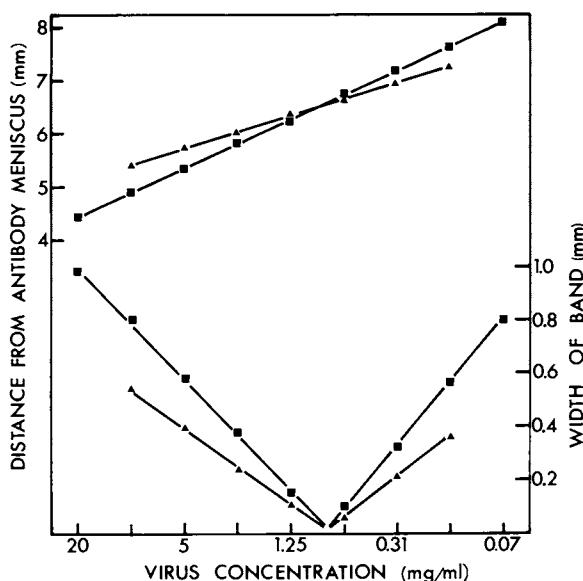


Fig. 6.9. Determination of diffusion coefficient of BMV by a double diffusion test with the apparatus shown in Figs. 6.7 and 6.8. Readings were taken after 3 days (\blacktriangle) and after 6 days (\blacksquare). From the position of the band at optimal proportions (where all the lines intersect, i.e., at 0.8 mg/ml virus), the diffusion coefficient can be calculated. In this text $x_g + x_b = 10.2$ mm (see text). D_g was found to be 1.49×10^{-7} cm 2 sec $^{-1}$ and the diameter of the virus particle was calculated to be 29 nm (Van Regenmortel, 1966b).

concentration changes may cause artifacts. This is best done by pouring a layer of liquid paraffin or light mineral oil over the gel surface. Precipitin lines can be clearly seen by examining the dish against a dark background over a box with a circular light source. Records of precipitin lines may be obtained by simple contact printing onto ordinary photographic paper (Almeida *et al.*, 1965) or by photography using annular illumination and ordinary or ultraviolet light (Thomson, 1964). Precipitin lines can also be stained with a variety of stains (Crowle, 1973; Simmonds and Cumming, 1979).

b. Precipitation Patterns

When cylindrical wells and a balanced system of reactants are used, the position and curvature of precipitin lines is a clear indication of the size of the reacting antigen. An antigen such as fraction I protein, with a diffusion coefficient of the same order of magnitude as that of rabbit IgG, will form a straight precipitin line situated midway between the two wells (Fig. 6.10). An antigen such as a monomeric viral protein subunit with a diffusion coefficient of about 10 Fick units (10^{-7} cm 2 sec $^{-1}$) will form a line further away from the antigen well

that curves around the antiserum well. Virus particles with diffusion coefficients of 0.3–1.6 Fick units will form lines near the antigen well and will curve around it (Fig. 6.10). These characteristic positions and curvature effects are much reduced or abolished when the reactants are present in extremely unbalanced conditions. In this case the zones of precipitation broaden and move away from the well containing the reactant in excess.

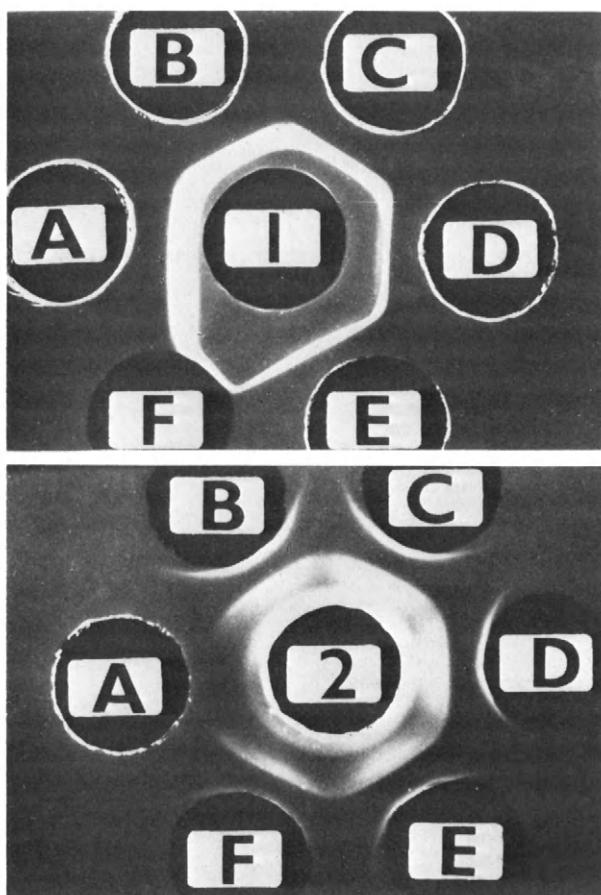


Fig. 6.10. Double diffusion tests illustrating the influence of antigen size on the appearance of precipitin lines. In the top section the central well 1 was filled with an antiserum to fraction I protein of *Cucurbita pepo*. Surrounding wells A to E contain serial twofold dilutions of *C. pepo* plant sap. In the bottom section, central well 2 was filled with PVX antiserum. Surrounding wells B to F contain serial twofold dilutions of crude tobacco sap from plants infected with PVX. Well A contains sap from a healthy tobacco plant. Note the progressive disappearance of the precipitin band formed by intact PVX with increasing dilution. The second precipitin band near the central well is caused by PVX subunits.

When the reactants are initially present in optimal proportions in their respective wells, the precipitin line will remain thin and sharp and will have maximum intensity. This line acts as an immunospecific barrier for the two reactants and only allows the crossing of unrelated antigen and antibody molecules. This selective permeability is only absolute when the two reactants are present in equivalent concentrations.

When two antigens diffuse from neighboring wells in a gel toward the same antibody source, different precipitation patterns can be observed at the position where the lines meet. Three basic patterns have been recognized, namely coalescence, partial fusion (or spur formation), and crossing of precipitin lines (Figs. 6.10 and 6.11). These patterns have also been described as representing reactions of identity, partial identity, and nonidentity of the corresponding antigens (Ouchterlony, 1968). These labels, however, represent an interpretation of the likely properties of the antigens based on the appearance of the precipitation patterns, and in many instances, the interpretation is in fact erroneous. For instance, when two identical antigens present at different concentrations are allowed to diffuse from neighboring wells, patterns of partial fusion or intersection instead of coalescence can be obtained (Feinberg, 1957). The use of descriptive names instead of interpretative labels will help to minimize confusion by clearly differentiating between observation and interpretation.

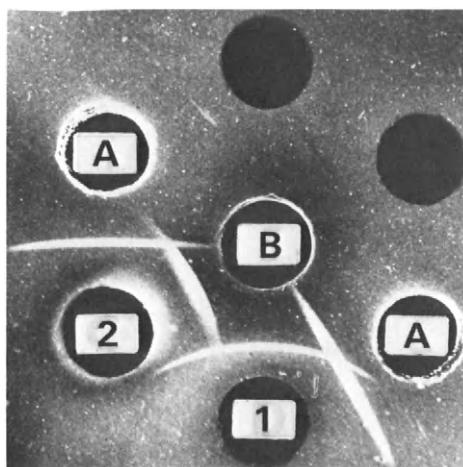


Fig. 6.11. Double diffusion test illustrating the pattern of partial fusion and crossing of precipitin lines. Well 1 contains a mixture of WCMV and fraction I protein of *C. pepo*; well 2 contains a mixture of TYMV and fraction I protein. Both wells A have been filled with antiserum to fraction I protein and well B with antiserum to WCMV. The crossing of precipitin lines caused by fraction I protein and virus demonstrates that the two reacting antigens are unrelated. The partial fusion of the lines caused by TYMV and WCMV demonstrates that these two viruses are serologically related.

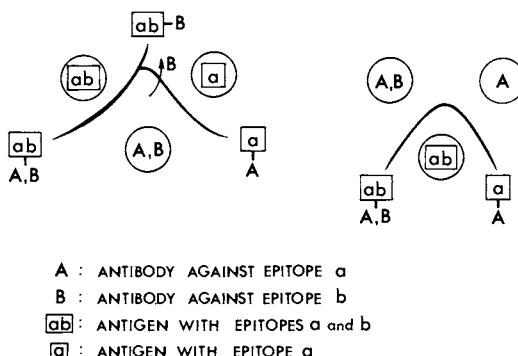


Fig. 6.12. Two double diffusion tests illustrating the importance of the correct placement of reactants for reliable antigenic comparisons. On the left side, the spur caused by the reaction between antibodies B and antigen ab demonstrates that the two antigens a and ab are related but not identical. On the right, the pattern of coalescence does not imply that the antigens a and ab used for obtaining the two antisera are identical. The two epitopes a and b are present on the same molecule and cannot diffuse independently to form a spur.

With elongated viruses that diffuse poorly in gels, precipitin lines with a pronounced curvature are formed very close to the antigen well (Fig. 6.10). In such a case, lines from neighboring antigen wells may still have a chance to fuse, and conclusions regarding possible relationships may still be made, if the antigen wells are placed very close to one another.

It is important to recognize that useful information is obtained only when two antigens are tested against a single antiserum. When two antisera prepared against different cross-reacting antigens are tested against only one of the antigens, a pattern of coalescence of precipitin lines is always obtained (Jennings, 1956; Grogan *et al.*, 1964; Van Regenmortel, 1966b; Scott, 1973). The fusion of precipitin lines in this case simply reflects the fact that different antigenic regions on the surface of the virion cannot diffuse independently. This phenomenon, illustrated in Fig. 6.12, is not always appreciated. As a result, the misnomer "reaction of identity," used to describe the merging of precipitin lines, sometimes leads authors to the erroneous conclusion that two related antigens are serologically identical.

c. Intragel Cross-Absorption

Serological comparisons between related antigens often require that antisera be cross-absorbed with heterologous antigens. This is most conveniently done by the procedure known as intragel absorption (Feinberg, 1957; Van Regenmortel, 1967). The method is illustrated in Fig. 6.13. The antigen preparation used for absorption is allowed to diffuse into the gel from the central well and will thereby establish a concentration gradient around the well. When at a later stage, the

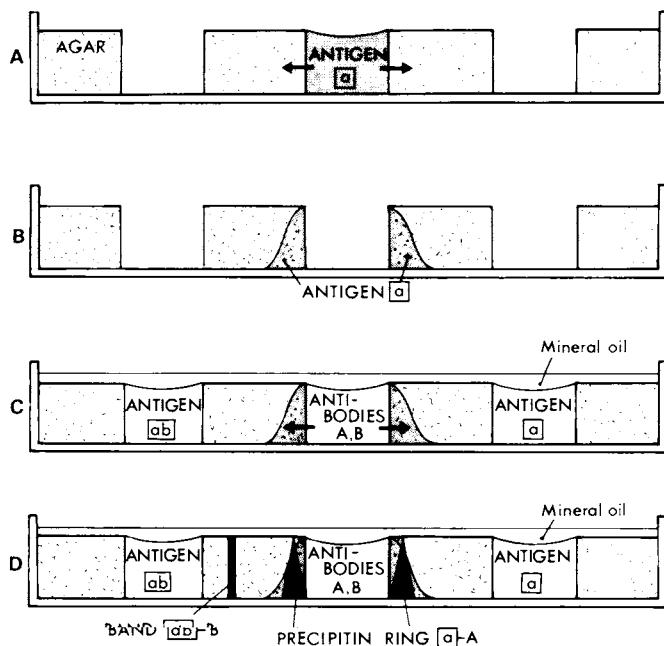


Fig. 6.13. Intragel cross-absorption test. In stage A, the cross-absorbing or heterologous antigen a is allowed to diffuse from the central well. After 18 hours, a concentration gradient of this antigen has been established inside the agar (stage B). The antiserum prepared against antigen ab is then placed in the central well and the homologous and heterologous antigens placed in surrounding wells (stage C). A layer of mineral oil is added to prevent evaporation. Antibodies A are fully precipitated in the immediate vicinity of the central well as they encounter the optimal concentration of the heterologous antigen. This ring of precipitation is permeable to antibodies B which form a precipitin band with the homologous antigen (stage D) (from Van Regenmortel, 1967).

antiserum is allowed to diffuse from the same well, the cross-reacting antibodies will be fully precipitated in the gel adjacent to the well. Unabsorbed antibodies will diffuse freely and will form a normal precipitin line with the homologous antigen. The results of intragel cross-absorption experiments with several TMV strains are illustrated in Fig. 6.14. This method of serological absorption is very convenient for demonstrating the presence of separate antigenic specificities in different virus strains (Van Regenmortel, 1966b, 1967; Wang and Knight, 1967; Wetter and Luisoni, 1969; Granett and Shalla, 1970; Gotlieb and Berbee, 1973; Jones and Diachun, 1977) and it represents the simplest way of identifying an individual serotype (Kado and Knight, 1968; Wetter and Bernard, 1977; Van Regenmortel, 1981a). The method of intragel absorption has also been used for establishing that the capsids of some viruses possess antigenic determinants that are absent on the corresponding depolymerized protein subunits (Van Regenmortel, 1966c).

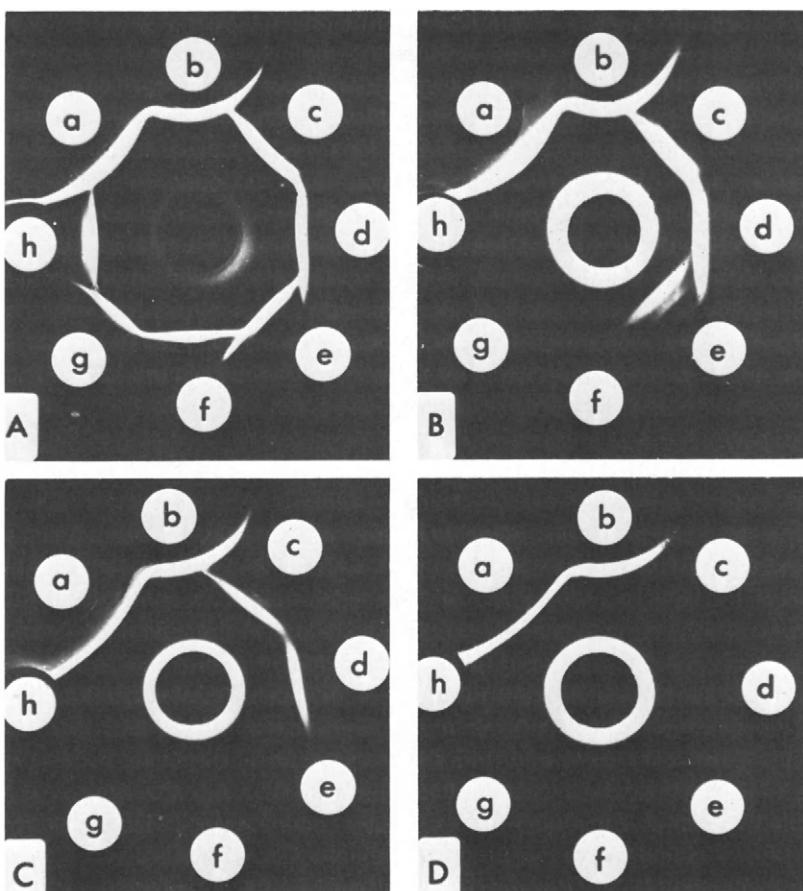


Fig. 6.14. Results of intragel cross-absorption experiments with TMV strains and mutants. The different strains have been arranged clockwise (starting in well a) in order of decreasing relationship to the homologous strain. (A) Central well filled with antiserum to U2 strain of TMV. Well a, U2; b, G-TAMV; c, Y-TAMV; d, SJ; e, TMV; f, mutant 371; g, mutant 414; h, HR. Wells a-f contain the same antigens in B, C, and D. (B) Central well filled initially with mutant 414, then 24 hours later with U2 antiserum. (C) Central well filled initially with TMV then 24 hours later with U2 antiserum. (D) Central well filled initially with Y-TAMV, then with U2 antiserum (from Van Regenmortel, 1967).

tel, 1966b, 1978) as well as for demonstrating the presence of heteroclitic antibodies in TMV antisera (Van Regenmortel, 1966b, 1967; Chapter 10).

d. Tests with Degraded Viruses

Filamentous viruses with a length above 500 nm diffuse poorly in 0.5–1% agar gel. In order to make the many viruses that are even longer amenable to im-

munodiffusion analysis, many workers used chemical treatments to dissociate the virions into smaller diffusible fragments down to the level of single protein subunits. Sodium dodecyl sulfate (SDS) (Gooding and Bing, 1970; Garnsey *et al.*, 1979) and several other dissociating agents (Purcifull and Gooding, 1970; Shepard and Shalla, 1970; Langenberg and Ball, 1972) have been used for this purpose in recent years. Purcifull and Batchelor (1977) have listed 35 plant viruses that have been dissociated with SDS in order to permit immunodiffusion studies.

Serological relationships between several potyviruses have been studied with antisera prepared against their dissociated protein subunits. In many cases it was found that the subunits from different viruses were more closely related serologically than the corresponding intact virions (Shepard *et al.*, 1974a). This may be due to the fact that a larger surface area of the viral subunit is immunochemically expressed in the monomer, compared with the capsid (Chapter 7). Another possible contributory factor may be that the partial denaturation of the protein uncovers internal sequence homologies that are buried in the native molecule (Arnon and Maron, 1971).

In many cases, the dissociated viral proteins react as well with antisera prepared against untreated virus as with antisera prepared against viral proteins dissociated by various treatments. Sometimes, however, antisera to the whole virus do not react with the dissociated subunits, in which case it is necessary to immunize animals with the chemically degraded protein. With TurMV, it was found that degraded protein obtained by pyrrolidine treatment had different antigenic properties from protein prepared with SDS (Hiebert and McDonald, 1976).

For the detection of many viruses, the test can be carried out simply by placing crude sap from infected plants into wells cut in agar containing a dissociating agent. A commonly used medium consists of 0.8% agar, 0.5% SDS, and 1% sodium azide (Purcifull and Batchelor, 1977). Less sodium azide may be used, but sodium chloride must then be added to the medium to raise the ionic strength. With some viruses it may be necessary to add the dissociating agent to the crude extract or even to boil the treated plant extract before introducing it into the wells. Lyophilization of crude extracts from infected plants has been found to be a convenient way of maintaining a bank of reference viral antigens for this type of immunodiffusion test (Purcifull *et al.*, 1975b).

In tests with chemically degraded viruses, nonspecific precipitation occurs more frequently than with other immunodiffusion tests. Nonspecific reactions are especially prevalent when SDS-treated antigens are tested against sera in agar media which lack SDS (Palmer *et al.*, 1971; Cho and Feng, 1974; Luisoni *et al.*, 1975). These unwanted reactions seem to be caused by the interaction of SDS with certain antiserum components and can be prevented by using purified immunoglobulins instead of whole antiserum. Lectins in potato tuber extracts may

also produce nonspecific precipitin lines that resemble normal antigen-antibody reactions (Shepard, 1970a).

Another application of immunodiffusion tests with degraded viruses is to be found in the analysis of proteins extracted from gels after polyacrylamide gel electrophoresis (Shalla and Shepard, 1970a; Wolf and Casper, 1971; Shepard and Secor, 1972; Purcifull *et al.*, 1973; Hiebert and McDonald, 1973). It is in fact not necessary to elute the protein from the gel beforehand since pieces of the polyacrylamide gel containing the antigen can be introduced directly in suitably cut wells of the immunodiffusion plate.

C. IMMUNOELECTROPHORESIS

Immunoelectrophoretic techniques are one of the most powerful analytical tools for resolving complex mixtures of antigens as they differentiate between antigens on the basis of two independent criteria: electrophoretic mobility and antigenic specificity. The antigen mixture is first separated into its components by electrophoresis in agar gel. Antiserum is then placed in a trough parallel to the path of electrophoretic migration and immunodiffusion precipitin lines are allowed to develop.

Alkaline buffers of pH 7.5-8.6 are mostly used, and under these conditions proteins and viruses will be negatively charged and move toward the anode (Van Regenmortel, 1972). However, a phenomenon known as electroendosmosis may cause some negatively charged proteins to migrate toward the cathode. Electroendosmosis arises as a result of the negative charges present on one of the two constituents of agar: the sulfated polysaccharide agarpectin. The other constituent of agar is the uncharged galactose polymer agarose (Hjerten, 1961; Brishammer *et al.*, 1961). The negatively charged agarpectin induces a flow of liquid, together with all substances dissolved in it, toward the cathode. The electroendosmotic flow reduces the anodic mobility of all antigenic components of the mixture, and in the case of weakly charged antigens, it may lead to a net cathodic mobility. Agaroses with different amounts of residual charges that produce various degrees of electroendosmosis are available commercially and can also be prepared from agar by precipitation with polyethylene glycol (Russell *et al.*, 1964). The use of agarose instead of agar for immunoelectrophoresis experiments is not necessarily always an advantage, since electroendosmosis sometimes leads to a superior separation of the various components.

Some applications of immunoelectrophoresis include the characterization of plant viruses (Devergne and Cardin, 1967a; Wagner and Bancroft, 1968), the differentiation between virus strains, e.g., with TBSV (Bercks and Lovisolo, 1965; Hollings and Stone, 1975), SBMV (Grogan and Kimble, 1964), TNV (Babos and Kassanis, 1963), red clover mottle virus (RCMV) (Devergne and

Cardin, 1968), PSV (Devergne and Cardin, 1976), the differentiation between complete virions and their dissociated protein subunits (Hamilton, 1961; Allen and Tremaine, 1965; Atabekov *et al.*, 1968a; Rappaport and Zaitlin, 1970; Van Regenmortel and Lelarge, 1973), and the control of virion integrity following various treatments (Koenig, 1969b, 1970; Paul and Querfurth, 1979).

1. Procedure

A convenient apparatus to use is the LKB 6800 immunoelectrophoresis apparatus, which allows the simultaneous handling of 18 microscope slides. About 10 ml of 1–1.5% agar or agarose in buffers of ionic strength 0.05–0.025 is spread evenly over a row of three slides. When the gel has set, 2–5 μ l of the antigen suspension is placed in small wells that are positioned centrally on the slide. Contact between the buffer vessels and the slides can be made with cellulose acetate wicks impregnated with buffer. The voltage gradient across the length of three 2.5×7.5 -cm slides can be measured with a voltmeter and should be 2–6 V/cm. The precise voltage, amperage, and length of time needed (1–3 hours) depend on the buffer used and on the antigens being separated, and must be determined empirically for each system. The strength of the buffer must be a compromise between the need to have enough ions to prevent local variations in pH and the need to keep ionic strength sufficiently low to avoid excessive conductivity and the concomitant heating and low particle mobility (Polson and Russell, 1967; Williams, 1971; Van Regenmortel, 1972). Some buffers that have given satisfactory results in studies with plant viruses are 0.025 M acetate-NaCl, pH 5–5.7 (Wagner and Bancroft, 1968); 0.025 M Tris-barbital, pH 8.8 (Van Regenmortel and Lelarge, 1973); 0.005 M phosphate, pH 7; 0.025 M Tris-HCl, pH 8.2 (Paul and Querfurth, 1979). The buffer in the electrophoresis vessels should have twice the molarity of the buffer in the gel. A dye such as bromophenol blue can be used as a reference for comparison between different runs. At the termination of the electrophoretic separation, antiserum is placed in lateral troughs cut in the gel. When precipitin lines have developed, the slides can be washed to remove unreacted protein and stained with Coomassie blue or Amido black (Crowle, 1973). Immunoelectrophoretic separations of different polymerization states of TMV protein are illustrated in Fig. 6.15.

2. Immuno-osmophoresis

This technique, also known as electrosyneresis or counter-immunoelectrophoresis, is based on the migration of negatively charged antigens toward the anode and the simultaneous movement of antibodies toward the cathode by electroendosmotic flow (Ragetli and Weintraub, 1964; John, 1965). The reactants are placed in small wells cut in a gel such as 1% agarose and

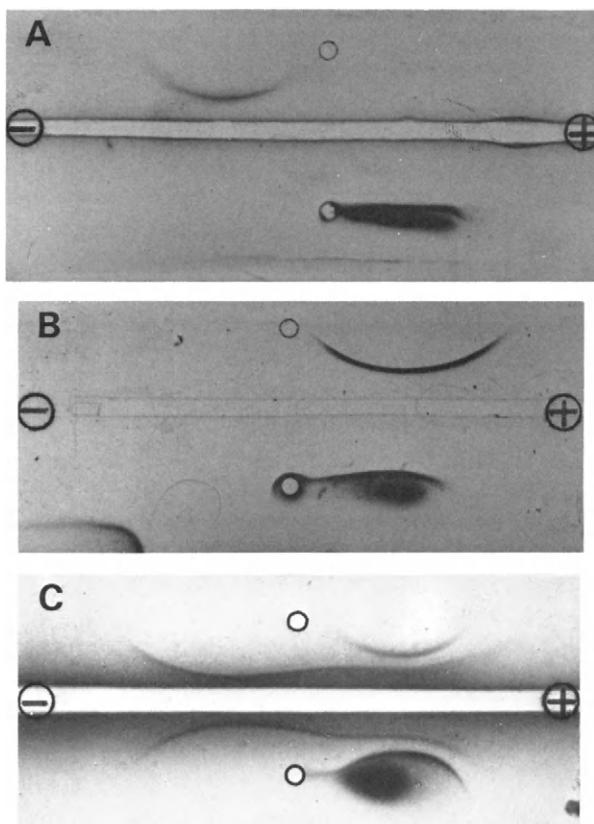


Fig. 6.15. (A) Immunoelectrophoresis in agar in 0.005 M, pH 7 phosphate buffer of TMV protein (250 µg/ml) in upper well and of formalinized TMV (5 mg/ml) in bottom well. TMV antiserum (undiluted) was placed in the central trough. The migration of the protein subunits toward the cathode is due to electroendosmosis. (B) Immunoelectrophoresis in agar in 0.005 M phosphate buffer pH 7. Upper well contains a preparation of TMV protein disks (4 mg/ml) and the bottom well TMV (10 mg/ml). Antiserum prepared against formalinized TMV was placed in the trough. Note the change in mobility of the protein upon aggregation to 20 S disks. (C) Immunoelectrophoresis in agar in 0.025 M barbital buffer, pH 8.8. Same antigens as in B. Antiserum prepared against unstabilized TMV was placed in the trough. Both the disks and virus particles partly dissociated at pH 8.8 during the course of the experiment and gave rise to a trailing precipitin line caused by protein subunits (from Van Regenmortel and Lelarge, 1973).

separated by a distance of about 2 cm. The gel-coated slides are connected to buffer vessels by means of filter paper strips. When a pH of 6-7 and a field strength of 10-12 V/cm are used, precipitin lines usually appear between the wells within 5-60 minutes. The main advantages of this technique are its rapidity and a sensitivity of detection of about 0.1 µg virus (Ragettli and Weintraub, 1965; Weintraub *et al.*, 1967; Clark and Barclay, 1972; Grauballe *et al.*, 1977).

3. Rocket Immunoelectrophoresis

This is a quantitative method for the measurement of antigen concentration (Laurell, 1966; Weeke, 1973) in which the antigen is allowed to migrate in an electric field in a layer of antibody-containing agarose. Antigen samples to be compared are placed in a series of small wells cut side by side in 1% agarose gel at pH 8.6 in which antiserum has been incorporated. When an electric current is applied (4–10 V/cm for 2–6 hours), the migration of the antigen toward the anode leads to the formation of rocket-shaped precipitation patterns. When all the antigen at the moving front has been complexed with antibody, the precipitation line remains stationary and the area under the rocket is proportional to the antigen concentration. The concentration of virus samples can be calculated from a standard curve based on known dilutions of a reference preparation (Havranek, 1978c). The extent to which the antiserum must be diluted may vary considerably and has to be determined empirically (Havranek, 1978a). The height of rockets is inversely proportional to the concentration of antibody in the gel. The method has been used with CMV and TMV (Havranek, 1978b).

A variation of the rocket electrophoresis procedure is the crossed immunoelectrophoresis method. This technique 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. This method is particularly suited to the analysis of very complex antigen-antibody systems and has been used for instance for separating the various antigenic components of herpes simplex virus (Vestergaard, 1975; Vestergaard *et al.*, 1977).

D. AGGLUTINATION

It is customary to speak of agglutination instead of precipitation when the size of the reacting antigenic particle is approximately equal to that of a cell. When either the antigen or the antibody is attached to the surface of red blood cells or carrier particles of similar size (latex, bentonite, barium sulfate), it is possible to induce visible serological clumping with a lower concentration of reactants than is needed for precipitation.

1. Passive Hemagglutination

Passive hemagglutination, also called indirect hemagglutination, utilizes erythrocytes to which virus particles or antibodies have been coupled by various chemical treatments. Prior to the coupling reaction the cells are stabilized with tannic acid (Stavitsky, 1977), sulfosalicyl acid (Becht, 1968), formaldehyde, or glutaraldehyde (Daniel *et al.*, 1963; Becht and Malole, 1975). Stabilized cells may be kept in the cold for several months. Virus particles may be adsorbed to

the cells simply by mixing the tanned cells with a virus preparation (Cunningham *et al.*, 1966; Richter, 1969; Reddy *et al.*, 1969) or by using coupling reagents such as bisdiazobenzidine (Anderer *et al.*, 1971b; Arquilla, 1977) and carbodiimides (Johnson *et al.*, 1966). Bisdiazobenzidine should be used with care as it is carcinogenic.

Virus-coated erythrocytes can be agglutinated by specific antibody and this reaction can be inhibited by prior incubation of the test serum with a suspension of the homologous virus. Such an inhibition of passive hemagglutination lends itself to the measurement of very small amounts of viral antigen.

Alternatively, when red cells coated with antibody are used, it is possible to detect the presence of as little as 10^{-5} mg of virus (Abu-Salih *et al.*, 1968a). With erythrocytes sensitized with antibody it is also possible to determine antiserum titers by hemagglutination inhibition tests.

Compared with tube precipitin tests, the increase in sensitivity for detection of elongated and isometric viruses is approximately 100- and 500-fold, respectively (Saito and Iwata, 1964; Richter, 1971). Antiserum titers up to 1000-fold higher than corresponding precipitin titers can also be obtained (Richter, 1967; Anderer and Ströbel, 1972a,b; Ghanekar *et al.*, 1979). The sensitivity of the passive hemagglutination technique makes it well-suited for studying weak cross-reactions between viruses and for demonstrating the weak antigenic activity of short peptides that correspond to antigenic determinants of a viral protein. Gamez *et al.* (1967) used the technique to demonstrate that there was no serological relationship between wound tumor and animal reoviruses. Anderer and Ströbel (1972a,b) coupled the C-terminal hexapeptide of TMV protein to erythrocytes and were able to detect antibodies reactive with the hexapeptide in TMV antisera.

Procedure

Fresh sheep red blood cells are washed several times with saline and a volume of 25 ml of cells is suspended in 200 ml phosphate-buffered saline (PBS), pH 7.2. A dialysis bag filled with 50 ml of 35% formaldehyde is submerged in the cell suspension for 3 hours. After this time the bag is punctured and the cells are left overnight at room temperature. The cells are then washed several times with saline and brought to a final concentration of 25% suspension of cells in saline for storage at 4°C.

When tannic acid is used for coupling, 1 ml of 0.1 mg/ml tannic acid is added to a 2.5% suspension of cells resuspended in PBS pH 7.2. After 10 minutes incubation at room temperature the cells are washed with PBS, resuspended in 1 ml and mixed with an equal volume of 0.5 mg/ml of a purified virus preparation, or with 1 ml of 0.1 mg/ml of specific IgG. For different plant viruses, the optimal pH for the coupling reaction varies between 5.0 and 8.0 and the pH can be adjusted by diluting the cells in PBS of a suitable pH. After 30 minutes incubation, the mixture is centrifuged and the sediment is washed twice with normal

rabbit serum diluted 1:200 in PBS. The final concentration of erythrocytes should be 1.25–2.5% in 1:200 dilution of normal rabbit serum.

When bis-diazotized benzidine is used for coupling (Herbert, 1978), 46 mg of benzidine is dissolved in 9 ml of 0.2 N HCl and cooled in an icebath. A solution of 35 mg NaNO₂ in 1 ml distilled water is also cooled and added to the benzidine solution with constant stirring. After 30 minutes, the mixture is distributed in 0.5-ml quantities in small tubes that are sealed after freezing at –20°C. The coupling reaction is performed by mixing equal volumes of 2.5% formalinized cells and of a virus preparation with 0.5 ml of the diluted benzidine reagent. Optimal dilution of the benzidine reagent as well as the optimal virus concentration have to be determined in each system. With TMV, best results were obtained with a 2 mg/ml purified virus preparation and 1:25 dilution of the benzidine reagent. After 15 minutes of coupling time, the cells are washed and suspended in 1:200 dilution of normal rabbit serum.

The passive hemagglutination test is usually performed in microtiter plates using a grid titration. Volumes of 50 µl of antiserum dilutions and of 1% suspension of virus-coated cells are added to each well. For inhibition studies, the two reactants are incubated for 1 hour prior to addition to the erythrocyte suspension. The plates are covered and read after overnight incubation. Erythrocytes that did not agglutinate will settle to the deepest point of the conical well (Fig. 6.16) and will form a small button. Agglutinated cells will form a network and will cover a large area of the well surface. It is important to include appropriate controls in the test to establish the specificity of the observed reactions (Abu-Salih *et al.*, 1968a).

2. Latex Test

In this test either the antigen or the antibody is adsorbed onto commercially available polystyrene latex particles (Difco Laboratories, Detroit, Michigan, or Sigma Chemical Co., St. Louis, Missouri). Optimal attachment of antibodies to

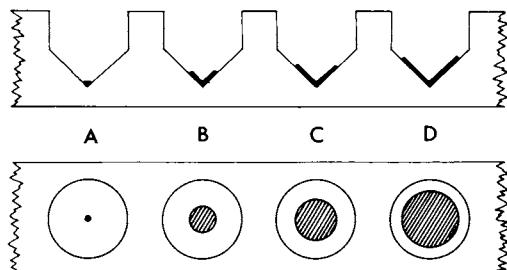


Fig. 6.16. Hemagglutination patterns of erythrocytes formed in the wells of a microtitration plate. In (A) the small button of sedimented cells indicates a negative reaction. (B), (C), and (D) correspond to increasing degrees of cell agglutination.

the latex occurs only within a narrow range of concentration of antiserum or purified immunoglobulin (Bercks, 1967a; Mumford, 1977; Litwin, 1977). Not all IgG preparations obtained by ammonium sulfate precipitation are equally effective for sensitizing latex particles, mainly because of variable degrees of contamination with albumin, α - and β -globulins (Bercks and Querfurth, 1969a). When IgG purified by preparative electrophoresis or by the Cohn fractionation method was used for sensitization, the test was found to be more specific and more sensitive (Bercks and Querfurth, 1969a; Augier de Montgrémier and Larroque, 1972).

Using antibody-coated latex, several authors succeeded in detecting 100- to 1000-fold smaller quantities of virus than was possible by microprecipitin tests or immunodiffusion tests (Bercks, 1967a; Aapola and Rochow, 1971; Koenig and Bode, 1978; Koenig *et al.*, 1979). Although other workers failed to obtain such a large increase in sensitivity of virus detection (Abu-Salih *et al.*, 1968b; Maat, 1970; Oertel, 1977a,b), there is general agreement that the method is well suited to the routine detection of viruses in crude plant extracts (Schade, 1971; Schade and Schimanski, 1974; Lundsgaard, 1976; Fuchs, 1976; Koenig and Bode, 1978; Khan and Slack, 1978).

Virus particles can also be attached to latex and such virus-coated particles have been used to detect distant serological relationships in the potex- and tymovirus group (Bercks and Querfurth, 1971a). Best results were obtained when an excess of antigen was adsorbed onto latex via an intermediate layer of previously adsorbed antibodies. Homologous antiserum titers showed a maximum increase of 300-fold over titers obtained in microprecipitin and immunodiffusion tests. In contrast, heterologous titers did not increase as much as homologous titers, and in some cases, heterologous titers did not increase at all when unfractionated antisera were used. This may be due to the fact that, in heterologous combinations, the reactions are inhibited by the large amount of interfering proteins present at the low dilutions of antiserum used (Bercks and Querfurth, 1971a).

The unsuitability of low titered antisera for the latex test can be overcome by coupling antibody molecules to latex via an intermediate layer of staphylococcal protein A (Querfurth and Paul, 1979; Torrance, 1980a). This modification of the test has been called PALLAS (for protein A-coated latex-linked antiserum) and is easier to perform, mainly because the amount of antibody needed for sensitization of the latex is not critical. Compared with the passive hemagglutination technique, the main advantages of the latex test are the ready commercial availability of the carrier particles and the long storage life of the sensitized reagent.

Procedure

A suspension of latex particles (about 0.8 μm diameter, Difco Laboratories) is diluted 1:15 with saline, and equal volumes of this suspension and suitably

diluted purified immunoglobulin are incubated for 1 hour. The optimal dilution of the globulin fraction of antiserum to be used for sensitization lies in the region 1:50 to 1:2000 and is determined by trial and error. The diluent is 0.05 M Tris-HCl buffer, pH 7.2. The sensitized latex is recovered by low-speed centrifugation and washed twice in an equal volume of 0.05 M Tris-HCl buffer, pH 7.2, containing 0.02% polyvinylpyrrolidone as a stabilizer. The final sediment is suspended in the same buffer containing 0.02% sodium azide, and is brought to a volume identical to that of the diluted immunoglobulin originally used. The

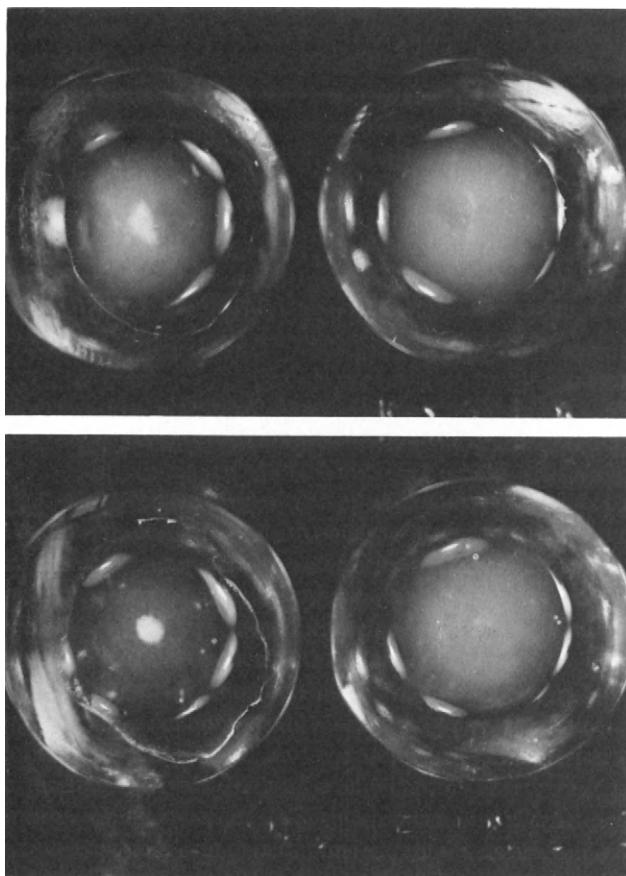


Fig. 6.17. Results of latex flocculation tests with ApMV. A volume of 0.025 ml of latex (sensitized with globulin obtained from an antiserum to ApMV) was mixed with 0.05 ml of plant sap (diluted 1:16) from healthy cucumber (right) and cucumber infected with ApMV (left). (Top) Flocculation observed 10 min after mixing. (Bottom) Flocculation observed 1 hour later, showing the settling of floccules. Note that the appearance of the controls on the right remains unchanged (photographs by courtesy of Dr. R. M. Lister).

sensitized latex preparation can be stored for more than 6 months in the cold without any loss in activity.

The latex test is performed in plastic microtiter plates by mixing 50 μl of the antigen preparation (crude sap or purified preparation) with 25 μl of the sensitized latex suspension. Antigen dilutions are made up with Tris-HCl buffer, pH 7.2, containing 0.02% polyvinylpyrrolidone. The plates are shaken for 15 minutes on a rotary shaker at 200 rpm. Results can be read immediately, although clearer agglutinations are visible after about 1 hour. Positive reactions appear as distinct flocculations of white latex particles that are clearly visible against a black background (Fig. 6.17).

Instead of using the wells of polystyrene plates, the test can also be performed in 100 λ disposable capillary pipes by mixing 10 μl of sensitized latex with 20 μl of antigen suspension (Marcussen and Lundsgaard, 1975; Khan and Slack, 1978). The agglutinations are observed under a dissecting microscope with dark-field illumination.

3. Bentonite Flocculation Test

In this test, either antigen or antibody is adsorbed to sodium bentonite particles (Bozicevich, 1977). The bentonite suspension is prepared by mixing 0.5 g of bentonite with 100 ml distilled water in a blender. After washing several times with water, the bentonite suspension is mixed either with a suitably diluted immunoglobulin preparation or with an antigen suspension (Bozicevich *et al.*, 1960, 1963; Bercks, 1967a). A 0.1% solution of methylene blue is added to increase the visibility of subsequent positive agglutinations. The sensitized bentonite suspension is washed twice with 0.02 M phosphate-buffered saline, pH 7.2, and finally resuspended in the same buffer containing 0.02% polyvinylpyrrolidone. The test is performed in plastic microtiter plates by mixing two volumes of the antigen or antiserum with one volume of sensitized bentonite suspension. After shaking for 10 minutes, the wells are examined at low magnification for the appearance of flocculation.

The test, which is slightly less sensitive than the latex test, has been used mainly for detecting viruses in crude plant extracts (Scott *et al.*, 1964; Kahn *et al.*, 1967; Abu-Salih *et al.*, 1968a; Maat, 1970).

E. COMPLEMENT FIXATION

1. Principle of Complement Fixation Tests

Complement is a multicomponent system of enzymatically active proteins, which is present in an inactive state in any serum. This system becomes activated

when a specific site of the Fc portion of certain antibody molecules becomes exposed as a result of antigen binding, and is then able to bind the Clq component of complement (Osler, 1976). When the antibody is directed against membrane antigens of erythrocytes, the binding of the Clq component leads to a cascade of enzymatic reactions, which finally result in the lysis of the cells and a concomitant release of hemoglobin. This phenomenon is known as immune hemolysis.

When the antibody is directed against a virus particle, complement will also be irreversibly bound upon formation of the virus–antibody complex; this leads to the effective removal of complement from the antiserum. When a second indicator system consisting of erythrocytes and antierythrocyte antibodies is now added to the virus system, the disappearance of complement as a result of the virus–antibody reaction will be revealed by the absence of red cell lysis. The extent to which complement has been used up in the first reaction can be quantitated by measuring the amount of lysis that any unused complement left in the system is able to produce. The amount of hemoglobin released from the lysed cells can be measured photometrically and is inversely proportional to the extent to which virus and antibody have interacted and fixed complement. The absence of a reaction in the virus system is indicated by complete cell hemolysis, whereas increasingly positive reactions result in diminishing amounts of hemolysis. It is clear that the variable amounts of endogenous complement present in both the test antiserum and antisheep erythrocyte serum have to be destroyed beforehand, usually by heating to 56°C. The assay is standardized by adding to the test system an amount of guinea pig complement just sufficient to cause complete lysis of the red cells present in the indicator system.

The attachment of at least two adjacent IgG molecules to a multivalent antigen molecule is required for the activation of complement. The binding of IgG to monovalent haptens, for instance, does not lead to complement fixation. However, the antigenic activity of monovalent fragments of viral proteins can be measured by their ability to inhibit complement fixation by the intact antigen. Such inhibition of complement fixation assays has been used to localize epitopes in viral coat proteins (Benjamini *et al.*, 1964; Milton and Van Regenmortel, 1979; Pratt *et al.*, 1980). The complement fixation curves observed with constant antibody and increasing amounts of antigen are similar to quantitative precipitin curves with zones of antibody and antigen excess. However, their characteristic bell-shaped appearance is not conditioned by the same factors that govern the shape of precipitin curves. When the complement fixation curve obtained with TMV (Fig. 6.18) is compared with the precipitin curve for the same virus (Fig. 6.3), it is clear that the two assays are measuring different parameters. The rapid decrease in complement fixation when more antigen is added is probably caused by the diminished probability for two IgG molecules to bind in close proximity to each other. The presence of bound adjacent antibody molecules is essential for complement fixation. In the case of viral protein

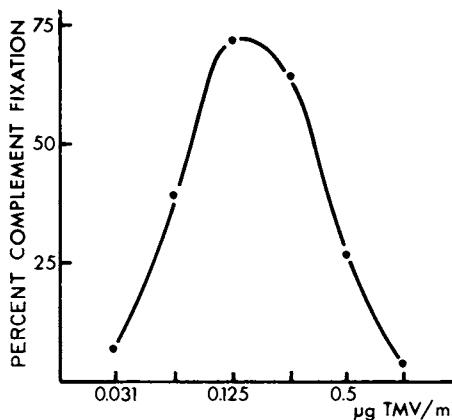


Fig. 6.18. Complement fixation curve of the reaction of TMV with specific antiserum (diluted 1 = 1800).

subunits where each epitope is present only once on each molecule, the peak of the complement fixation curve (Fig. 6.19) probably corresponds to aggregates of the composition $\text{Ag}_4\text{-Ab}_3$ and $\text{Ag}_3\text{-Ab}_2$. The addition of more antigen then leads to the formation of $\text{Ag}_2\text{-Ab}$ complexes where individual IgG molecules are no longer in close apposition, and thus no longer capable of fixing complement.

The complement fixation curves of TMV and TMV protein subunits are remarkably similar for antigens of such different sizes (Figs. 6.18 and 6.19). The range of antigen concentration associated with maximal fixation was approximately 0.1–1 $\mu\text{g}/\text{ml}$ antigen in both cases. When the peak height of the complement fixation curve is plotted against the logarithm of the antiserum dilution, a straight line with a slope characteristic for the particular immune system is obtained (Champion *et al.*, 1974). This slope m is defined by the equation $y = m \log x + b$, where y is percentage complement fixed at the peak of the curve, x is reciprocal of antiserum dilution, and b is the y intercept (Fig. 6.19).

2. Applications of Complement Fixation Tests

The test is used extensively in the diagnosis of virus infections of man and animals (Schmidt and Lennette, 1973; Mayr *et al.*, 1977) but has never been very popular with plant virologists. In the 1950s, semi-quantitative complement fixation tests were used by a few workers, mainly for the purpose of differentiating between virus strains (Tall *et al.*, 1949; Weaver and Price, 1952; Moorhead and Price, 1953; Moorhead, 1956, 1961; Wright and Hardy, 1961). However, the tests were fairly complicated and yielded information that could be obtained more easily by simpler methods. In subsequent years, the sensitivity and accu-

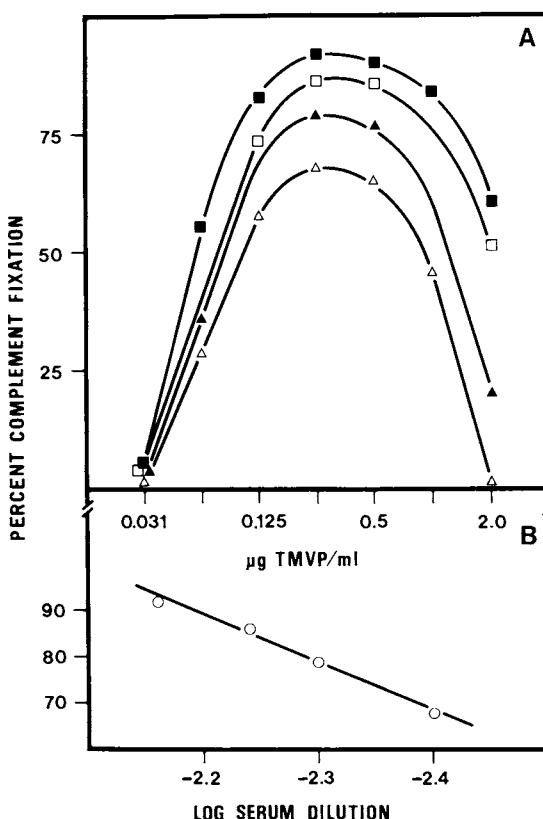


Fig. 6.19. (A) Complement fixation curve of the reaction between TMV protein and different dilutions of TMV protein antiserum. ■, 1 = 150; □ 1 = 175; ▲, 1 = 200; △, 1 = 250. (B) Dependence of the peak height of curves obtained in A on antiserum concentration. A slope $m = 111$ (see text) was calculated for the TMV protein system (from Milton and Van Regenmortel, 1979).

racy of the assay were improved (Wright, 1963; Wright and Stace-Smith, 1966), but the possibility of detecting 1 $\mu\text{g}/\text{ml}$ of virus (as compared with 1–2 $\mu\text{g}/\text{ml}$ virus in precipitin tests) must have seemed a small advantage to most workers, in view of the complexity of the test.

The real advantage of complement fixation assays over precipitin tests, namely a much greater accuracy in measuring small differences in antigen and antibody concentration, was clearly demonstrated by the results of Tremaine and Wright (1967). These authors showed that the extent of cross-reactivity between two SBMV strains, as measured by complement fixation, was strictly proportional to antiserum titer, and furthermore that the proportion of cross-reactive antibodies in the antisera increased during the course of immunization.

The very high accuracy of complement fixation data makes the assay particularly suited for the study of antigenic relationships (Prager and Wilson, 1971a,b; Champion *et al.*, 1974) and for the analysis of the structure of viral antigenic determinants (Milton and Van Regenmortel, 1979). A bewildering variety of different ways of performing the assay have been described, and this may dissuade the uninitiated from attempting to use the technique. For this reason, a detailed description of a procedure that was successfully used for analyzing the antigenic structure of plant viruses will be presented in Section E,4.

3. Some Definitions

a. *Hemolysin*

In the older literature also known as hemolytic amboceptor, hemolysin is a rabbit antiserum prepared against sheep red blood cells.

b. *Hemolytic Unit of Complement*

The CH_{50} unit or 50% hemolytic unit of complement is the amount necessary for 50% lysis of a given number of red blood cells, under standardized conditions of temperature and hemolysin concentration.

c. *Anticomplementary Activity*

Antisera are said to be anticomplementary when they are able to initiate the complement cascade reaction, i.e., to fix complement, in the absence of any specific interaction between antibody and the test antigen. Anticomplementary activity is usually caused by the presence in antisera of immunoglobulin aggregates or preformed antigen-antibody complexes. Such antisera are not suitable for complement fixation assays. Anticomplementary activity is especially prevalent at low serum dilutions, and the high dilutions at which virus antisera are normally used minimize this type of problem. It is possible to remove anticomplementary activity by a preliminary incubation of the antiserum with guinea pig complement, followed by heating at 60°C (Mayr *et al.*, 1977).

4. Procedure

a. *Preparation of Reagents*

In order to give meaningful quantitative data, complement fixation tests require careful standardization of all reagents and experimental conditions (Levine *et al.*, 1961; Levine and Van Yunakis, 1967).

i. Diluent. A stock buffer of 1.4 M NaCl, 0.1 M Tris, 0.005 M MgCl₂, and 0.0015 M CaCl₂ adjusted to pH 7.4 with concentrated HCl is stored at 4°C

(Levine, 1978). A 10-fold dilution of this stock buffer containing 0.1% ovalbumin is used in all the dilution steps of the assay.

ii. Complement. Complement is widely available commercially as lyophilized guinea pig serum and is reconstituted with the supplied diluent. It can be stored at -20°C in 1-ml quantities.

iii. Sheep Erythrocytes. Sterile sheep blood diluted with an equal volume of Alsever's solution is available commercially and can be stored at 4°C for about a month. The red cells are washed three times with diluent and 1.0 ml of the packed cells is suspended in 19 ml of diluent. This suspension is standardized by lysing 1 ml with 14 ml of 0.1% Na_2CO_3 . The absorbance of this lysate at 541 nm should be 0.680, which corresponds to a suspension containing 10^9 cells/ml. If the observed absorbance is somewhat higher than 0.680, the suspension should be diluted slightly so that lysis of 1 ml does produce such an absorbance.

iv. Hemolysin. Hemolytic rabbit antisheep cell serum is available commercially and should be used at a dilution of 1:100–1:500 for optimum sensitization of sheep cells. The optimal dilution can be determined by a grid titration of complement dilutions (1/100, 1/150, 1/200, 1/250, etc., to 1/500) versus dilutions of hemolysin (1/100, 1/150, 1/200, etc., to 1/400). This titration is performed as follows: 1 ml of the complement dilutions is added to 5 ml of diluent in a 15-ml centrifuge tube, and the mixture is incubated overnight at 4°C . The following day, sensitized erythrocytes are prepared by adding 5 ml of the hemolysin dilutions, dropwise and with constant swirling, to 5 ml of the standardized red cell suspension. These mixtures are incubated for 15 minutes at 37°C , and are then diluted with 90 ml of diluent to make suspensions of 5×10^7 sensitized cells/ml (0.25% sensitized cell suspension). Volumes of 1 ml of these cells sensitized with various dilutions of hemolysin are now added to the 6 ml of complement dilutions prepared the previous day. After 1 hour incubation at 37°C , the reaction is stopped by placing the tubes in iced water. The tubes are centrifuged at 2000 rpm for 10 minutes and the absorbance at 413 nm is measured. Controls with a twofold excess of complement as well as with no complement, are included in order to determine the extent of complete lysis and the background lysis of the sensitized cells. The lowest dilutions of complement and of hemolysin that give about 90% lysis are selected for use. An alternative way of determining complement and hemolysin titers, expressed in 50% hemolysis units, has been described in detail by Garvey *et al.* (1977).

v. Sensitized Erythrocytes. Hemolysin is very stable and can be stored at 4°C for several months. Once the titer of a particular lot of hemolysin has been determined, a 2.5% suspension of sensitized erythrocytes can be prepared with

the same dilution of hemolysin by the procedure outlined above. Red cells must be sensitized on the day they are to be used for the assay, and should be kept on ice at 0°C. Since the only source of complement in the assay must be that provided by the guinea pig serum, endogenous complement in the hemolytic serum must be inactivated beforehand by incubation at 56°C for 15 minutes.

b. Complement Fixation Assay

For assaying a virus-antibody system, the specific antiserum must first be heated at 56°C for 15 minutes to inactivate endogenous complement. Antiserum dilutions in the range 1:500-1:10,000 are prepared for assaying viruses, but lower dilutions (1:100-1:500) are usually required with viral subunits. Serial twofold dilutions of antigen in the range 0.1-4 µg/ml are also prepared.

The assay is performed in centrifuge tubes kept in an ice bath by adding successively 1 ml of the antiserum dilution, 3 ml of diluent, 1 ml of the appropriate complement dilution, and 1 ml of the antigen dilution. The following controls are also included: (1) tubes containing each antiserum dilution and complement but no antigen, to test for anticomplementary activity of the serum; (2) a tube containing the highest concentration of antigen and complement, but no antibody, to test for complement binding or inactivation by the antigen; (3) two tubes with a single and double dose of complement, respectively, but no antigen nor antibody, to determine the level of complete lysis; (4) two tubes containing only buffer diluent to check for spontaneous cell lysis without complement (background reading).

After incubation of all assay and control tubes for 18 hours at 4°C, 1 ml of sensitized cells is added to each tube which is then incubated for 60 minutes at 37°C. The hemolysis is stopped by placing the tubes in an ice bath. After low speed centrifugation the absorbance of the supernatants is measured at 413 nm. The results are expressed as percentage of complement fixed (%CF) as in

$$\%CF = \frac{[(A_{413} \text{ total lysis}) - (A_{413} \text{ background})] - [(A_{413} \text{ test}) - (A_{413} \text{ background})]}{(A_{413} \text{ total lysis}) - (A_{413} \text{ background})} \times 100$$

The percentage of complement fixation is then plotted against antigen concentration (Figs. 6.18 and 6.19). If the antiserum that has been used in the assay is too concentrated, the %CF will decrease only very slowly with increasing antigen concentration. Best results are obtained when the peak of the curve corresponds to about 80% fixation. This is especially important for inhibition studies aimed at demonstrating the antigenic activity of short peptides (Milton and Van Regenmortel, 1979).

An example of the use of such inhibition assays for demonstrating the antigenic activity of a heptapeptide isolated from TMV protein is shown in Fig. 6.20.

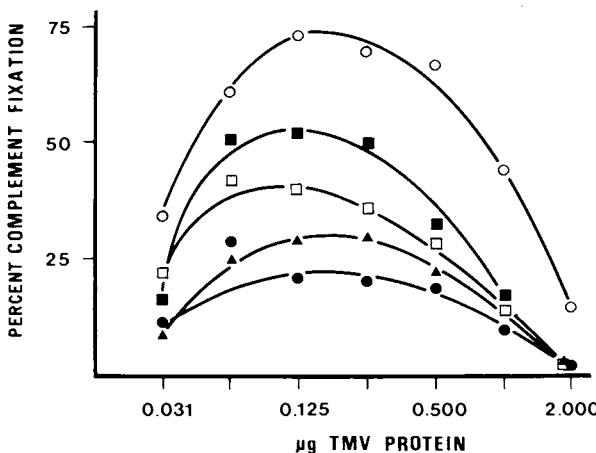


Fig. 6.20. Inhibition of complement fixation in the TMV protein - anti-TMV protein system, by increasing quantities of a heptapeptide corresponding to residues 62-68 of TMV protein. The TMV protein antiserum was diluted 1 = 200 and was incubated for 15 minutes at 37°C with various amounts of the peptide, prior to addition of TMV protein. ○, No peptide; ■, 0.29 nanomoles of peptide equivalent to a 40 molar excess of peptide over TMV protein; □, 0.57 nanomoles of peptide; ▲, 2.86 nanomoles of peptide; ●, 5.7 nanomoles of peptide equivalent to an 800 molar excess of peptide over TMV protein (from Milton, 1979).

F. LABELED ANTIBODY TECHNIQUES

The sensitivity of detection of antigen-antibody reactions can be increased by attaching to either of the two reactants a label that can be detected in minute quantities (Feteanu, 1978). In this section, the discussion will be limited to the three types of marker that have been most commonly used for labeling antibody molecules: enzymes, fluorescent dyes, and radioactive materials.

The use of enzymes for labeling antibodies was first reported in 1966 and was originally developed for localizing antigens in histological preparations, both at the optical and electron microscope level (Nakane and Pierce, 1966; Wicker and Avraméas, 1969), as well as for identifying precipitin lines in immunodiffusion tests (Avraméas and Uriel, 1966). It was soon found, however, that when the reactants were attached to a solid phase, enzyme immunoassays were particularly suitable for the quantitative measurement of antigens and antibodies (Engvall and Perlmann, 1971; Van Weemen and Schuurs, 1971; Feldmann *et al.*, 1976; Schuurs and Van Weemen, 1977). As a result, the method known as Enzyme-Linked Immunosorbent Assay (ELISA) was rapidly adopted by many laboratories for the diagnosis of virus diseases and for the measurement of very low concentrations of virus and specific antibody (Voller *et al.*, 1976; Voller and Bidwell, 1977).

1. ELISA (Enzyme-Linked Immunosorbent Assay)

Numerous variations of ELISA have been developed and some are presented schematically in Table 6.1. For the solid phase, most investigators use plates of polystyrene or polyvinylchloride that are commonly used for hemagglutination and complement fixation tests.

The indirect ELISA method (procedure 2 in Table 6.1), which is used for measuring viral antibody, consists of the following steps: (1) coating of wells of microtiter plate with a standard virus preparation; (2) binding of specific virus antibody present in test serum; (3) binding of enzyme-labeled antiglobulin conjugate; (4) addition of substrate for colorimetric revelation of bound enzyme conjugate. The initial coating step requires the presence of 0.05 M bicarbonate buffer, pH 9.6, and between each successive incubation with different reagents, the wells are washed several times with phosphate-buffered saline containing 0.05% Tween-20. The enzyme most commonly used for preparing conjugates is alkaline phosphatase, but horseradish peroxidase and glucose oxidase are also suitable (Feteanu, 1978; King and Kochoumian, 1979). The presence of bound enzyme in the well is revealed by a chromogenic substrate, which is initially colorless but yields a colored product after enzymatic degradation. Besides a simple visual scoring, which may be adequate for field screening of virus-infected samples, accurate quantitative readings can be made with a spectrophotometer.

For the detection of plant viruses, most workers use the "double antibody sandwich" form of ELISA (procedure 3 in Table 6.1) described by Clark and

TABLE 6.1
Different Types of Enzyme-Linked Immunosorbent Assay (ELISA)

Reactant attached to solid phase	Successive steps of assay	Reference
1 Virus	Labeled antivirus conjugate	Feteanu, 1978
2 Virus	Virus antibody + labeled antiglobulin conjugate	Voller and Bidwell, 1977
3 Antivirus globulin	Virus + labeled antivirus conjugate	Clark and Adams, 1977
4 Antivirus globulin (e.g., goat)	Virus + virus antibody (e.g., rabbit) + labeled antirabbit globulin conjugate	Scherrer and Bernard, 1977
5 Virus	Virus antibody (rabbit) + antirabbit globulin (goat) + complex of peroxidase-antiperoxidase (rabbit)	Butler et al., 1978a
6 C1q	Virus-antibody mixture + labeled antiglobulin conjugate	Torrance, 1980b

Adams (1977). In this method, the wells are first coated with antivirus globulin and the virus in the test sample is then trapped by the adsorbed antibody. The presence of virus is revealed by an enzyme-labeled antivirus conjugate. Unlike the indirect procedure, this method necessitates the preparation of a different antibody conjugate for each virus to be tested. However, it has the advantage of securing a standardized attachment of different viruses to the initial antibody coating of the well. This cancels out any differences in affinity for the solid phase shown by individual viruses.

a. Procedures

i. *Double Antibody Sandwich Method.* The various stages of the test, which is performed in polystyrene microtiter plates (Cooke M 129 B, Dynatech, Alexandria, Virginia), are shown in Fig. 6.21. If bacterial contamination is likely to occur, 0.02% sodium azide may be added to all the buffers used in the assay. The wells are coated at 37°C by incubation (15 minutes to several hours) with 1-10 µg/ml antivirus globulins (300 µl) diluted in 0.05 M sodium carbonate buffer, pH 9.6. The globulins can be prepared by precipitation from antiserum with an equal volume of 4 M ammonium sulfate. There appears to be no advan-

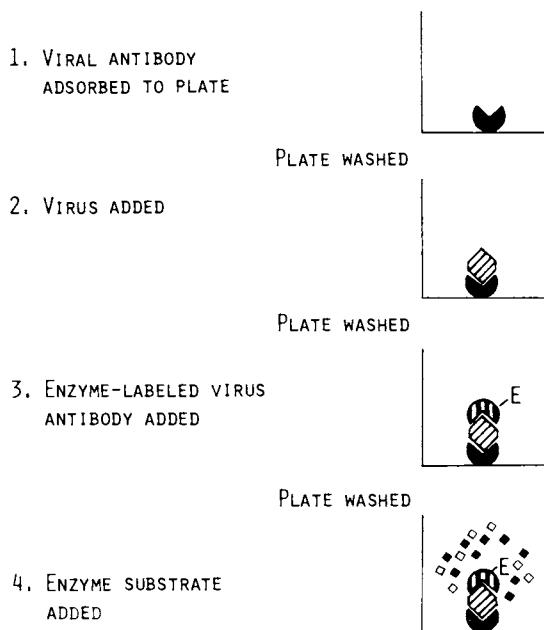


Fig. 6.21. Double antibody sandwich ELISA method.

tage in using highly purified IgG. Plates are then rinsed three times with phosphate-buffered saline pH 7.4, containing 0.05% Tween-20 (PBS-T) and once with 1% bovine serum albumin in PBS-T. This last washing step may not be necessary in all systems.

Antigen preparations (300 μ l) in PBS-T are added to the coated wells for 1–16 hours at 37°C. The addition to crude plant extracts of 1–2% polyvinyl pyrrolidone (Clark and Adams, 1977), 1 M urea, and reducing agents (Gugerli, 1979) may reduce nonspecific reactions and increase the sensitivity of virus detection. With unstable viruses that are easily degraded in PBS-T, it may be advantageous to combine the antigen incubation step with the subsequent incubation with conjugate (Flegg and Clark, 1979). The virus extract is first mixed with the diluted conjugate, before being added to the wells. The formation of virus-antibody complexes counteracts the breakdown of unstable viruses and may thereby prevent the attachment of viral protein subunits to the antibodies on the solid phase (Korpraditskul *et al.*, 1980). When viral subunits are bound to such antibodies, the surfaces of the antigen molecules that remain available for further binding of enzyme conjugate are unlikely to be reactive with antivirus antibodies since these surfaces will probably harbor cryptotopes (see Chapter 7).

After rinsing the wells, the antivirus enzyme conjugate diluted in PBS-T (300 μ l) is added for 1–3 hours at 37°C. The enzyme conjugate most commonly used is prepared with alkaline phosphatase (Boehringer, Mannheim; or Sigma, St. Louis, Missouri) by coupling the globulins with enzyme at ratios ranging between 1:1.5 and 8:1 (w/w globulin:enzyme) using 0.06% glutaraldehyde (Avraméas, 1969). The optimal dilution of conjugate (usually 1:200–1:1000) must be determined empirically. The conjugate should be stored at 4°C in the presence of 1% bovine serum albumin.

After further rinsing, the bound enzyme conjugate is detected by the addition of 300 μ l of the substrate *p*-nitrophenyl phosphate at 1 mg/ml in 0.1 M diethanolamine buffer, pH 9.8. After 1–3 hours hydrolysis, the reaction is stopped by the addition of 50 μ l of 3 M NaOH to each well. Results are scored visually by the appearance of a yellow color, or absorbances at 405 nm are read in a spectrophotometer (Fig. 6.23). Results are considered positive if the absorbance is twice that found with healthy controls, or alternatively, if it is two standard deviation units higher than the mean of a negative control curve.

The various incubation times that have been mentioned in this description of the procedure can be varied considerably, depending on the concentration of available reactants and on the speed at which results are needed (Korpraditskul *et al.*, 1979).

ii. Indirect Double Antibody Sandwich Method. The various stages of the test are shown in Fig. 6.22. Wells are coated with goat or chicken antivirus globulins (1–10 μ g/ml) as described above. After rinsing and incubation with antigen,

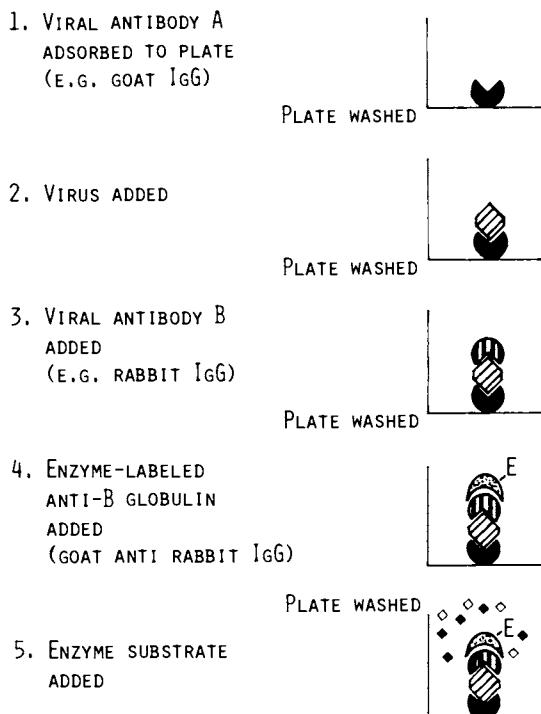


Fig. 6.22. Indirect ELISA method combined with the double antibody sandwich procedure. The viral antibody used for coating the plate may be obtained from chickens, goats, or rabbits. The viral antibody used in step 3 should be obtained from another animal species than that used for producing the coating antibody.

antivirus rabbit globulins are allowed to react for 1–3 hours with the trapped antigen. After further rinsing, the wells are filled for 2 hours with an antirabbit globulin conjugate prepared with immunoglobulins obtained from a goat immunized with rabbit IgG. The bound enzyme conjugate is detected as described above. The main advantages of this procedure are that a single conjugate can be used for all virus systems, and that all serotypes of a virus will be detected with antiserum to one strain (Van Regenmortel and Burckard, 1980). The requirement for virus antiserum prepared in two different animal species can be circumvented by using bovine C1q for coating the microtiter plates (Torrance, 1980b). Since the C1q component of complement (see Chapter 2, Section C,2) binds immune complexes, the virus and its specific antiserum are incubated together in the wells; the resulting virus–IgG complexes are trapped by the C1q molecules and detected by an enzyme-labeled antiglobulin conjugate. A simple method for preparing bovine C1q has been described (Hudson and Hay, 1980).

b. Strain Specificity of Different ELISA Procedures

Numerous authors have reported that the double antibody sandwich method of ELISA is extremely strain specific (Koenig, 1978; Barbara *et al.*, 1978; Lister and Rochow, 1979; Bar-Joseph and Salomon, 1980; Uyemoto, 1980). Enzyme conjugates prepared with antibodies against one virus strain often do not react with closely related strains. This may be an advantage when it is important to distinguish between different strains, but more often it is a drawback for diagnostic work. It has been suggested, for instance, that if a broad range of virus strains is to be detectable, a pool of immunoglobulins prepared against a number of different serotypes would have to be used. For screening field-collected samples for the presence of BYDV, for instance, Rochow and Carmichael (1979) suggested that at least five different conjugated antisera would be needed to detect the presence of the five major types of barley yellow dwarf virus.

When it is necessary to detect various unidentified serotypes of a virus, the indirect form of ELISA which utilizes an antiglobulin conjugate is much to be preferred. This was clearly demonstrated in comparative studies of the value of two ELISA procedures for the detection of a wide range of TMV strains (Van Regenmortel *et al.*, 1980; Van Regenmortel and Burckard, 1980). By combining the indirect and double antibody sandwich procedures (procedure 4 in Table 6.1), it was possible to detect distantly related serotypes with antisera to one strain only (Fig. 6.23). Since the serological distance between the various TMV strains used in these studies was as great as is likely to be encountered in any group of related serotypes, it is clear that the indirect ELISA is the best method for detecting serologically distinct strains. Crook and Payne (1980) and Rybicki and Von Wechmar (1981) reached similar conclusions in their studies of the suitability of different ELISA procedures for detecting relationships among insect baculoviruses and bromoviruses, respectively.

The procedure used for overcoming the narrow strain specificity of the reaction (Fig. 6.23) requires that two virus antisera be prepared in different animal species (Scherrer and Bernard, 1977). The most convenient animals in this respect are rabbits, chickens, rats, and goats. Chicken antibodies do not cross-react with mammalian immunoglobulins (Leslie and Clem, 1969; Esteves and Binaghi, 1972). This is an advantage since it excludes any binding between the chicken antibody used for coating and the goat antirabbit globulin conjugate (Van Regenmortel and Burckard, 1980). Another advantage of using chicken antibody is that the immunoglobulins can be obtained easily from the eggs of immunized hens (Polson *et al.*, 1980; Bar-Joseph and Malkinson, 1980; see Chapter 4, Section A,3,c).

The narrow strain specificity associated with the use of antivirus enzyme conjugates is probably caused by a reduction in antibody avidity following conjugation with the enzyme (Koenig, 1978; Ghabrial and Shepherd, 1980). It is known that ELISA is particularly sensitive to differences in antibody affinity

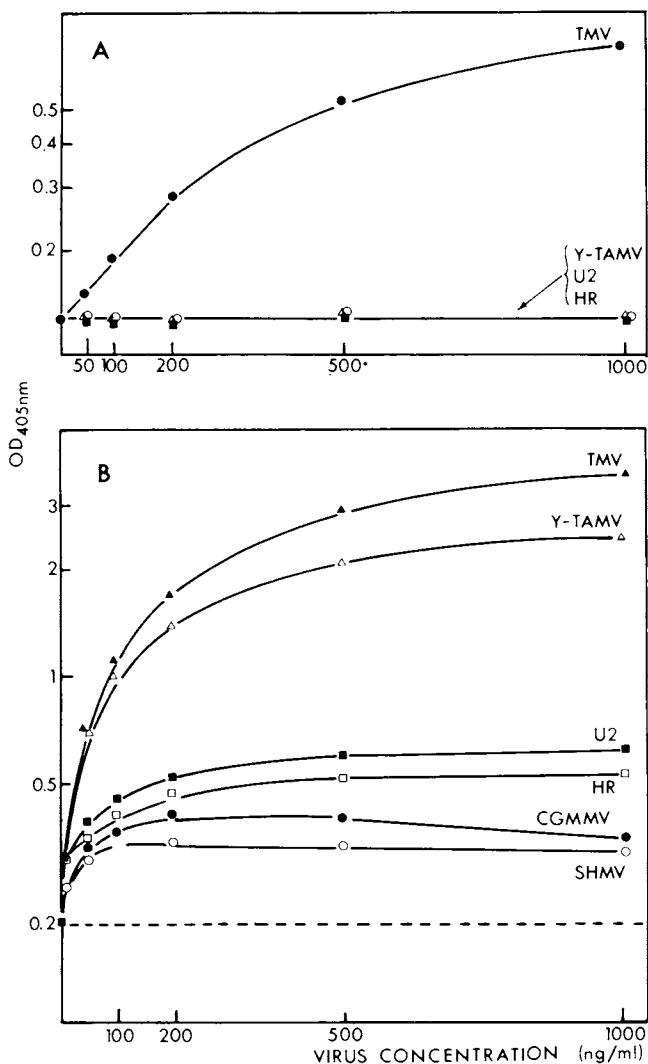


Fig. 6.23. Comparison between the two ELISA procedures illustrated in Figs. 6.21 and 6.22 for their ability to detect related tobamoviruses. (A) Double antibody sandwich method using an anti-TMV enzyme conjugate only detects the homologous TMV strain. (B) Indirect procedure (Fig. 6.22) using an antiglobulin enzyme conjugate allows the detection of five heterologous strains with antibody directed to the TMV strain (Van Regenmortel and Burckard, 1980).

(Butler *et al.*, 1978b). However, no impairment of the normal degree of cross-reactivity between strains is observed when the heterologous antibodies are used for coating the wells instead of for conjugate preparation (Barbara *et al.*, 1978; Koenig, 1978; Bar-Joseph and Salomon, 1980). In the one-step glutaraldehyde procedure that is commonly used for labeling the antibody (Avraméas, 1969), only a relatively small proportion of the available enzyme and globulins becomes conjugated. Since the conjugated molecules are not separated from the free enzyme and free globulins (Clark and Adams, 1977), the unconjugated antibody which has retained its initial affinity will preferentially bind to heterologous antigens and may mask any cross-reaction with the labeled antibody.

The relatively low avidity of conjugated antibody molecules can be put to good use in that it may allow the elimination of unwanted reactions between certain virus antisera and plant antigens. Contaminating antihost antibodies, if present in the enzyme-labeled virus antiserum, can be prevented from contributing to the absorbance readings by the prior addition of an excess of unlabeled antiserum to plant antigens (Koenig, 1978). It is also possible to eliminate contaminating reactions with host antigens by adding extracts of healthy plants to the enzyme conjugate (Lister, 1978; Thomas, 1980).

c. Applications of ELISA

The extreme sensitivity of ELISA approaches that of radioimmunoassays and allows the detection of as little as 1–10 ng/ml virus (Clark *et al.*, 1976a; Gugerli, 1978; Reeves *et al.*, 1978; Hariri and Lapierre, 1979; Devergne *et al.*, 1978). This high sensitivity made it possible, for instance, to detect CMV in single viruliferous aphids (Gera *et al.*, 1978) and to demonstrate the presence of PSbMV, SoyMV, bean common mosaic virus (BCMV), and LMV in seed lots containing 0.1–25% infected seed (Lister, 1978; Hamilton and Nichols, 1978; Jafarpour *et al.*, 1979). In several instances it was shown that detection of viruses by ELISA was more sensitive than by the classical infectivity assays on indicator plants (Clark *et al.*, 1976a; Gugerli, 1978; Bossenec and Maury, 1978; Jafarpour *et al.*, 1979). Enzyme immunoassays are extremely convenient for examining large numbers of samples and they are, therefore, the method of choice for large-scale investigations of the incidence of virus infection in crops (Thresh *et al.*, 1977). Typical applications include the routine indexing of fruit trees in the field for the presence of apple mosaic virus (ApMV) (Clark *et al.*, 1976b), PPV (Adams, 1978), PNRSV (Barbara *et al.*, 1978), and citrus tristeza virus (CTV) (Bar-Joseph *et al.*, 1979a) and the testing of potato leaves, tubers, and sprouts for the presence of potato virus S (PVS) (Richter *et al.*, 1977), PVY and potato virus A (PVA) (Maat and De Bokx, 1978b), potato leafroll virus (PLRV) (Maat and De Bokx, 1978a; Gugerli, 1980; Tamada and Harrison, 1980a), and PVX (De Bokx *et al.*, 1980). In addition to sensitivity and suitability for mass indexing, other outstanding advantages of ELISA are its simplicity

and the fact that the reagents used in the test are relatively cheap and stable and possess a long shelf-life. Important savings of enzyme conjugate can be achieved by collecting the conjugate from the wells after use and reutilizing it three to four times. With some virus systems, it is also possible to reuse several times microtiter plates that have been coated with antivirus globulin. This is achieved by dissociating the virus-antibody complex at pH 2.2 after completion of the test (Bar-Joseph *et al.*, 1979b).

2. Tests with Fluorescent Antibody

Antibodies labeled with fluorescent dyes are one of the most useful reagents for studying the intracellular location and distribution of plant viruses within the tissues of host plants (Nagaraj and Black, 1961; Worley and Schneider, 1963; Nagaraj, 1965; Mumford and Thornley, 1977; Tsuchizaki *et al.*, 1978; Thornley and Mumford, 1979) as well as in insect vectors and vector-cell monolayers (Sinha and Black, 1962, 1963; Sinha and Reddy, 1964; Sinha, 1965; Chiu and Black, 1969; Peters and Black, 1970; Kimura and Black, 1971; Reddy and Black, 1972; Hsu, 1978).

Immunofluorescence techniques have been used, for instance, to follow the distribution and synthesis of TMV protein in different plant tissues (Schramm and Röttger, 1959; Hirai and Hirai, 1964; Nagaraj, 1965; Carroll, 1966; Schönbeck and Spengler, 1979) and to assay its multiplication in tobacco protoplasts (Otsuki and Takebe, 1969). It should be noted, however, that the proportion of protoplasts that could be shown by fluorescent antibody staining to be infected with PVX was lower than the proportion found by electron microscopy (Shalla and Petersen, 1973). Other applications of fluorescent antibody stains include the study of virus-induced inclusion bodies (Rao *et al.*, 1978) and various methods for improving the sensitivity of virus detection (Murayama and Yokoyama, 1966; Sinha and Thottappilly, 1974; Gingery, 1978).

The multiplication of WTV in its leafhopper vector has been studied extensively with fluorescent antibodies (Nagaraj *et al.*, 1961; Sinha, 1965; Chiu and Black, 1969). Sinha *et al.* (1964) showed that a large percentage of insects survived when small samples of hemolymph were collected in order to assess if an insect is viruliferous.

Procedure

The general methodology of immunofluorescent staining is described in several reviews (Peters and Coons, 1976; Kawamura, 1977; Feteanu, 1978; Sternberger, 1979). In the direct staining procedure, the viral antibody is conjugated to the fluorescent dye. Although it is not as sensitive as the indirect method, this procedure is somewhat less subject to nonspecific background stain-

ing. In the indirect procedure, viral antigens are first allowed to react with unlabeled virus antiserum. After washing the preparation to remove extraneous serum proteins, the presence of bound antibodies is revealed by a second reaction with fluorescent antigen-globulin antibodies. Although the indirect procedure is more sensitive than the direct one, its tendency to give rise to nonspecific staining necessitates very stringent specificity controls (Emmons and Riggs, 1977).

Labeled antibody is prepared by adding 0.5 mg of fluorescein isothiocyanate to 1 ml of 10 mg/ml purified immunoglobulin in 0.05 M sodium carbonate buffer, pH 9.5. Conjugation is allowed to proceed for 18 hours at 4°C. Alternatively, the 1% immunoglobulin preparation may be placed in dialysis tubing and dialyzed for 24 hours at 4°C against 10 volumes of 0.1 mg/ml fluorescein isothiocyanate, in the same carbonate buffer, pH 9.5 (Clark and Shepard, 1963; Goding, 1976). The conjugated globulin preparation is then dialyzed against phosphate-buffered saline, pH 7.0. Excess unreacted dye and overlabeled proteins are a major source of nonspecific staining and should be removed by successive passages through columns of Sephadex G-25 and DEAE-cellulose (Emmons and Riggs, 1977; Feteanu, 1978). The specificity of staining can also be improved by absorbing the conjugate preparation with host tissue powder (Nagaraj and Black, 1961; Rao *et al.*, 1978).

3. Radioisotope-Labeled Antibody

Antibodies labeled with ^{125}I have been used to locate viral antigen in plant tissues infected with TMV (Langenberg and Schlegel, 1967, 1969), SMV (Powell and Schlegel, 1970a,b), and CIYMV (Schlegel and Delisle, 1971). In addition to histological localization studies where the labeled antibody is revealed by autoradiography, other applications of ^{125}I -labeled antibodies include the detection of virus by radioimmunosorbent assay (Ghabrial and Shepherd, 1980) and the identification of precipitin lines in immunodiffusion (Schlegel and Hudson, 1969; Salzmann and Moss, 1969).

Procedure

^{125}I -Labeled antibody can be prepared by labeling either purified total immunoglobulins (Ghabrial and Shepherd, 1980), purified virus antibodies obtained by dissociation of the immune complex at pH 2.5 (Schlegel and Delisle, 1971), or the washed virus-antibody precipitate (Langenberg and Schlegel, 1967; Powell and Schlegel, 1970a). In the latter case, the iodinated antibody is separated from the precipitate at low pH, at the completion of the labeling procedure. The iodination method of Tsuzuku *et al.* (1967) utilizing chloramine T and sodium sulfite, as well as the more gentle procedure developed by Fraker and Speck (1978), has been used successfully. The general methodology of

labeling and radioimmunoassay is described extensively in several texts (Parker, 1976; Daugherty and Ziegler, 1977; Thorell and Larson, 1978; Hunter, 1978; Feteanu, 1978).

Another labeling method that is used increasingly in immunochemistry deserves mention in this section. This is the labeling of viral antibodies *in situ* (Moar *et al.*, 1979) or on a solid phase (Cleveland *et al.*, 1979; Langone, 1980) by ^{125}I -labeled protein A of *Staphylococcus aureus*. The ability of protein A to bind specifically and with a very high affinity to the Fc region of IgG makes it a powerful analytical reagent for the quantitative determination of antibodies present in solution or on a solid phase (Goding, 1978).

G. TESTS WITH RADIOISOTOPE-LABELED VIRAL ANTIGENS

1. Immunoprecipitation

Studies on the replication of plant viruses in plants or in cell-free systems often require the isolation of viral proteins in minute quantities in situations where the synthesis of other proteins cannot be inhibited. By precipitating specific viral proteins with an antiserum prepared against the virus or the degraded viral subunits, it is possible to isolate them and to follow their rate of synthesis (Hirai and Wildman, 1967; Faed and Matthews, 1972). Alternatively, the viral proteins may be incubated with a specific rabbit antiserum and the soluble complexes precipitated with either an antiglobulin serum or protein A (Kessler, 1975). The labeled viral antigen may also be isolated by coprecipitation after addition of large amounts of the cold antigen and specific antibody (Horwitz and Scharff, 1969b; Shapiro and August, 1976). The immune precipitates can be dissolved in buffers containing sodium dodecyl sulfate and analyzed by polyacrylamide gel electrophoresis. This approach was used, for instance, in a study of the *in vitro* translation products of carnation mottle virus (CarMV) RNA (Salomon *et al.*, 1978). Methods for the *in vivo* labeling of TMV and TYMV with ^3H , ^{35}S , and ^{32}P have been described by Matthews *et al.* (1963), Hirai and Wildman (1967), and Singer (1971).

2. Radioimmunoassay

A solid phase radioimmunoassay for assaying TMV, SBMV, wheat streak mosaic virus (WSMV), BSMV, and CPMV in the 0.01–5 $\mu\text{g}/\text{ml}$ concentration range has been described by Ball (1973, 1974). In a competitive type of assay, unlabeled antigen is first incubated in antibody-coated polystyrene centrifuge tubes, and ^{125}I -labeled antigen is added afterward to measure the amount of residual binding of the labeled material to the tubes. In view of the recent

development of simpler ELISA techniques for measuring viruses in the same concentration range, this type of radioimmunoassay is unlikely to find many adepts in the future.

It seems likely, however, that other types of radioimmunoassay will continue to be useful for elucidating the antigenic structure of viral proteins. Direct binding assays with ^{14}C - or ^3H -acetylated peptides corresponding to various regions of the viral protein have contributed much to our knowledge of the antigenic structure of TMV protein (Benjamini *et al.*, 1965, 1969; Stewart *et al.*, 1966; Young *et al.*, 1967; Milton *et al.*, 1977). Following the incubation of radioactive peptides with rabbit globulins specific for anti-TMV or anti-TMV protein, the bound peptides can be precipitated at 50% saturation of ammonium sulfate or, alternatively, with goat antiserum to rabbit IgG. Since the percentage of peptide that is bound in this type of assay is rather small, it is essential to include adequate controls with normal globulins to establish the extent of nonspecific binding.

The antigenic activity of short peptides isolated from a viral protein can also be determined by their ability to inhibit the precipitation reaction between radioactively labeled viral protein and specific antibodies. Suitable conditions for the inhibition assay correspond to a ratio of antibody to labeled protein close to the equivalence zone (Fig. 6.24). The application of such inhibition studies for

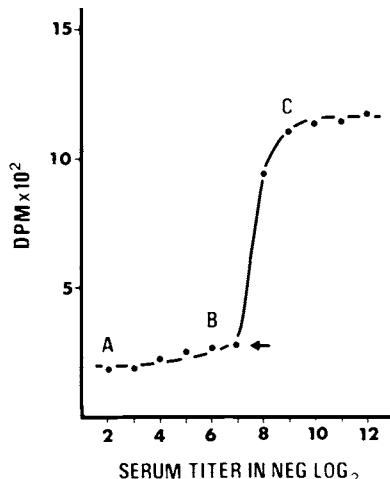


Fig. 6.24. Precipitin curve of 0.5 μg quantities of ^3H -TMV protein and increasing dilutions of specific antiserum. The ordinate represents the radioactivity found in 1.0 ml of the supernatant, after removal of the immune precipitate. The region of the curve between A and B corresponds to the zone of antibody excess, and between B and C to the zone of antigen excess. The arrow indicates the region of slight antigen excess chosen for performing inhibition assays with viral peptides (Milton *et al.*, 1980).

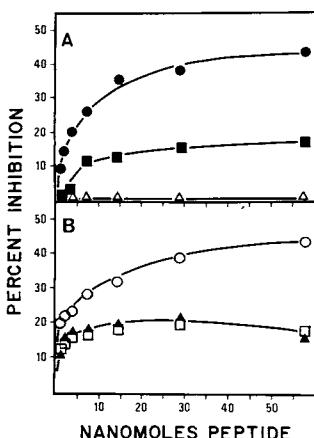


Fig. 6.25. (A) Inhibition of the precipitin reaction between 0.5 μ g of radiolabeled TMV protein and its specific antiserum, by increasing amounts of tryptic peptide 4 (residues 62–68) from homologous strain TMV (●), from a mutant with an exchange at position 65 (■), and from a mutant with an exchange at position 63 (△). The replacement of proline by serine at position 63 totally abolishes the inhibitory activity of tryptic peptide 4. (B) Inhibition of the precipitin reaction between 0.5 μ g of radiolabeled TMV protein and specific antiserum, by increasing amounts of tryptic peptide 12 (residues 142–158) from homologous TMV (○), and from two mutants with an exchange at position 156 (□ and △). In this case, the replacement of proline by leucine at position 156 does not totally abolish the inhibitory activity of tryptic peptide 12.

comparing the antigenic activity of tryptic peptides of wild type TMV protein with that of the corresponding peptides of TMV mutants is illustrated in Fig. 6.25. It was found that a single exchange in the antigenically active region of a peptide could considerably decrease or even totally abolish its inhibitory activity.

H. IMMUNOELECTRON MICROSCOPY

The visualization of immunological reactions on electron microscope grids is one of the most sensitive serological techniques (Doane and Anderson, 1977). Two different approaches can be distinguished, depending on whether the viral antigen is in suspension or is visualized in thin sections of infected tissue. Histological methods of immunocytology entail the use of labeled antibodies as a stain, the most common labels being ferritin (Shalla and Amici, 1967; Shalla and Shepard, 1972; Shepard *et al.*, 1974b; Kishida *et al.*, 1975; Rifkind, 1976) and enzymes such as horseradish peroxidase (Kraehenbuhl and Jamieson, 1976). Enzyme-labeled antibodies can be applied after embedding and thin sectioning of the tissue, or they can be allowed to diffuse inside fixed cells and to interact with antigenic sites prior to thin sectioning. The use of immunocytological techniques

for the localization of viral antigens in infected cells has been reviewed by Howe *et al.* (1969), Kurstak and Morisset (1974), and Kurstak *et al.* (1977).

The only techniques that will be described in detail are those used when the virions are in suspension, and the virus-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: clumping, antibody coating, and trapping on antibody-coated grids.

1. Clumping of Virus Particles

When a virus preparation is mixed with a suitable dilution of specific antiserum, the formation of virus-antibody complexes will be visualized in the electron microscope by the appearance of clumps of variable size (Anderson and Stanley, 1941; Black *et al.*, 1946; Watson *et al.*, 1966; Ball and Brakke, 1968, 1969; Milne and Luisoni, 1975). The complexes can be centrifuged to a pellet to obtain the maximum sensitivity of detection, and aggregates can be visualized with as little as 10^6 virions present in a suspension (Almeida and Waterson, 1969). Negative staining increases the ease of detection, and an agar surface can be used to absorb impurities (Kelen *et al.*, 1971). The clumping phenomenon is especially valuable when the virus concentration is too low for the particles to be seen directly.

When sufficient antibody is present in the mixture, the virions will be covered by a more or less continuous coat of antibody molecules, a phenomenon also known as decoration (Milne and Luisoni, 1977a). The halo of antibody molecules that surrounds the virions leads to a fuzzy outline of the particles that is very characteristic (Almeida and Waterson, 1969). An excess of antibody may inhibit clumping in the same way as it would inhibit a precipitation reaction. The visualization of a coat of antibody molecules around virus particles incubated with heterologous antiserum has been used by Randles *et al.* (1976) to confirm the distant serological relationships that exist between several tobamoviruses.

2. Antibody Coating

The phenomenon of decoration corresponds to the antibody coating of virions and can be observed independently of the clumping reaction if the virions are first immobilized on the microscope grids prior to the addition of antibody (Milne and Luisoni, 1977a,b). When an excess of antibody is mixed directly with the virus preparation, the virions will be covered by a continuous layer of antibody molecules and little clumping will result.

Antibody coding for identifying viruses present in a mixture is illustrated in Figs. 6.26 and 6.27. In the procedure known as leaf-dip serology (Ball and

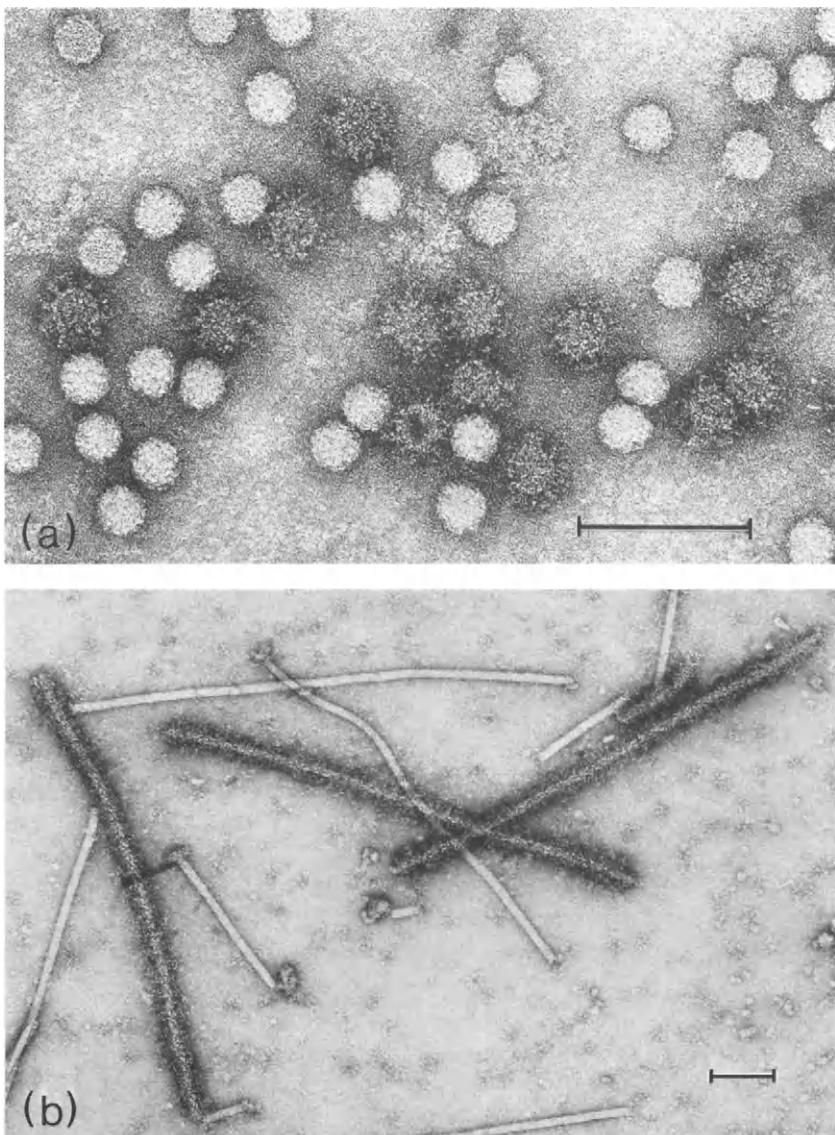


Fig. 6.26. Identification of viruses by antibody coating. The bars represent 100 nm. (a) A mixture of purified tomato bushy stunt and carnation mottle viruses was treated with antiserum to carnation mottle virus. (b) A mixture of two potyviruses found in naturally infected white bryony plants was treated with antiserum to one of them (photographs by courtesy of Dr. R. G. Milne).

Brakke, 1968; Ball, 1971), the particles are usually fairly evenly covered with antibodies. In this method, the leaf-dip procedure developed by Brandes (Brandes, 1964; Brandes and Bercks, 1965) is made more specific by using drops of dilute virus antiserum instead of virus. The antiserum should be diluted in a volatile buffer such as ammonium acetate. The edge of a cut leaf is dipped into a drop of antiserum placed on a microscope grid, and after drying and negative staining, it is possible to distinguish the antibody-coated virus from another serologically unrelated virus with similar morphology that will remain uncoated (Langenberg, 1974).

Antibody coating can be used as an analytical tool (Van der Lubbe *et al.*, 1979), especially with viruses that possess a complex morphology, since the site of antibody attachment can be localized (Yanagida and Ahmad-Zadeh, 1970; Tosi and Anderson, 1973). This approach was followed for the identification of different antigenic structures present in the particles of MRDV (Luisoni *et al.*, 1975).

When antibodies specific for a particular virus strain are prepared by cross-absorption, a further analytical refinement can be introduced. In mixed reconstitution experiments in which the RNA of a TMV strain was sequentially reconstituted first with coat protein from one strain and then with protein of another serologically distinct strain, it was possible by means of the corresponding strain-specific antibodies to locate the regions encapsidated with the respective proteins (Otsuki *et al.*, 1977; Fukuda *et al.*, 1980). The same method made it possible to show that protoplasts doubly infected with two strains of TMV produced progeny virus with mixedly coated particles (Otsuki and Takebe, 1978).

3. Trapping (Immunosorbent Electron Microscopy or ISEM)

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. Although this terminology has gained acceptance (Paliwal, 1977; Beier and Shepherd, 1978; Hamilton and Nichols, 1978), it will not be used here to avoid confusion with other microscopical procedures that also rely on the visualization of virus-antibody interaction. All forms of immunoelectron microscopy can be said to be "serologically specific" and it seems preferable to use a special term to refer to the technique by which virus particles are attached to grids previously coated with antiserum. "Immunosorbent electron microscopy" (Roberts and Harrison, 1979) or "serological trapping" (Nicolaïeff and Van Regenmortel, 1980; Nicolaïeff *et al.*, 1980) seem more satisfactory terms for describing this procedure.

The method consists of the following steps. Freshly prepared microscope grids with a parlodion-carbon film are allowed to float, film side down, on 10–50 μ l

drops of diluted virus antiserum for about 30 minutes. During this time a layer of serum proteins is adsorbed to the film. Excess protein is then removed by floating the grids for several minutes on a buffer solution. The coated grids are drained by a brief contact with filter paper and are then placed for 1–2 hours 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 with distilled water, and the virions are visualized after metal shadowing or negative staining.

The ISEM technique has been applied successfully to the detection of numerous elongated and isometric plant viruses (Derrick and Brlansky, 1976; Paliwal, 1977; Barker and Harrison, 1977; Hamilton and Nichols, 1978; Brlansky and Derrick, 1979; Roberts and Harrison, 1979) as well as for revealing the presence of double-stranded RNA in extracts of tobacco infected with TMV (Derrick, 1978).

The main advantages of ISEM are its sensitivity, which equals that of ELISA and local lesion assays (Beier and Shepherd, 1978; Hamilton and Nichols, 1978) and the fact that it can be used for quantitative assay of viruses in crude plant extracts. 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).

Not all authors agree as to which conditions are optimal for the test. Although parlodion or Formvar–carbon films are frequently used, it has been claimed that films, made with carbon only, give better results (Roberts and Harrison, 1979). Such films, however, appear to be somewhat more fragile. Most workers have found that when the antiserum used for coating the film is diluted less than about 1:1000, the trapping of virions is inhibited (Milne and Lesemann, 1978). With certain viruses, however, a lower antiserum dilution may produce better results (Nicolaiéff and Van Regenmortel, 1980). Other factors that have been shown to affect the number of particles trapped are the temperature (Paliwal, 1977; Roberts and Harrison, 1979), the ionic strength, the type of buffer used (Derrick and Brlansky, 1976; Beier and Shepherd, 1978; Roberts and Harrison, 1979), and the time during which antiserum-coated grids are exposed to the virus preparation (Fig. 6.28). A simplified method for obtaining comparative counts of virus particles in electron microscopy has been described by Roberts (1980).

It is important to use adequate control grids to assess the specificity of the trapping reaction. With purified preparations of different viruses, a large but variable number of virions become adsorbed to uncoated carbon grids. However, by using grids coated with normal serum, this adsorption can be completely prevented (Nicolaiéff and Van Regenmortel, 1980). When virus in crude plant sap is examined, the plant sap constituents prevent the adsorption of virus particles onto uncoated grids, although they do not affect the specific trapping of

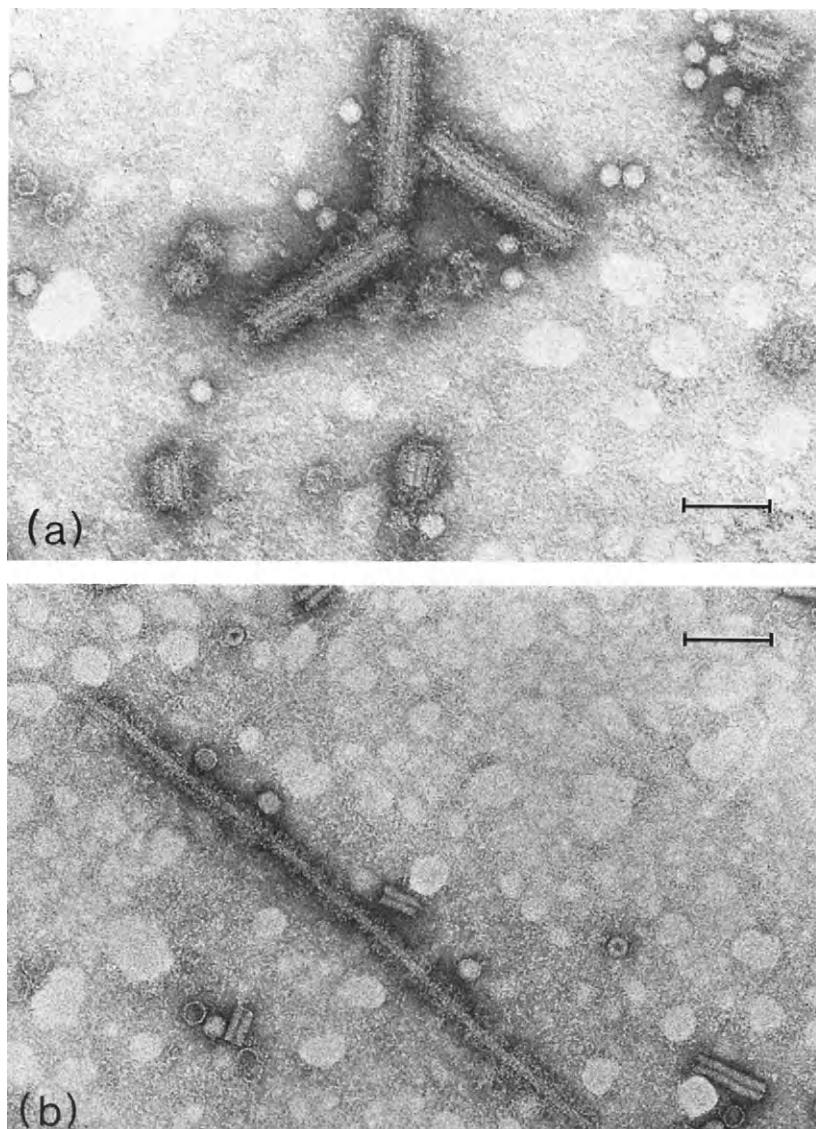


Fig. 6.27. Identification of virus by antibody coating. The bars represent 100 nm. (a) A mixture of raspberry ringspot and tobacco rattle viruses was incubated for 1 hour with antiserum to tobacco rattle virus, diluted 1:256. Grids stained with 2% ammonium molybdate, pH 6.0. (b) A mixture of raspberry ringspot, tobacco rattle, and celery mosaic viruses was incubated for 1 hour with an antiserum to celery mosaic virus diluted 1:256 (photographs by courtesy of I. M. Roberts).

virions on antibody-coated grids (Lesemann *et al.*, 1980). Since the influence exerted by serum proteins on the adsorption of different viruses may vary, the most reliable control consists of grids coated with normal serum diluted to the same degree as the antiserum. This ensures that it is solely antibodies present in the antiserum that are responsible for the observed trapping. It should also be mentioned that specific attachment via antibody molecules can only be observed when the grid is not swamped with an excess of virus (e.g., 0.2–1 mg/ml virus). The most adequate range of virus concentration for observing specific trapping reactions appears to be 0.01–10 µg/ml.

When grids are pretreated with staphylococcal protein A at a concentration of 10–100 µg/ml, prior to coating with antiserum, somewhat higher particle counts can be obtained (Shukla and Gough, 1979; Gough and Shukla, 1980; Lesemann and Paul, 1980; Nicolaïeff *et al.*, 1980). The improvement resulting from the use of protein A is especially noticeable in the detection of heterologous serological reactions (Van Regenmortel *et al.*, 1980; Lesemann and Paul, 1980) and is linked to the fact that antiserum can be used at a higher concentration than usual

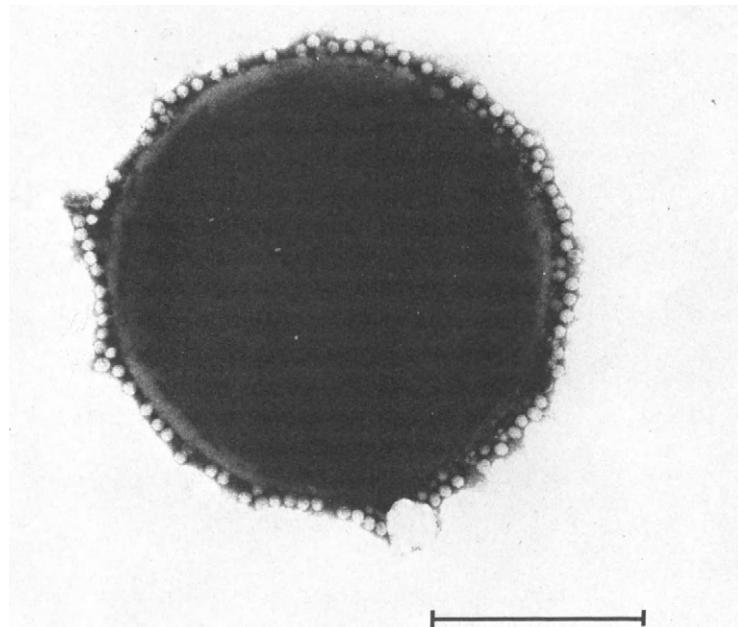


Fig. 6.28. Electron micrograph of *Staphylococcus aureus* cell coated with a 10^{-5} dilution of TYMV antiserum and incubated with 1 µg/ml TYMV. The virus particles are clearly visible at the periphery of the cell after staining with phosphotungstate. The bar represents 500 nm. (Courtesy A. Nicolaïeff).

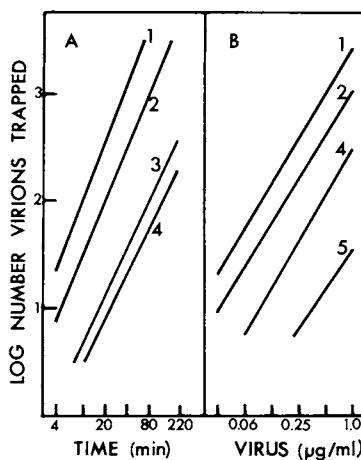


Fig. 6.29. Trapping of five TMV strains on electron microscope grids coated with TMV antiserum diluted 1:3000. 1, TMV; 2, Y-TAMV; 3, HR; 4, U2; 5, CV4. (A) Relationship between the log of the number of virions trapped per unit area of the grid, and time of reaction between the purified virus preparations (0.5 $\mu\text{g/ml}$) and antiserum coated grids. (B) Relationship between the log of the number of virions trapped and the virus concentration.

without producing an inhibition of the trapping reaction. The ability of protein A to bind to antibodies and to lead to the entrapping of virions is illustrated in Fig. 6.28. When *Staphylococcus aureus* cells are incubated with diluted antiserum, IgG molecules bind via protein A to the surface of the bacteria; the resulting antibody-coated cells are then able to "fish out" the homologous virus particles from a dilute suspension (Katz *et al.*, 1980). The serological trapping reaction has been used successfully for detecting different serotypes of a virus with a single antiserum (Nicolaïeff and Van Regenmortel, 1980), as well as for demonstrating serological relationships between different viruses (Roberts *et al.*, 1980). With a series of strains that are increasingly distantly related, the extent of heterologous serological trapping was found to be proportional to the serological distance between the strains (Fig. 6.29). It is possible that the failure to detect heterologous reactions with tymoviruses reported by Lesemann *et al.* (1980) was due to the short 15 minute trapping period used by these authors.

As pointed out by Milne and Luisoni (1977a), the coating of virus particles with a layer of antibody molecules is the most reliable criterion obtainable by electron microscopy, of a specific immune reaction. Clumping and trapping of particles may occur for reasons other than the presence of specific antibody on the grid, and the specificity of these reactions is not directly demonstrable. On the other hand, when the trapping reaction is combined with a subsequent decoration step, the number of observable particles is increased, whereas the specificity of the reaction is demonstrated by the coating of antibody molecules. This

procedure, therefore, combines the advantages of both systems (Milne and Luisoni, 1977a; Kojima *et al.*, 1978).

I. NEUTRALIZATION

Virus neutralization tests measure the loss of virus infectivity resulting from the binding of antibody molecules to the surface of virions. Specific neutralization must be distinguished from the nonspecific inactivation of virus caused by normal serum and which occurs at a lower serum dilution than the specific neutralization effect (Mulvania, 1926; Kassanis, 1943; Rappaport and Siegel, 1955; Kassanis *et al.*, 1978).

Although neutralization tests are widely used with animal viruses and bacteriophages (Fazekas de St Groth, 1962; Svehag, 1968; Osterrieth, 1972; Mandel, 1979) they have limited application in plant virology, mainly because of the lack of sufficiently accurate and sensitive assays for measuring plant virus infectivity.

Many characteristic features of the neutralization reaction of animal viruses have also been described in studies with TMV (Rappaport, 1957b, 1959; Rappaport *et al.*, 1957). When the percentage of surviving virus is plotted against time, an initial linear exponential decline in virus infectivity is followed by a rapid decrease in the rate of inactivation. Eventually a point is reached when no further decrease in infectivity occurs. The residual infectivity, known as the persistent fraction, has been shown not to be due to the presence of genetic variants (Rappaport, 1959). The existence of a small fraction of the viral population that escapes neutralization in spite of the presence of excess antibody has been ascribed to various causes such as (1) aggregation of virus particles prevents virions in the center of the aggregate from coming into contact with antibody; (2) steric hindrance by neighboring antibodies prevents critical sites on the virion from being reached; (3) dissociation of virus-antibody complexes releases infective virus.

Various mechanisms of neutralization have been proposed, such as a single hit model (Svehag, 1968; Trautman and Harris, 1977), a multihit model (Daniels, 1975; Della-Porta and Westaway, 1978), and an allosteric transition model (Mandel, 1976). The last model proposes that the binding of one antibody molecule stabilizes the metastable viral capsid and makes the virus resistant to uncoating.

In the case of TMV, it has been estimated that 4% of the antigenic sites are critical and must remain free of antibody for infectivity to be retained (Rappaport, 1959). This figure of 4% is close to the 3% of the surface represented by the rod extremities of the TMV particle, and it is tempting to speculate that antibody molecules present at the end of the rod simply obstruct the uncoating

process. Such an interpretation is not necessarily in conflict with the neutralization data of Rappaport *et al.* (1964) obtained with TMV particles partly degraded with detergent, since the antibody may still have interfered with subsequent uncoating in a way similar to that found with intact particles.

1. Serological Blocking of Aphid Transmission

This test consists in incubating virus with antiserum, allowing aphids to feed through membranes on the mixture, and determining whether or not the aphids can transmit the virus after the acquisition feeding period (Gold and Duffus, 1967; Rochow and Ball, 1967; Duffus and Gold, 1973). Since the aphids do not always feed well on the virus-antiserum mixture, better results are often obtained when the reactants are first subjected to density gradient centrifugation prior to the feeding of the insects (Duffus and Russell, 1972). A positive serological test then consists of the failure to observe infectivity with material obtained from the normal virus zone. Virus neutralization can also be studied by injecting virus-antiserum mixtures into insect vectors (Black and Brakke, 1954; Rochow, 1970; Rochow and Muller, 1975).

Neutralization tests have been used to demonstrate serological relationships between plant viruses when the usual *in vitro* methods cannot be applied, for instance, with viruses that are not transmissible mechanically. These tests have been of considerable help in clarifying the relationships between members of the luteovirus group (Duffus and Gold, 1969; Duffus and Russell, 1975; Rochow and Duffus, 1978).

2. Modified Phage Assay

The inactivation of bacteriophage by means of specific antiphage serum is probably the most sensitive method for detecting small amounts of antibody. By covalently attaching antigenic groups to the phage surface, it is possible to extend the use of this sensitive test to the detection of a wide variety of antigens, since the modified phage can be neutralized by antibody directed against the antigenic group that is coupled to the phage (Haimovich *et al.*, 1970a,b; Blank *et al.*, 1972). It is also possible to inhibit the neutralization of the modified phage by prior incubation of antiserum with the free antigenic group, and this can serve as a sensitive method for quantitating antigens (Maron and Bonavida, 1971). Sarvas and Mäkelä (1970) have shown that $10^{-13} M$ antibody could be detected in a test using 10^{-17} modified phage.

The method has been used by Du Plessis and Van Regenmortel (1977) to detect small amounts of TMV protein and specific antibody, as well as for serological comparisons between different tobamoviruses.

Role of Quaternary Structure on Viral Antigenicity

A. SOLUBLE ANTIGENS

It has been known for many years that purified virus preparations as well as extracts from infected plants often contain, in addition to intact virions, a variable amount of small proteins antigenically related to the virus. These so-called soluble antigens represent subunits of the coat protein of the virus that are produced in excess in the infected cell and are not encapsidated, or they arise from the degradation of virus particles (Doke, 1972). Soluble antigens are usually recognized by the presence of fast diffusing precipitin lines in immunodiffusion tests and have been described, for instance, with TMV (Kleczkowski, 1957), BMV (Hamilton, 1961), PNRSV (Willison *et al.*, 1961; Allen and Tremaine, 1965), CMV (Grogan *et al.*, 1963; Scott, 1968; Ziemiecki and Wood, 1975), tobacco necrosis virus (TNV) (Babos and Kassanis, 1963), CIYMV (Purcifull and Shepherd 1964), pea enation mosaic virus (PEMV) (Izadpanab and

Shepherd, 1966), BSMV (Hamilton and Ball, 1966), PVX (Van Regenmortel, 1966b; Shalla and Shepard, 1970b), Cacao necrosis virus (CNV) (Tremaine, 1972), and ACLSV (Chairez and Lister, 1973a,b). Soluble antigens may undergo proteolytic degradation in tissue extracts, and this may alter their antigenic specificity (Hiebert and McDonald, 1976). If valid comparisons between the epitopes of virions and their protein subunits are to be made, it is thus essential to establish that the immunogen and test antigens have not been chemically degraded (Chapter 3, Section A,3,c).

Since soluble antigens can usually be detected with virus antisera, it follows that most virus preparations used for immunization contain antigens related to the subunits. However, it has been demonstrated in a few instances that there is very little or no serological relationship between a particular virus and its soluble antigen. The most likely explanation for the presence of subunit-specific antibodies in these cases is that the unrelated epitopes arose inside the body of the animal as a result of capsid dissociation.

B. CRYPTOTOPES AND NEOTOPES

The antigenic specificity of viral proteins, like that of all globular proteins is largely dependent on the three-dimensional folding of the polypeptide chain (see Chapter 1). In viral capsids the bonds between adjacent subunits lead to conformational changes in the protein monomers and these may induce the formation of different conformational epitopes in the polymerized and depolymerized states of the coat protein. It is clear that changes in the folding of the polypeptide chain may alter the respective positions of the residues that contribute to the structure of epitopes.

As shown in Fig. 7.1, the aggregation of subunits into a polymeric structure leads to the effective disappearance of those surfaces of the protein that are turned inward in the capsid. Since the epitopes situated on these hidden surfaces are no longer manifest after polymerization, they have been called hidden epitopes or *cryptotopes* (Jerne, 1960). Such hidden epitopes are likely to be a feature of the antigenic specificity of all macromolecular structures built up from subunits (Bårtel and Campbell, 1959). In addition to such buried cryptotopes, the subunit surface that is exposed to the solvent in both the capsid and the monomer (A and A' in Fig. 7.1) may be sufficiently altered by conformational change to harbor epitopes of different specificity in the polymerized and depolymerized forms of the protein. It may be operationally difficult if not impossible to distinguish cryptotopes buried on hidden surfaces from those that are no longer found on the outside surface because of conformational change.

The reverse situation is somewhat easier to interpret, since any new epitopes that are found only in the capsid must necessarily arise on a surface common to

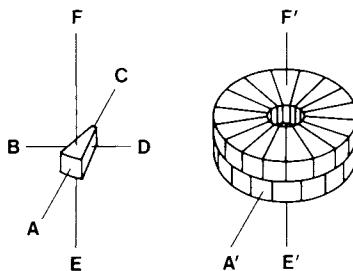


Fig. 7.1. A model of the protein subunits of tobacco mosaic virus in monomeric form and as a double layer disk. Surface A' harbors neotopes, whereas surfaces A, B, C, and D possess cryptotopes. Surface E' and F' represent an important part of the disk surface but a relatively insignificant part of the 300-nm-long virions (from Van Regenmortel and Lelarge, 1973).

monomers and polymerized protein. Such capsid-specific epitopes that are not present on the corresponding surfaces of the monomeric subunit have been called new epitopes or *neotopes* (Van Regenmortel, 1966b, 1967). Neotopes could arise either through conformational change only, or by the creation of a totally new structure through the juxtaposition of amino acid residues from neighboring subunits. In the latter case, the neotope would correspond to a type of discontinuous determinant (as defined by Atassi and Smith, 1978; see Chapter 1), in which the juxtaposed residues originate from separate polypeptide chains.

The existence of neotopes in viral capsids has been demonstrated for all major groups of viruses (Neurath and Rubin, 1971; Norrby and Wadell, 1972; Eppstein and Thoma, 1977). With animal viruses, antibodies directed to neotopes could play an important role in virus neutralization, in which case antibodies formed as a result of immunization with monomeric subunits would fail to neutralize the virus efficiently. This is of considerable practical importance in view of current attempts to produce subunit vaccines against several human and animal viruses (Wiktor *et al.*, 1973; Rubin and Tint, 1975; Parks and Rapp, 1975; Bachrach *et al.*, 1978). 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 (Rapp and Reed, 1976) and second because it reduces the pyrogenic activity and toxic side effects of some whole virus vaccines. In general, it is found that the immunogenicity of subunit vaccines is much lower than that of whole virus vaccines. On the other hand, immunization with the product of a limited reassociation of subunits often increases the neutralizing activity of the vaccine compared with the poor efficacy of isolated monomers (Morein *et al.*, 1978). In some cases, relatively small aggregates already give rise to a neotope-like conformation, and a knowledge of the factors that control neotope formation is thus of considerable importance.

Immunodiffusion tests have revealed the presence of neotopes on the surface of many plant viruses, e.g., TMV, PNRSV, TYMV, TEV, BMV, AMV,

BSMV, CMV, and tobacco rattle virus (TRV) (Van Regenmortel, 1966b; Moed and Veldstra, 1968; Devergne and Cardin, 1970; Gugerli, 1976). In some cases a total lack of serological cross-reactivity between protein monomers and capsids was observed, but in others a limited cross-reactivity was found to exist. However, interpretation is often made difficult by the presence of aggregates of various sizes in the subunit preparation.

A few other terms should be introduced at this stage, since their use can simplify discussions of the serological properties of viral proteins. Particularly useful are the terms *paraneotope* and *paracryptotope*, which describe the antibody combining sites (or paratopes) specific for neotopes and cryptotopes, respectively. *Metatope* has been used sometimes to refer to epitopes common to both capsids and dissociated subunits (Van Regenmortel, 1966b; Devergne and Cardin, 1970).

C. STUDIES WITH INDIVIDUAL VIRUSES

1. Tobacco Mosaic Virus

More studies have been devoted to this virus than to any other plant virus (Rappaport, 1965; Van Regenmortel, 1981b), and this applies also to the analysis of the influence of polymerization on the antigenic specificity of viral proteins. In order to facilitate the subsequent discussion, our current knowledge of the aggregation states of TMV protein will be briefly summarized.

TMV protein is usually found in solution in the form of oligomers ranging from trimers to closed two-ring disks of 34 subunits (Durham and Klug, 1971). Only under conditions of low concentration, low temperature, and low ionic strength, is the protein obtained in monomeric form (Ansevin and Lauffer, 1959). Caspar (1963) argued on thermodynamic grounds that the first stable aggregate of the protein subunits should be a trimer, which corresponds approximately to a structure with sedimentation coefficient 4 S. The next stable aggregate is believed to be a cyclic heptamer with a sedimentation coefficient of 8 S (Durham *et al.*, 1971; Durham and Klug, 1971; Lonchampt *et al.*, 1972). The next larger aggregate is the 20 S two-turn disk (Fig. 7.1), which is used for initiation of virus assembly (Butler and Klug, 1971). The disk serves as a pre-formed array of nucleotide binding sites that interact with a specific nucleation sequence of 170 nucleotides situated about 900 residues from the 3' extremity of the RNA (Butler and Durham, 1977; Hirth and Richards, 1980). Following the initial nucleation step, the rods elongate in two opposite directions by further addition of protein subunits. This leads to the formation of a helical array of protein molecules. The mechanism of self-assembly has proven to be much more complex than originally thought, since it now appears that the virus builds itself

from the inside out, i.e., that the RNA inserts itself into the helix from within the central channel of the already completed portions of the rod (Lebeurier *et al.*, 1977; Butler *et al.*, 1977). Addition of protein occurs at the end of the growing rod, where the RNA folds back as it is being drawn up along the central channel. Once the RNA is completely encapsidated, further addition of subunits stops. However, the last layers of subunits are probably in a metastable situation and are able to "peel off" readily. This would explain the finding that purified virus preparations, obtained by sedimenting the virus several times in an ultracentrifuge, continue to release small amounts of protein subunits. Only by stabilizing the particles with formaldehyde can this dissociation of subunits be prevented (Van Regenmortel and Lelarge, 1973).

In the older literature, TMV protein subunits were either referred to as A protein, because they can be obtained from intact virions by alkaline degradation (Schramm, 1947; Schramm *et al.*, 1955) or as X protein, when they are isolated from the sap of TMV-infected leaves (Takahashi and Ishii, 1952, 1953; Jeener and Lemoine, 1953).

Differences in antigenic specificity between TMV and its protein subunits have been known for many years. The presence of neotopes on the surface of virions was demonstrated by Jeener *et al.* (1954), Starlinger (1955), Aach (1959), and Takahashi and Gold (1960), but could not be confirmed by Kleczkowski (1961, 1966). The method used for demonstrating a new antigenic specificity on the capsid consisted in first absorbing TMV antiserum with subunits and then testing for any residual reaction with the virus. Kleczkowski (1966) who could not demonstrate the presence of paraneotopes in TMV antiserum, used TMV protein at a concentration of 50 mg/ml to absorb his antiserum. Since such highly concentrated protein preparations must have contained a considerable amount of disks, it is not possible to draw any conclusions from such experiments. The presence of aggregated TMV protein in Kleczkowski's preparations is confirmed by the appearance of the viral protein precipitin curves obtained by this author (Fig. 6.2).

The presence of cryptotopes on TMV subunits was demonstrated by the residual precipitin reaction observed when the subunits were tested with TMV antiserum absorbed with virus particles (Aach, 1959; Van Regenmortel, 1967). In this case again, the results could not be confirmed by other workers (Kleczkowski, 1961; Rappaport *et al.*, 1965) who found that absorption of TMV protein antiserum with virus resulted in the removal of all antibodies capable of reacting with the subunits. Subsequent studies showed that the failure of these workers to demonstrate the presence of paracryptotopes in their antisera was probably due to the presence of depolymerized protein in their virus preparations (Van Regenmortel and Lelarge, 1973). The main lesson to be learned from these discrepant reports is that this type of cross-absorption test cannot give reliable

results unless the polymerization state of the antigen used for absorption is carefully controlled.

The rod extremities of the TMV particle represent a special case, since they possess antigenic specificities that are absent over 97% of the virion surface. Although early work by Malkiel (1947b) failed to detect additional specificities on the rod extremities of sonic-treated TMV, there is evidence that the epitopes present on these surfaces can play a role in certain cross-absorption experiments (Van Regenmortel and Lelarge, 1973).

Another controversy was generated by the report of Kleczkowski (1957, 1961) that a large number of precipitin lines were formed by TMV protein in immunodiffusion tests. He suggested that preparations of TMV protein contained as many as seven antigenically different components, a conclusion challenged by Van Regenmortel (1967) who argued that Kleczkowski's double diffusion tests had been performed under conditions (large antigen excess, diffusion time of 1 month) known to produce artifacts. Kleczkowski (1968) subsequently argued that his own experiments had been performed in the neighborhood of optimal proportions, with an antibody-antigen ratio corresponding to equivalence in tube precipitin tests. However, since the antigen precipitated in tube precipitin tests was partly aggregated, whereas the precipitin lines in gel diffusion tests are formed by monomers (polymers are dissociated by the dilution effect that accompanies diffusion), the effective equivalence point in the two tests is not the same (Van Regenmortel and Lelarge, 1973).

The presence of multiple precipitin lines was also reported by Rappaport and Zaitlin (1967, 1970) who analyzed TMV protein by immunoelectrophoresis. These authors interpreted their results in terms of a single determinant model, which assumes that only one epitope is present on the surface of TMV protein. It was proposed that this single epitope stimulates the production of a heterogeneous population of antibodies, some of which are capable of recognizing different conformational states of the protein induced by polymerization. This single determinant model was subsequently refuted by the finding that the antigenic valence of monomeric TMV protein is at least three (Van Regenmortel and Lelarge, 1973). The location of some cryptotopes on surfaces of the subunit that are buried in the capsid has also been established (Benjamini *et al.*, 1972a; Milton and Van Regenmortel, 1979).

When immunodiffusion tests with nonaggregated TMV protein are performed under balanced reactant conditions, two precipitin lines corresponding to antigens of low molecular weight are commonly observed (Jeener *et al.*, 1954; Kleczkowski, 1957; Van Regenmortel, 1967; McCarthy, 1968; Van Regenmortel and Lelarge, 1973). The diffusion coefficient of these antigens determined by double diffusion tests (Chapter 6, Section B,3) was found to be $11.4\text{--}14.6 \times 10^{-7} \text{ cm}^2 \text{ sec}^{-1}$, which corresponds to an antigen of about 15,000 molecular

weight (Lelarge, 1974). When TMV protein was isolated in the monomeric state from a single band obtained after electrophoresis in polyacrylamide gels, this material still gave rise to the same two precipitin lines (Lelarge, 1974). A possible explanation of this phenomenon is that the binding of antibody to some cryptotopes can induce, in the monomer, a conformational change similar to that observed during the polymerization of the subunit into capsids. Since antibodies specific for each conformational state are usually present in most antisera, it is tempting to speculate that they are able to react separately with the native and induced conformational forms of the epitope. It should be noted, incidentally, that in heterologous reactions with protein of other TMV strains, only one of the two precipitin lines is obtained. An alternative explanation for these two lines was proposed by Knuhtsen (1972) who suggested that they were due to monomers and dimers of TMV protein. However, attempts made to confirm the presence of significant amounts of dimers in preparations of TMV protein failed (Lelarge, 1974). With some strains that contain cysteine, SDS-polyacrylamide gel electrophoresis revealed the presence of a dimer band that represented less than 1% of the monomer band. In the presence of a reducing agent, or when strains devoid of cysteine were examined, a single monomer band was observed, although such preparations induced the usual two precipitin lines in immunodiffusion tests.

When TMV subunits are polymerized to the 8 S or 20 S aggregation states, the protein has an electrophoretic mobility characteristic of viral rods and is able to react with some of the paraneotopes present in TMV antisera. However, in some antisera antibodies are present that react only with subunits in the helical configuration found in virus rods, and not with disks possessing cylindrical symmetry (Van Regenmortel and Lelarge, 1973). These results demonstrate the very high discriminating power of antibodies for recognizing subtle conformational alterations in the capsid surface, and suggest that immunochemical studies could be useful for probing such phenomena as the swelling of isometric viruses (Sehgal *et al.*, 1979) and the change in length and flexuousness induced in filamentous viruses by magnesium ions (Govier and Woods, 1971).

2. Barley Stripe Mosaic Virus

The presence of neotopes in BSMV has been demonstrated by cross-absorption experiments by Hamilton and Ball (1966) and Atabekov *et al.* (1968a,b). Aggregates of BSMV protein with approximate sedimentation coefficients of 10 S, 20 S, and 30 S were found to possess the same neotope specificity as intact BSMV particles (Atabekov *et al.*, 1968b). The 30 S aggregate appears to be the double disk equivalent of the 20 S polymer found with TMV protein. The molecular structure of the 10 S and 20 S aggregates of BSMV protein is unknown, but it has been inferred from calculations that neither of them corre-

sponds to a single layer disk (Atabekov *et al.*, 1968c). The monomeric 2 S BSMV subunit is likely to possess cryptotopes, but cross-absorption experiments demonstrating a separate monomer specificity have not been reported (Gumpf and Hamilton, 1968; Atabekov *et al.*, 1968a).

3. Potato Virus X

Crude sap from plants infected with PVX has been shown to contain variable amounts of soluble antigens (Van Regenmortel, 1966b; Shalla and Shepard, 1970b). The antigenic properties of PVX subunits (in the form of dimers) and whole virions have been compared by Shepard and Shalla (1970) and Shalla and Shepard (1970a). These authors ascribed the observed antigenic differences between subunits and capsids to the conformational changes that occur during dissociation of the virions. They took the view, already propounded by Rappaport (Rappaport, 1965; Rappaport *et al.*, 1965; Rappaport and Zaitlin, 1970) that there are two, necessarily mutually exclusive explanations for the presence of novel antigenic specificities on viral subunits, namely, conformational change or the uncovering of hidden determinants. Shalla and Shepard (1970a) stated that since there was no evidence that conformational changes did not occur when the capsids of different viruses were depolymerized, the entire concept of cryptotopes as buried determinants was not very plausible and should be viewed as "highly hypothetical." The authors' own data, however, showed that the two explanations are not mutually exclusive, since they provided evidence for both a conformational change in the PVX subunit and for the existence of genuine "buried" cryptotopes in their system. Indeed, they showed that about four antibody molecules could bind to one PVX subunit, and since it is impossible to accommodate so many IgG molecules on the one extremity of the viral subunit that is exposed in the capsid, these results in fact represent good experimental evidence for the presence of cryptotopes in PVX protein.

The difference in antigenic specificity between PVX subunits obtained by degrading the virus with pyridine and the native soluble antigens, reported by Shalla and Shepard (1970b), remains unexplained. An irreversible denaturation of the subunits obtained by chemical degradation appears to be ruled out, as an explanation, since functional subunits that can be reassembled into PVX-like particles were found to be serologically indistinguishable from subunits obtained by pyridine degradation (Goodman, 1975b).

4. Brome Mosaic Virus

Since BMV is unstable at neutral pH, it could be expected that the virus will become degraded in the body of animals used for immunization. It has indeed been observed that most antisera to unstabilized BMV contain large quantities of

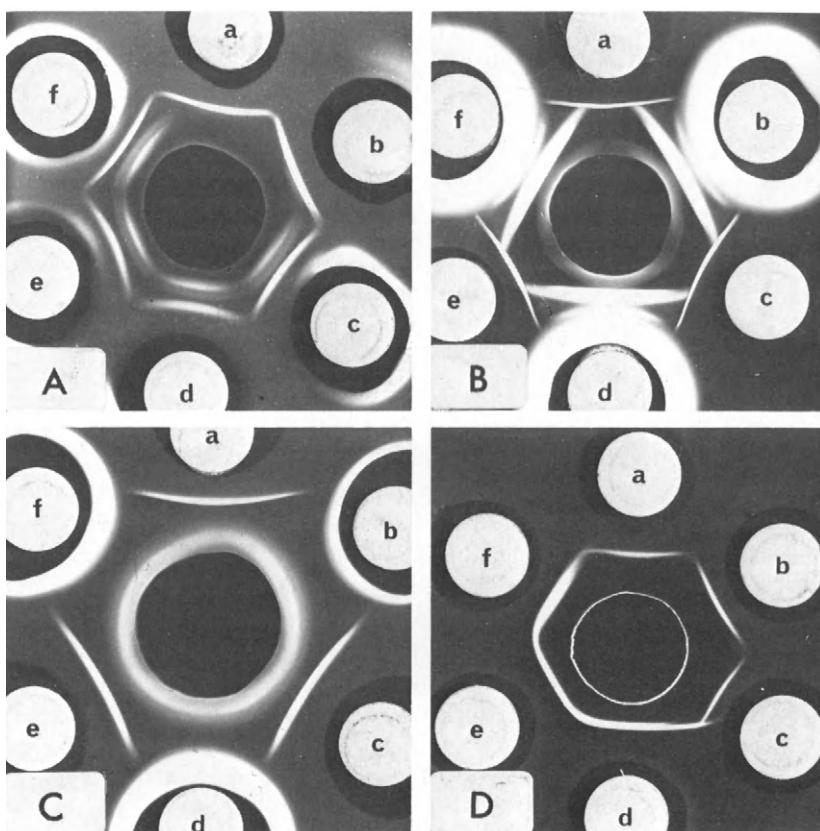


Fig. 7.2. Immunodiffusion precipitin patterns illustrating the presence of neotopes and cryptotopes in different aggregation states of brome mosaic virus protein (from Von Wechmar and Van Regenmortel, 1968) (A) Central well contains antiserum prepared against unstabilized BMV. Wells a and b contain BMV at pH 4.0. No reaction with subunits is visible. Wells c to f contain various BMV preparations at pH 7.0. Reaction with subunits that dissociate from the virus at pH 7.0 gives rise to additional precipitin lines. (B) Central well filled with same antiserum as in A. Wells a, c, and e contain BMV in acetate buffer at pH 4.0. Wells b, d, and f contain dissociated BMV subunits in 0.01 M CaCl_2 , pH 6.0. Note that spurs correspond to a reaction between neotopes on BMV particles and paraneotopes. (C) Central well contains antiserum prepared against BMV stabilized with 0.2% formaldehyde. Wells a, c, and e contain BMV at pH 4.0. Wells b, d, and f contain dissociated BMV subunits in 0.1 M Tris buffer pH 7.0.(D) Central well filled with antiserum prepared against dissociated BMV subunits stabilized with 0.2% formaldehyde. Wells a, b, and c contain BMV at pH 4.0, at 1.0, 0.5, and 0.25 mg/ml, respectively. Note the spurs between wells a-f and wells c-d, indicating the presence of paracryptotopes in the antiserum.

paracryptotopes in addition to paraneotopes and antibodies specific for both capsids and dissociated subunits (Von Wechmar and Van Regenmortel, 1968). Antibodies specific for neotopes and cryptotopes have been prepared from such antisera by absorption with the appropriate antigens. When BMV stabilized with 0.2% formaldehyde was used for immunization, an antiserum resulted that did not react with dissociated subunits, and contained, therefore, only paraneotopes (Figs. 7.2A-C). When formalinized subunits were used for immunization, the resulting antiserum contained paracryptotopes as well as antibodies reacting with both capsids and dissociated subunits (Fig. 7.2D). The neotope specificity could be regained by letting the subunits reassociate in an immunodiffusion medium prepared with cacodylate-buffered saline (Von Wechmar and Van Regenmortel, 1968). It has been shown by Tremaine and Chidlow (1972) that when dissociated protein of the related bromovirus CCMV is reassembled into spherical particles two-thirds the size of virions, a neotope specificity identical to that of the virus is also regained. Evidence for the presence of some cross-reactivity between the neotopes of BMV and CCMV as well as between the cryptotopes of BMV and CCMV proteins has been presented by Scott and Slack (1971). By means of indirect ELISA, Rybicki and Von Wechmar (1981) recently showed that the coat proteins of bromoviruses were more closely related serologically than the parent viruses.

5. Potyviruses

The antigenic specificity of dissociated subunits of potyviruses such as PVY, TEV, and SoyMV is markedly different from that of the corresponding intact virions (Shepard *et al.*, 1974a; Soong and Milbrath, 1980). Compared with the distant serological relationships that exist among the virions of PVY, BCMV, LMV, MDMV, TurMV, and watermelon mosaic virus (WMV), the dissociated subunits of these viruses were found to be much more closely antigenically related. In immunodiffusion tests with antisera against PVY and TEV subunits, the heterologous cross-reaction with 11 other potyviruses consisted of precipitin lines that fused completely (Shepard *et al.*, 1974a). This indicates that a common antigenic group present in many potyviruses becomes expressed only when the virions dissociate. This enhanced cross-reactivity may result from the uncovering of hidden surfaces of the subunits, or from the fact that sequence similarities often become immunochemically expressed when molecules are partly denatured (Arnon and Maron, 1971; Arnheim *et al.*, 1971). This type of cross-reaction between the subunits of different potyviruses became measurable only when antisera obtained after long immunization periods were used (Uyemoto *et al.*, 1972; Shepard *et al.*, 1974a).



Virus Identification

A. INTRODUCTION

The antigenic properties of virions represent the single most useful criterion for reliable virus identification. The main advantages of the serological diagnosis of virus infections are its specificity, rapidity, and reliability. In the past, indexing of virus-infected plant material was mostly done by inoculation to indicator hosts that produce characteristic symptoms. Although this approach remains valuable today (Hakkaart, 1969; Horst and Lawson, 1975; Ramsdell *et al.*, 1979), it is time-consuming and expensive with respect to man power and plant growing facilities. Bioassay indexing methods are also cumbersome for testing a large number of samples. Furthermore, symptoms can be greatly modified by such factors as the host cultivar, environmental conditions such as light and temperature, time of infection, strain of virus, as well as the presence of other viruses. As a result, the same virus is often capable of inducing a confusing variety of host responses.

In contrast, specific antibodies always react in a reproducible fashion with the homologous virus, and they represent an unvarying standard reference for virus identification. It should be pointed out, however, that major differences in the

pathogenicity of strains are not always reflected in the antigenic properties of the corresponding virions. Studies on host range and symptoms will thus always be needed for assessing the disease-producing characteristics of a virus (Hollings, 1974; Gooding, 1975; Devergne *et al.*, 1978).

Other properties that have been used for characterizing and identifying viruses are the morphological and physicochemical properties of virions, and the so-called *in vitro* properties of viruses in plant sap. Francki (1980) argued cogently for abandoning the classical *in vitro* properties of viruses in plant sap as an aid in virus identification. A literature search revealed considerable variability in reported values of thermal inactivation point, longevity *in vitro*, and dilution end point for individual viruses, and in most cases the data did not allow any conclusive taxonomic assignments.

As far as morphological properties are concerned, these are mainly useful for assigning a virus to a particular group (Brandes and Bercks, 1965). Furthermore, electron microscopy is not suited for the large-scale analysis of many samples. The same limitation applies to the study of virus-induced inclusions, which is useful mainly for virus diagnosis at the group level (Edwardson and Christie, 1978). These inclusions are found in the cytoplasm or nucleus of virus-infected cells and consist of large aggregates of virus-coded nonstructural proteins (Dougherty and Hiebert, 1980a,b,c; Shepherd *et al.*, 1980). The inclusion proteins of potyviruses (Shepard and Shalla, 1969; Hiebert *et al.*, 1971; Hiebert and McDonald, 1973; Purcifull *et al.*, 1973; Knuhtsen *et al.*, 1974), tobamoviruses (Granett and Shalla, 1970), and PVX (Shalla and Shepard, 1972) have been shown to be antigenically unrelated to the capsid protein. Recently, immunoprecipitation studies with translation products of potyvirus RNA demonstrated that the inclusion protein genes represent a major part of the total viral genome (Dougherty and Hiebert, 1980b,c). It seems likely, therefore, that in the future, immunochemical studies of inclusion proteins will widen the range of virus-coded gene products that can be analyzed serologically for the purpose of virus identification and classification. Recently, Lawson and Hearon (1980) showed that carnation etched ring virus (CERV) infection could be diagnosed by means of an antiserum specific for the inclusion protein associated with this virus.

Another major advantage of diagnostic procedures based on serology is that they allow virus identification to be made at both the group and strain level. Differentiation between virus strains is usually accomplished by means of antisera that have been cross-absorbed with heterologous strains. Serologically distinguishable strains of TMV, AMV, and CMV have been used, for instance, for localizing the cistron coding for the coat protein of these viruses, as well as for studying virus replication and self-assembly. In complementation experiments with RNA fragments from different strains (Kado and Knight, 1968) or in hybridization experiments with different components from multipartite virus strains (Dingjan-Versteegh *et al.*, 1972), it was possible to show that the coat protein

serotype of a recombinant was specified by a particular piece of RNA (Mossop and Francki, 1977; Thongmeearkon and Goodman, 1978). Phenotypically mixed virions of TMV strains have been recognized by immunoprecipitation (Taliansky *et al.*, 1977) and in mixed reconstitution experiments with two TMV serotypes, the regions of the virus rods encapsidated with the two serologically distinct proteins have been identified by immunoelectron microscopy (Otsuki *et al.*, 1977; Fukada *et al.*, 1980).

B. DIAGNOSIS OF VIRUS DISEASES

From the earliest serological studies on plant viruses (Dvorak, 1927; Purdy, 1929; Purdy-Beale, 1931, 1934; Gratia, 1933, 1934; Chester, 1934, 1935) it became apparent that the antigenic specificity of individual viruses would be extremely useful for the diagnosis of virus diseases (Chester, 1937a,b,c; Malkiel, 1947a; Matthews, 1957).

One of the main economic advantages to be gained from research on plant viruses is linked to the improved yields of agricultural crops brought about by an early diagnosis of virus infections.

Since there is no cure for virus-diseased plants, prevention is the only remedy and most efforts have concentrated on the selection of healthy propagating material. In annuals such as cereal and vegetable crops that are not propagated vegetatively, the major practical advantage of a rapid serological diagnosis of virus infection is limited to the detection of seed-borne viruses in seed lots (Scott, 1961; Hamilton, 1965; Carroll *et al.*, 1979).

In the case of vegetatively propagated crops such as potatoes, flowerbulbs, and fruit trees, infected plants must be recognized at the earliest possible time and rapid methods of diagnosis applicable to large scale use are thus essential. The first successful certification program for virus-free seed potatoes was operated 30 years ago in the Netherlands. Millions of potato plants were tested annually by the microprecipitin technique (Van Slogteren and Van Slogteren, 1957) and this led to a considerable improvement in seed potato quality.

Reliable immunodiffusion techniques based on the use of dissociated viral subunits were developed in the United States for PVX and PVS diagnosis on a mass scale (Shepard, 1972; Shepard and Claflin, 1975). The serological detection of the labile PVA has also become possible (Fribourg and De Zoeten, 1970; Gnutova and Krylov, 1975) and simplified procedures that eliminate tissue grinding and chemical treatment of the antigen have been developed (Sampson and Taylor, 1968; Slack and Shepherd, 1975; Richter and Polak, 1975; Hinostroza de Lekeu, 1979; Lima and Purcifull, 1980). The nonspecific precipitation reactions that occur with the sap of some plants can be prevented by using certain chemical additives or by replacing whole antisera with purified immunoglobulin

preparations (Sutic, 1960; Shepard, 1970a; Purcifull and Batchelor, 1977; Staszewicz, 1977).

False positive results can sometimes be obtained when virus antisera contain antibodies to host plant antigens. This problem can be overcome by including healthy plant controls in the testing procedure. For the reliable identification of an unknown virus sample, it is necessary to let the test antiserum react both with an extract from healthy plants and with the homologous virus antigen. This precaution will eliminate false negative results that could be due, for instance, to an imbalance of the reactants or to an insufficiently sensitive test.

In recent years, the use of increasingly sensitive serological methods has made it possible to detect viruses that are present in infected tissue in very low concentration, for instance, in seed and dormant potato tubers (Kahn *et al.*, 1967; Schade and Schimanski, 1974; Clark and Adams, 1977; Tamada and Harrison, 1980b). The ELISA technique especially has become widely used for the large-scale testing of field samples (Thresh *et al.*, 1977; Casper, 1977a,b; Lister, 1978; Barbara *et al.*, 1978; Gugerli, 1978, 1979, 1980). Several authors have shown that the sensitivity of virus detection by ELISA is comparable to that obtained by infectivity tests on indicator plants (Clark *et al.*, 1976a; Jafarpour *et al.*, 1979; Gonsalves, 1979; Ramsdell *et al.*, 1979). In the case of TMV infections in plants such as grapevines (Bercks, 1967c), fruit trees (Gilmer and Wilks, 1967; Opel *et al.*, 1969), red currant (Kleinhempel, 1969), and sugar beet (Putz *et al.*, 1981), the use of sensitive methods of serodiagnosis made it possible to detect very low concentrations of virus in the original infected plant material. Only through such a direct identification can the possibility be excluded that the virus (when it is as infectious as TMV) was introduced as a contaminant during experimental transmission.

C. IDENTIFICATION OF INDIVIDUAL VIRUSES

A list of all plant viruses that have been identified by serological means appears in Table 8.1. The different techniques that have been used with each virus are abbreviated in the table as follows: PRE, liquid precipitation tests; ID, immunodiffusion tests; IE, immunoelectrophoresis test; PHA, passive hemagglutination test; LAT, latex test; CF, complement fixation test; EIA, enzyme immunoassay (ELISA); RIA, radioimmunoassay; FLU, fluorescent antibody test; IEM, immunoelectron microscopy; NEU, neutralization test.

The references in Table 8.1 have been selected either because of their methodological interest, or because they clearly illustrate the value of serodiagnosis for the corresponding virus diseases. Recent papers from as many laboratories as possible have been included, since this may help the reader to localize likely possible sources of diagnostic antisera. The CMI/AAB numbers refer to the

TABLE 8.1

Virus	Abbreviation	Group	Serological techniques	Reference	Number of CMI/AAB description
Agropyron mosaic virus					
Alfalfa latent virus	AgMV	POTY	PRE	Slykhuis and Bell, 1966	118
Alfalfa mosaic virus	ALV	CARLA	PRE; ID	Veerisetty and Brakke, 1977	211
	AMV		ID; EA	Bancroft et al., 1960;	46; 229
				Dingjan-Veststeegh et al., 1972; Bercks et al., 1973; Vuittenez et al., 1974;	
Andean potato latent virus	APLV	TYMO	ID; LAT; EIA; IEM	Gooding, 1975; Marco and Cohen, 1979	
				Fribourg et al., 1977a; Koenig and Bode, 1978;	
				Koenig et al., 1979;	
Andean potato mottle virus	APMV	COMO	LAT; ID	Lesemann et al., 1980	203
				Fribourg et al., 1977a,b;	
Apple chlorotic leaf spot virus	ACLSV	CLOSTERO	PRE; ID; EIA; LAT	Koenig and Bode, 1978; Salazar and Harrison, 1978c	
				Saksena and Mink, 1969;	
				Lister and Hadidi, 1971;	
				Chairez and Lister, 1973a,b;	
				Fleegg and Clark, 1979;	
Apple mosaic virus	ApMV	ILAR	PRE; ID; EIA	Fuchs et al., 1979	
				De Sequeira, 1967; Fulton, 1968; Seneviratne and Posnette, 1970; Casper, 1973; Barbara et al., 1978	
Apple stem grooving virus	ASGV	CLOSTERO	PRE; LAT	De Sequeira and Lister, 1969a,b	31

Arabis mosaic virus	ArMV	NEPO	ID; IE; LAT; EIA; IEM	Hollings, 1963; Bock, 1966; Tomlinson and Walkley, 1967a; Bercks, 1973; Bercks et al., 1976; Thresh et al., 1977; Thomas, 1980	16
Arracacha virus A	AVA	NEPO	ID	Jones and Kenten, 1978	216
Artichoke Italian latent virus	AILV	NEPO	ID	Majorana, 1974	176
Artichoke mottle crinkle virus	AMCV	TOMBUS	ID; IE	Martelli et al., 1971;	
Barley stripe mosaic virus	BSMV	HORDEI	ID; IEM; IE; LAT	Hollings and Stone, 1975 Hamilton, 1964; Hamilton and Ball, 1966; Atabekov et al., 1968b; Slack and Shepherd, 1975; Lunsgaard, 1976; Bransky and Derrick, 1979; Carroll et al., 1979	68
Barley yellow dwarf virus	BYDV	LUTEO	ID; EIA; IEM	Apolo and Rochow, 1971; Derrick and Bransky, 1976; Paliwal, 1977, 1979; Rochow and Duffus, 1978; Lister and Rochow, 1979	32
Barley yellow mosaic virus	BarYMV	POTY	CF PRE; ID; EIA; IEM	Usugi and Saito, 1976 Bercks, 1959, 1960b; Zaumeyer and Goth, 1964; Uyemoto et al., 1972;	143
Bean common mosaic virus	BCMV			Meiners et al., 1978; Jafarpour et al., 1979	73
Bean golden mosaic virus	BGMV	GEMINI	ID	Goodman and Bird, 1978	192
Bean mild mosaic virus	BMMV		ID	Waterworth et al., 1977	
Bean pod mottle virus	BPMV	COMO	ID; PRE	Scott et al., 1961; Bancroft, 1962; Shepherd, 1963,	108
				1964; Agrawal and Maat, 1964; Campbell, 1964; Moore and Scott, 1971	

(continued)

TABLE 8.1—Continued

Virus	Abbreviation	Group	Serological techniques	Reference	Number of CMV/AAB description
Bean yellow mosaic virus	BYMV	POTY	PRE; ID; EIA	Bercks, 1960a,b; Bercks, 1961, Taylor and Smith, 1968; Uyeda et al., 1975; Jones and Diachun, 1977; Stein et al., 1979	40
Bearded iris mosaic virus	BIMV	POTY	PRE; LAT ID; LAT; FLU	Barnett et al., 1971	147
Beet curly top virus	BCTV	GEMINI		Mumford, 1977; Mumford and Thornley, 1977	210
Beet mild yellowing virus	BMYV	LUTEO	NEU	Duffus and Russell, 1975	
Beet necrotic yellow vein virus	BNYVV		PRE; ID	Putz, 1977; Tamada and Baba, 1973	144
Beet western yellows virus	BWYV	LUTEO	NEU	Duffus and Gold, 1969; Duffus and Russell, 1970, 1972, 1975; Duffus and Rochow, 1978	89
Beet yellow stunt virus	BYSV	CLOSTERO	PRE	Duffus, 1972	
Beet yellows virus	BYV	CLOSTERO	PRE; NEU	Polak, 1971	207
Belladonna mottle virus	BelMV	TYMO	ID; IE; LAT; IEM	Jankulowa et al., 1968; Koenig, 1969b; Bercks and Querfurth, 1969; Lesemann et al., 1980	13
Bidens mottle virus	BidMV	POTY	PRE; SDS; ID	Purcifull and Zitter, 1973;	161
Blackeye cowpea mosaic virus	BICMV	POTY	ID; IEM	Shepard et al., 1974a	
Black raspberry latent virus	BRLV	ILAR	PRE	Lima et al., 1979; Lima and Purcifull, 1980	
				Converse and Lister, 1969; Lister and Converse, 1972	106

Blueberry shoestring virus	BBSVV	ID	Lesney et al., 1978
Broad bean mottle virus	BBMV	ID	Scott and Slack, 1971
Broad bean necrosis virus	BBNV	PRE	Inouye and Nakasone, 1980
Broad bean stain virus	BBSV	PRE; ID; IE	Gibbs et al., 1968; Moghal and Franck, 1974; Goyer, 1975; Fischer and Lockhart, 1976
Broad bean true mosaic virus	BBTMV	COMO	ID; IE
Broad bean wilt virus	BBWV	PRE; ID	Taylor et al., 1968; Froud and Tomlinson, 1972b; Sahambi et al., 1973; Barker, 1976
Broccoli necrotic yellow virus	BNYV	RHABDO	Uyemoto and Provvidenti, 1974; Doe, 1975
Brome mosaic virus	BMV	BROMO	ID; IE; PHA
Cacao necrosis virus	CNV	NEPO	Lin and Campbell, 1972
Cacao swollen shoot virus	CSV	RHABDO	Hamilton, 1961; Richter, 1967; Von Wechmar and Van Regenmortel, 1968;
Cacao yellow mosaic virus	CYMV	TYMO	Scott and Slack, 1971; Richter et al., 1973
Cactus virus X	CX	POTEX	Kenten, 1972
Cacao necrosis virus		PRE; ID	Brunt, 1970
Cacao swollen shoot virus		PRE; ID	Brunt et al., 1965
Cacao yellow mosaic virus		ID; PRE	Milicic et al., 1966; Bercks, 1967a; Koenig, 1969a;
Cactus virus X		PRE; ID; LAT	Attathom et al., 1978
Carnation etched ring virus	CERV	CAULIMO	Lawson and Civerolo, 1976
Carnation Italian ringspot virus	CIRSV	TOMBUS	Hollings et al., 1970; Hollings and Stone, 1975
Carnation latent virus	CLV	CARLA	PRE; ID; LAT
			Kassanis, 1956; Bagnall et al., 1959; Hakkkaart et al., 1962; Wetter, 1967b; Oertel, 1977b

(continued)

TABLE 8.1—Continued

Virus	Abbreviation	Group	Serological techniques	Reference	Number of CMI/AAB description
Carnation mottle virus	CarMV	ID; IE; PRE; LAT	Hollings and Stone, 1964; Devergne and Cardin, 1967b; Tremaine, 1970; Waterworth and Kaper, 1972; Poupet et al., 1973; Orelle, 1977a	7	
Carnation necrotic fleck virus	CNFV	CLOSTERO	PRE; ID	Poupet et al., 1975; Bar-Joseph and Smookler, 1976, Bar-Joseph et al., 1976	136
Carnation ringspot virus	CRSV		ID; PRE; IE	Hollings and Stone, 1965a; Devergne and Cardin, 1967b; Tremaine et al., 1976	21
Carnation vein mottle virus	CVMV	POTY	PRE; IEM	Milne and Luisoni, 1975; Hollings et al., 1977a	78
Carnation yellow fleck virus	CYFV	CLOSTERO	PRE; ID	Smookler and Loebenstein, 1974; Poupet et al., 1975; Bar-Joseph and Smookler, 1976; Short et al., 1977	
CMotV		ID	Frowd and Tomlinson, 1972a	Frowd and Tomlinson, 1972	137
Carrot mottle virus		CTLV CasLV CasCMV CaMV	PRE PRE; ID PRE ID; IEM	Howell and Minck, 1976 Bock et al., 1978b Kitajima et al., 1965 Pitone et al., 1961; Beier and Shepherd, 1978	218 90 24

Celery mosaic virus	CeMV	POTY	ID; PRE	Shepard and Grogan, 1967a,b; Sutabutra and Campbell, 1971; Wolf and Schmelzer, 1972	50
Cereal tillering disease virus	CTDV	REO	ID; IEM	Milne and Luisoni, 1977b	
Cherry leaf roll virus	CLRV	NEPO	ID; IE	Cropley, 1961; Kegler et al., 1966; Jones and Murant, 1971; Waterworth and Lawson, 1973; Walkley et al., 1973	80
Cherry rasp leaf virus	CRLV	NEPO	ID	Hansen et al., 1974	
Chicory yellow mottle virus	ChYMV	NEPO	ID; PRE	Quacquarelli et al., 1972; Avgelis and Quacquarelli, 1974	132
Chloris striate mosaic virus	CSMV	GEMINI	ID	Franckii et al., 1979	221
Chrysanthemum virus B	CVB	CARLA	PRE	Hakkaart, 1969; Hakkaart and Maat, 1974; Oertel, 1974	110
Citrus leaf rugose virus	CiLRV	ILAR	ID	Gonsalves and Garnsey, 1975; Garnsey, 1975	164
Citrus trifoliata virus	CTV	CLOSTERO	SDS; ID; PRE; EIA; FLU	Gonsalves et al., 1978; Tsuchizaki et al., 1978 Garnsey et al., 1979;	33
Citrus variegation virus	CVV	ILAR	ID	Bar-Joseph et al., 1979a	
Clitoria yellow vein virus	CYVV	TYMO	ID	Gonsalves and Garnsey, 1975 Koenig, 1976; Bock et al., 1977	
Clover yellow mosaic virus	CIYMV	POTEX	PRE; ID	Pratt, 1961; Bercks and Brandes, 1963; Ford, 1964; Purcifull and Shepherd, 1964; Welsh et al., 1973	111

(continued)

TABLE 8.1—Continued

Virus	Abbreviation	Group	Serological techniques	Reference	Number of CM/IAAB description
Clover yellow vein virus	CYVV	POTY	PRE; IEM	Hollings and Nariani, 1965; Gibbs et al., 1966; Bos et al., 1977; Harville and Derrick, 1978	131
Cocksfoot mild mosaic virus	CMMV		IDC; IF; EIA	Paul and Huth, 1970; Bercks and Querfurth, 1972; Hariri and Lapierre, 1979; Paul et al., 1980a	107
Cocksfoot mottle virus	CoMV		PRE; ID	Serieant, 1967; Paul et al., 1980a	23
Cocksfoot streak virus	CSV	POTY	ID	Catherall, 1971	59
Columbia datura virus	CDV	POTY	PRE	Kahn and Battels, 1968	
Cowpea aphid-borne mosaic virus	CABMV	POTY	PRE	Bock, 1973	
Cowpea chlorotic mottle virus	CCMV	BROMO	ID	Kuhn, 1964; Scott and Slack, 1971; Fulton et al., 1975	134
Cowpea mild mottle virus	CPMMV	CARLA	PRE	Brunt and Kenten, 1973	49
Cowpea mosaic virus	CPMV	COMO	ID; IEM	Shepherd, 1963; Campbell, 1964; Valenta and Gressnevova, 1966;	140
				Gibbs et al., 1968; Swaans and Van Kammen, 1973; Beier and Shepherd, 1978	
Cowpea mottle virus	CPMotV		ID	Shoyinka et al., 1978	
Cowpea ringspot virus	CPRSV	CUCUMO	ID	Phatak et al., 1976	212
Cowpea severe mosaic virus	CPSMV	COMO	ID	Shepherd, 1963; Agrawal and Maal, 1964	209

Cucumber green mottle mosaic virus	CGMMV	TOBAMO	PRE	Nozu et al., 1971; Tochihara and Komuro, 1974;	154
Cucumber mosaic virus	CMV	CUCUMO	ID; IE; EA	Van Regenmortel, 1975 Kahn and Scott, 1964; Lawson, 1967a; Scott, 1968; Mink, 1969;	213
				Devergne and Cardin, 1970, 1973, 1975; Richter et al., 1972b, 1975a; Franciki and Habil, 1972;	
				Mink et al., 1975; Gera et al., 1978	
Cucumber necrosis virus	CuNV		ID	Dias and Doane, 1968; Tremaine, 1972	82
Cymbidium mosaic virus	CybMV	POTEX	LAT; EA; ID	Marcussen and Lundsgaard, 1975; Frowd and Tremaine, 1975; Zettler et al., 1978;	27
				Korpraditskul et al., 1979	
Cymbidium ringspot virus	CybRSV	TOMBUS	PRE; ID; IE	Hollings et al., 1977b	178
Dahlia mosaic virus	DMV	CAULIMO	PRE; ID	Brunt, 1966a, 1971a	51
Daphne virus X	DVX	POTEX	PRE	Forster and Milne, 1978	195
Dasheen mosaic virus	DasMV	POTY	ID, PRE	Abo El-Nil et al., 1977	191
Desmodium yellow mottle virus	DYMV	TYMO	PRE; ID; IE	Scott and Moore, 1972;	168
Dulcamara mottle virus	DuMV	TYMO	ID	Koenig, 1976	
				Gibbs et al., 1966; Koenig, 1976; Vassanyi and Beczner, 1978	
Eggplant mild mottle virus	EMMV	CARLA	PRE	Khalil et al., 1978	
Eggplant mosaic virus	EMV	TYMO	ID; PRE; IE	Gibbs and Harrison, 1969; Koenig, 1970; Waterworth et al., 1975	124

(continued)

TABLE 8.1—Continued

Virus	Abbreviation	Group	Serological techniques	Reference	Number of CMI/AAB description
Elderberry latent virus	ELV	ID	Jones, 1972	127	
Elm mottle virus	EMotV	ID	Schmeizer, 1969; Jones and Mayo, 1973	139	
Erysimum latent virus	EryLV	TYMO	ID	Shukla et al., 1980	222
Fiji disease virus	FDV	REO	ID	Ikegami and Francki, 1974	119
Foxtail mosaic virus*	FoMV	TOBAMO	PRE; ID	Pausen and Niblett, 1977	
Frangipani mosaic virus	FMV	POTY	PRE; ID	Francki et al., 1971	196
Gloriosa stripe mosaic virus	GSMV	NEPO	ID	Koenig and Lesemann, 1974	
Grapevine Bulgarian latent virus	GBLV	NEPO	ID	Martelli et al., 1977	186
Grapevine chrome mosaic virus	GCMV	NEPO	ID	Martelli and Quacquarelli, 1972	103
Grapevine fanleaf virus	GFLV	NEPO	PRE; ID; LAT	Dias and Harrison, 1963; Taylor and Hewitt, 1964; Bercks and Stellmach, 1966; Bercks and Querfurth, 1969a	28
Guinea grass mosaic virus	GGMV	POTY	PRE	Thouvenel et al., 1976a	190
Helenium virus S	HeLVs	CARLA	PRE; EIA	Kuschki et al., 1978	
Helenium virus Y	HeLVY	POTY	PRE	Kuschki et al., 1978	
Henbane mosaic virus	HMV	POTY	PRE; ID	Purcifull and Shepherd, 1964; Lovisolo and Bartels, 1970; Govier and Plumb, 1972	95
Heracleum latent virus	HLV		PRE; LAT	Bem and Murant, 1979	228
Hibiscus chlorotic ringspot virus	HCRSV		ID	Waterworth et al., 1976	227
Hippocrateum mosaic virus	HiMV		PRE	Brunt, 1973	117
Hydrangea ringspot virus	HyRSV	POTY	PRE	Bercks and Brandes, 1961	114
Hypochoeris mosaic virus	HyMV	POTEX	PRE	Brunt and Stace-Smith, 1978	

Iris mild mosaic virus	IMMV	POTY	PRE	Lawson, 1967b; Brunt, 1976b	116
Kennedy's yellow mosaic virus	KYMV	TYMO	ID	Koenig, 1976	193
Lettuce mosaic virus	LMV	POTY	PRE; EIA; IEM	Brlansky and Derrick, 1979;	9
Lettuce necrotic yellows virus	LNYV	RHABDO	ID	Jafarpour et al., 1979;	
Lilac chlorotic leafspot virus	LCLV	CLOSTERO	PRE	Harrison and Crowley, 1965;	
Lilac mottle virus	LiMV	CARLA	PRE	McLean et al., 1971	
Lilac ring mottle virus	LRMV	ILAR	ID	Brunt, 1978	202
Lily symptomless virus	LSV	CARLA	PRE; ID	Waterworth, 1972	
Lonicera latent virus	LLV	CARLA	PRE	Van der Meer et al., 1976	201
Lucerne Australian latent virus	LALV		ID	Van Slogteren, 1976;	
Lucerne transient streak virus	LTSV		PRE; ID	Simmonds and Cumming,	
Lychnis ringspot virus	LRSV	HORDEI	PRE	1979	
Maclura mosaic virus	MacMV		PRE	Van der Meer et al., 1980a	
Maize chlorotic dwarf virus	MCDV		IEM; FLU; EIA	Jones et al., 1979	225
Maize dwarf mosaic virus	MDMV	POTY	PRE; ID; IEM; EIA	Forster and Jones, 1979	224
Maize mosaic virus	MMV			Gibbs et al., 1963	
Maize rayado fino virus	MRFV	RHABDO	ID	Plese et al., 1979	
			PRE; ID; IEM	Derrick and Brlansky, 1976;	
				Gordon and Nault, 1977;	
				Gingery, 1978; Reeves	
				et al., 1978	
				Wagner and Dale, 1966;	
				Sehgal, 1968; Snazelle	
				et al., 1971; Langenberg	
				and Ball, 1972; Langenberg,	
				1973, 1974; Sum et al.,	
				1979	
				Derrick and Brlansky, 1976	94
				Gomez et al., 1979;	220
				Bradfute et al., 1980	

(continued)

TABLE 8.1—Continued

Virus	Abbreviation	Group	Serological techniques	Reference	Number of CMI/AAB description
Maize rough dwarf virus	MRDV	REO	ID; PRE; IEM	Wetter et al., 1969; Luisoni et al., 1973; Ikegami and Francki, 1973; Luisoni et al., 1975	72
Maize streak virus	MSV	GEMINI	ID; IE; PRE	Bock et al., 1974	133
Mirabilis mosaic virus	MIMV	CAULIMO	PRE; ID; IE	Brunt and Kitajima, 1973	
Mulberry latent virus	MLV	CARLA	CF	Tsuchizaki, 1976	
Mulberry ringspot virus	MRSV	NEPO	ID	Tsuchizaki, 1975	142
Myrobalan latent ringspot virus	MLRSV	NEPO	ID; IE	Delbos et al., 1976	160
Narcissus latent virus	NalV	CARLA	PRE	Brunt, 1977	170
Narcissus mosaic virus	NamV	POTEX	PRE; ID	Brunt, 1966b; Koenig et al., 1973; Koenig, 1975	45
Narcissus tip necrosis virus	NTNV		ID; IE	Mowat et al., 1977	166
Narcissus yellow stripe virus	NYSV	POTY	PRE	Brunt, 1971b	76
Nerine latent virus	NelV	CARLA	PRE; EIA	Maat et al., 1978a	
Nerine virus X	NeVX	POTEX	PRE	Maat, 1976; Phillips and Brunt, 1980	
Nicotiana velutina mosaic virus	NVMV		PRE; IEM	Randles et al., 1976	189
Oat blue dwarf virus	OBDV		ID	Bantari and Zeyen, 1973	123
Oat necrotic mottle virus	ONMV	POTY	PRE	Gill, 1976	169
Oat sterile dwarf virus	OSDV	REO	ID; IEM	Milne and Luisoni, 1977a; Milne and Lesemann, 1978; Luisoni et al., 1979	217
Odontoglossum ringspot virus	ORSV	TOBAMO	ID; PRE	Paul et al., 1965; Zettler et al., 1978	155
Okra mosaic virus	OkMV	TYMO	ID	Koenig and Givord, 1974; Bozarth et al., 1977	128

Union yellow dwarf virus	UYYV	ROSY	PRE	De wijs, 1953
Ononis yellow mosaic virus	OYMV	TYMO	IEM; ID	Koenig, 1976; Lesemann et al., 1980
Pangola stunt virus	PanSV	REO	IEM; ID	Milne and Luisoni, 1977a; Boccardo et al., 1979
Panicum mosaic virus	PMV		ID	Niblett et al., 1977
Papaya mosaic virus	PaMV	POTEX	ID	De Bokx, 1965; Koenig, 1975
Papaya ringspot virus	PRSV	POTY	ID; PRE	Story and Hallowell, 1969
Parsley virus 5	PV5	POTEX	PRE	Frowd and Tomlinson, 1972a
Parsnip mosaic virus	PanMV	POTY	PRE	Murant et al., 1970
Parsnip virus 3	PV3	POTEX	PRE; ID	Garrett and Tomlinson, 1966
Parsnip yellow fleck virus	PYFV		ID; PHA; LAT	Abu-Salih et al., 1968a; Murant and Goold, 1968
Passiflora latent virus	PLV	CARLA	PRE	Brandes and Wetter, 1963
Passionfruit woodiness virus	PWV	POTY	PRE	Taylor and Kimble, 1964
Pea early-browning virus	PEBV	TOBRA	PRE; ID	Maat, 1963; Gibbs and Harrison, 1964; Lockhart and Fischer, 1976
Pea emation mosaic virus	PEMV		PRE; ID	Izadpanah and Shepherd, 1966
Pea seed-borne mosaic virus	PSbMV	POTY	PRE; ID; EIA; IEM	Stevenson and Hagedorn, 1973b; Knesek et al., 1974; Hamilton and Nichols, 1978
Pea streak virus	PeaSV	CARLA	PRE	Wetter et al., 1962; Wetter, 1967b
Peach rosette mosaic virus	PRMV	NEPO	ID; EIA	Dias and Cation, 1976;
Peanut clump virus	PCV		PRE	Ramsdell et al., 1979
Peanut mottle virus	PeMotV	POTY	PRE; ID; PHA	Dollet et al., 1976; Thouvenel et al., 1976b
				Sun and Hebert, 1972; Paguio and Kuhn, 1973a,b; Bock et al., 1978a; Reddy et al., 1978

(continued)

TABLE 8.1—Continued

Virus	Abbreviation	Group	Serological techniques	Reference	Number of CMI/AAB description
Peanut stunt virus	PSV	CUCUMO	ID; IE; IEM	Waterworth <i>et al.</i> , 1973; Mink <i>et al.</i> , 1975; Devergne and Cardin, 1976; Fischer and Lockhart, 1978; Har- ville and Derrick, 1978; Douine and Devergne, 1978; Diaz-Ruiz <i>et al.</i> , 1979	92
Pelargonium flower-break virus	PFBV	TOMBUS	ID; IE	Stone and Hollings, 1973	
Pelargonium leaf-curl virus	PLCV		ID; IE	Hollings and Stone, 1965b, 1975	
Pepino mosaic virus	PepMV	POTEX	PRE	Jones <i>et al.</i> , 1980	
Pepper mottle virus	PeMoV	POTY	ID	Purcifull <i>et al.</i> , 1975a; Nelson and Wheeler, 1978	
PV/MV	POTY		PRE	Brunt and Kenten, 1971; De Wijs, 1973	
PTV	POTY		PRE; ID	Fribourg, 1979; Fernandez- Northcote and Fulton, 1980	
Pepper veinal mottle virus		TOMBUS	PRE; ID; IE	Hollings and Stone, 1975	
Peru tomato virus			PRE; ID	Catherall and Chamberlain, 1977	
Petunia asteroid mosaic virus	PAMV	TYMO	PRE	Peters and Derkx, 1974	
Phleum mottle virus	PMotV	TYMO	ID	Granett, 1973	
Physalis mosaic virus	PhMV	TYMO	PRE	Paulsen and Fulton, 1969	
Plantago mottle virus	PlMV	ILAR	ID	Schade, 1969, 1975; Noel	
Plum line pattern virus	PLPV	TYMO	PRE; EIA; IEM	<i>et al.</i> , 1978; Adams, 1978;	
Plum pox virus	PPV	POTY		Kerlan and Dunez, 1979	

Poa semilatent virus	PSLV	HORDEI	IEM; PRE; ID	Polak and Slykhuis, 1972; Slykhuis, 1972;
Pokeweed mosaic virus	PokMV	POTY	PRE	Langenberg, 1974
Poplar mosaic virus	PopMV	CARLA	EIA	Shepherd et al., 1969
Poamv	PoAMV	POTEX	PRE	Luisoni et al., 1976;
PBRSV	NEPO	ID		Van der Meer et al., 1980b
PLRV	LUTEO	EIA; IEM		Juo and Rich, 1969
Potato aucuba mosaic virus				Salazar and Harrison, 1978b,c
Potato black ringspot virus				Casper, 1977b; Maat and De Bokx, 1978a; Kojima et al., 1978; Gugerli, 1979;
Potato leafroll virus				De Bokx, 1977b; Maat and Casper, 1977b; Maat and De Bokx, 1978a; Kojima et al., 1978; Gugerli, 1979;
Potato mop-top virus	PMTV	TOBAMO	PRE; IEM	Roberts et al., 1980
Potato mosaic virus A	PVA	POTY	PRE; CF; LAT; PHA; EIA	Kasanis et al., 1972; Randles et al., 1976; Roberts and Harrison, 1979; Mehrad et al., 1978
Potato virus M	PVM	CARLA	PRE; ID	Bartels, 1964; Fribourg and De Zoeten, 1970; Gnutova and Krylov, 1975; Maat and Mierzwa, 1975; Maat and De Bokx, 1978b; Gugerli, 1979
Potato virus S	PVS	CARLA	PRE; LAT; ID; EIA	Wetter, 1967b; Ghena, 1970; Shepard et al., 1971
Potato virus T	PVT	CLOSTERO	PRE; EIA	Shepard et al., 1971; Beircks, 1967a; Kahn et al., 1967; Shepard, 1970b; Ghena, 1970; Goth and Webb, 1975; Richter et al., 1977; Khan and Slack, 1978
				Salazar and Harrison, 1977; Salazar and Harrison, 1978a

(continued)

TABLE 8.1—Continued

Virus	Abbreviation	Group	Serological techniques	Reference	Number of CMI/AAB description
Potato virus X	PVX	POTEX	ID; LAT; PRE; PHA	Bercks, 1963; Tomlinson and Walkley, 1967b; Shepard and Shalla, 1970, 1972; Shepard, 1972; Krylov and Gnutova, 1974; Fuchs and Richier, 1975; Khan and Slack, 1978	4
Potato virus Y	PVY	POTY	IEM; PRE; ID; EIA	Bartels, 1957; Sampson and Taylor, 1968; Ghena, 1970; Purcifull and Gooding, 1970; Shepard, 1972; Makkouk and Gumpf, 1976; Derrick and Briansky, 1976; Gugel, 1978	37
Potato yellow dwarf virus	PYDV	RHABDO	FLU	Wolcyrz and Black, 1956; Hsu, 1978	35
Prune dwarf virus	PDV	ILAR	ID; EIA	Waterworth and Fulton, 1964; Casper, 1977a	19
Prunus necrotic ringspot virus	PNRSV	ILAR	ID; EIA; LAT; IEM	Fulton and Hamilton, 1960; Tremaine and Willison, 1962b; Allen, 1964; Bock, 1967; Schade, 1967b, 1968; Fulton, 1968; Severirane and Posnette, 1970; Bozarth, 1971; Barbara et al., 1978, 1980; Thomas, 1980	5

Quail pea mosaic virus	QPMV	COMO	ID	Moore, 1973; Meiners et al., 1977
Radish mosaic virus	RaMV	COMO	ID; IE	Campbell, 1964; Campbell and Tochihara, 1969;
				Siefanac and Mamula, 1971; Quacquarelli and Avgelis, 1974; Plakoli and Stefanac, 1976
Raspberry bushy dwarf virus	RBDV		ID	Converse and Casper, 1977
Raspberry ringspot virus	RRV	NEPO	ID; LAT; PHA	Maat et al., 1962; Maat, 1965; Murant et al., 1968; Bercks, 1968; Abu-Salih et al., 1968a; Vuittenez et al., 1970
Red clover mottle virus	RCMV	COMO	ID	Bos and Maat, 1965; Devergne and Cardin, 1968; Gerhardson and Lindsten, 1973
Red clover necrotic mosaic virus	RCNMV	COMO	PRE; ID	Musil, 1969; Gerhardson and Lindsten, 1973; Hollings and Stone, 1977
Red clover vein mosaic virus	RCVMV	CARLA	REC; ID	Wetter et al., 1962; Freitag and Milne, 1970; Varma et al., 1970
Ribgrass mosaic virus	RMV	TOBAMO	PRE; EIA; ID; IEM	Juretic and Wetter, 1973; Hollings, 1974; Juretic, 1974
Rice black-streaked dwarf virus	RBSDV	REO	PRE; ID	Luisoni et al., 1973
Rice dwarf virus	RDV	REO	PRE; ID	Iida et al., 1972; Kimura and Miyajima, 1976
Rice necrosis mosaic virus	RNMV		PRE	Inouye and Fujii, 1977
Rice ragged stunt virus	RRSV	REO	ID; IEM	Milne et al., 1979

(continued)

TABLE 8.1—Continued

Virus	Abbreviation	Group	Serological techniques	Reference	Number of CMI/AAB description
Rice transitory yellowing virus	RTYV	RHABDO	PRE ID	Bakker, 1970	100
Rice yellow mottle virus	RYMV	CUCUMO	ID	Schmelzer, 1967; Richter et al., 1979	149
Robinia mosaic virus	RobMV			Fulton, 1967b; Basit and Francki, 1970; Gotlieb and Berbee, 1973	65
Rose mosaic virus	RosMV	ILAR	PRE; ID		
Ryegrass mosaic virus	RyMV	POTY	PRE; IEM ID	Langenberg, 1974	86
Saguaro cactus virus	SCV			Milbrath and Nelson, 1972; Nelson and Tremaine, 1975	148
Sammons opuntia virus	SOV	TOBAMO	PRE ID	Wetter and Paul, 1967	
Satellite virus	SV			Grogan and Uyemoto, 1967; Uyemoto and Grogan, 1969; Rees et al., 1970; Kassanis and Phillips, 1970	
Satsuma dwarf virus	SDV			Usgi and Saito, 1979	208
Scrophularia mottle virus	ScMV	TYMO	ID; CF ID; IEM	Bercks et al., 1971; Koenig, 1976; Lesemann et al., 1980	113
Shallot latent virus	SLV	CARLA	PRE	Bos et al., 1978	
Soil-borne wheat mosaic virus	SBWMV	TOBAMO	PRE; ID; IEM	Rao and Brakke, 1969; Tsuchizaki et al., 1973; Powell, 1976; Randles et al., 1976	77
Sonchus yellow net virus	SYNV	RHABDO	SDS; ID PRE; ID; IE; CF	Jackson and Christie, 1977	205
Southern bean mosaic virus	SBMV			Shepherd and Fulton, 1962; Kuhn, 1963; Grogan and	57

Sowbane mosaic virus	SowMV	PRE; ID	ID	Kimble, 1964; Tremaine and Wright, 1967 Bancroft and Tolin, 1967; Kado, 1967; Engelbrecht and Van Regenmortel, 1968; Bercks and Querfurth, 1969b Peters and Black, 1970 Kojima and Tamada, 1976 Ross, 1967; Quiniones and Dunleavy, 1970; Ross, 1975; Bossemee and Maury, 1978; Lister, 1978; Briansky and Derrick, 1979; Soong and Milbrath, 1980	64
Sowthistle yellow vein virus	SYVV	RHABDO	PRE	Peters and Black, 1970 Kojima and Tamada, 1976 Ross, 1967; Quiniones and Dunleavy, 1970; Ross, 1975; Bossemee and Maury, 1978; Lister, 1978; Briansky and Derrick, 1979; Soong and Milbrath, 1980	62
Soybean dwarf virus	SoyDV	LUTEO	PRE; ID; NEU	Peters and Black, 1970 Kojima and Tamada, 1976 Ross, 1967; Quiniones and Dunleavy, 1970; Ross, 1975; Bossemee and Maury, 1978; Lister, 1978; Briansky and Derrick, 1979; Soong and Milbrath, 1980	179
Soybean mosaic virus	SoyMV	POTY	PRE; IEM; EIA; ID	Peters and Black, 1970 Kojima and Tamada, 1976 Ross, 1967; Quiniones and Dunleavy, 1970; Ross, 1975; Bossemee and Maury, 1978; Lister, 1978; Briansky and Derrick, 1979; Soong and Milbrath, 1980	93
Squash mosaic virus	SMV	COMO	ID; IEE	Knuhtsen and Nelson, 1968; Lastra and Munz, 1969; Nelson and Knuhtsen, 1973a, b	43
Statice virus Y	StaVY	POTY	PRE	Lesemann et al., 1979	
Strawberry latent ringspot virus	SLRSV	NEPO	EIA; ID; LAT; IEM; PHA	Richter, 1969; Vuittenez et al., 1970; Thomas, 1980	126
Strawberry vein banding virus	SVBV	CAULIMO	EIA; IEM	Morris et al., 1980	
Sugar beet mosaic virus	SuBMV	POTY	PRE	Bercks, 1960a, b, 1961	219
Sugarcane mosaic virus	SCMV	POTY	PRE; LAT; ID	Taylor and Pares, 1968;	53
				Bond and Pitone, 1971;	88
				Baudin and Vuittenez, 1972; Teakle and Grylls, 1973; Von Wechmar and Hann, 1967	

(continued)

TABLE 8.1—Continued

Virus	Abbreviation	Group	Serological techniques	Reference	Number of CMI/AAB description
Sunn-hemp mosaic virus	SHMV	TOBAMO	PRE	Kassanis and McCarthy, 1967	153
Sweet potato mild mottle virus	SPMMV	POTY	PRE; ID	Hollings et al., 1976	162
Tobacco etch virus	TEV			Bartels, 1964; Purcifull, 1966; Purcifull and Gooding, 1970; Gooding, 1975; McLaughlin et al., 1975	55
Tobacco mosaic virus	TMV	TOBAMO	PRE; ID; IE; PHA; LAT; CF; EIA; IEM; NEU	Rappaport, 1965; Wetter, 1967a; Van Regenmortel and Lelarge, 1973; Hollings, 1974; Van Regenmortel and Burckard, 1980	14
Tobacco necrosis virus	TNV		PRE; ID	Grogan and Uyemoto, 1967; Cesati and Van Regenmortel, 1969; Kegler et al., 1969; Kassanis and Phillips, 1970; Uyemoto and Gilmer, 1972	14
Tobacco rattle virus	TRV	TOBRA	PRE; ID; LAT; IE; IEM	Maat, 1963; Harrison and Woods, 1966; Van Hoof et al., 1966; Ghabrial and Lister, 1973; Gugerli, 1976, 1977	12
Tobacco ringspot virus	TRSv	NEPO	PRE; ID; IEM; EIA	Kahn et al., 1962; Hollings, 1965a; Gooding, 1970; Khan and Maxwell, 1975; Fribourg, 1977; Lister,	17

Tobacco streak virus	TSV	ILAR	ID	1978; Brilansky and Derrick, 1979 Brunt and Paludan, 1970; Gooding, 1971; Converse, 1972; Chabrial and Lister, 1974; Edwardson and Purcifull, 1974; Ghanekar and Schwenk, 1980	44
Tomato aspermy virus	TAV	CUCUMO	ID	Hakkart, 1967; Lawson, 1967a; Oertel, 1968; Mink, 1969; Devergne and Cardin, 1975; Habili and Francki, 1975	79
Tomato black ring virus	TBRV	NEPO	ID; LAT	Harrison, 1964; Bercks and Mischke, 1964; Hollings, 1965b; Bercks, 1967a;	38
Tomato bushy stunt virus	TBSV	TOMBUS	ID; IE; LAT; IEM; EIA	Delbos et al., 1976 Bercks, 1967b; Allen, 1968; Wetter and Luisoni, 1969; Albrechtova et al., 1975; Hollings and Stone, 1975; Fisher and Lockhart, 1977; Fuchs et al., 1979	69
Tomato mosaic virus	TomMV	TOBAMO	PRE; ID; EIA; IE	McRitchie and Alexander, 1963; Wang and Knight, 1967; Burgyan et al., 1978	156
Tomato ringspot virus	TomRSV	NEPO	PRE; ID; EIA	Cadman and Lister, 1961; Gooding, 1963; Téliz et al., 1966; Fulton and Fulton, 1970; Converse, 1978	18

(continued)

TABLE 8.1—Continued

Virus	Abbreviation	Group	Serological techniques	Reference	Number of CMI/AAB description
Tomato spotted wilt virus	TSWV		ID; IEM; PHA; PRE	Best and Harilalrasubramanian, 1967; Feldman and Boninsegna, 1968; Joubert et al., 1974; Paliwal, 1974; Derrick and Bransky, 1976; Tas et al., 1977; Ghanekar et al., 1979	39
Tulare apple mosaic virus	TAMV	ILAR	ID	Fulton, 1967a; Lister and Saksena, 1976	42
Tulip breaking virus	TBV	POTY	PRE	Van Slooteren and De Vos, 1966; Bartels, 1971	71
Turnip crinkle virus	TCV		PRE; ID PRE; ID	Hollings and Stone, 1969 Brunt, 1976a; Tomlinson and Walkley, 1967b; McDonald and Hiebert, 1975; Lisa and Lovisolo, 1976	109
Turnip mosaic virus	TuMV	POTY			8
Turnip rosette virus	TRoSV		PRE; ID PRE; ID; PHA; IEM	Hollings and Stone, 1969 Abu-Sálih et al., 1968a; Lesemann et al., 1980	125
Turnip yellow mosaic virus	TYMV	TYMO		Duffus and Russell, 1972	2; 230
Turnip yellows virus	TYV	LUTEO	NEU	Lisa and DellaValle, 1977	
Viola mottle virus	VMV	POTEX	PRE	Van Regenmortel et al., 1962;	
Watermelon mosaic virus	WMV	POTY	ID; PRE	Webb and Scott, 1965; Milne and Crogan, 1969; Purcifull and Hiebert, 1979	63

Wheat streak mosaic virus	WSMV	POTY	PRE; ID; IE; IEM	Slykhuis and Bell, 1966; Ball and Brakke, 1968; Brakke and Ball, 1968; Langenberg and Ball, 1972; Langenberg, 1974	48
Wheat striate mosaic virus	WStMV	RHABDO	ID; PRE; FLU	Sinha, 1968; Sinha and Thottappilly, 1974; Thottappilly and Sinha, 1973; 1974	99
Wheat yellow leaf virus	WYLV	CLOSTERO	PRE	Inouye, 1976	157
White clover mosaic virus	WCIMV	POTEX	ID	Bercks and Brandes, 1961; Purcifull and Shepherd, 1964	41
Wild cucumber mosaic virus	WCMV	TYMO	ID; PRE; IE	McLeod and Markham, 1963; Van Regenmortel, 1966a	105
Wild potato mosaic virus	WPMV	POTY	PRE	Jones and Fribourg, 1979	
Wineberry latent virus	WLV	FLEXOUS	PRE; IEM	Jones, 1977	
Wound tumor virus	WTV	REO	FLU	Whitcomb and Black, 1961b; Whitcomb, 1964, 1966;	34
Zygocactus virus X	ZVX	POTEX	PRE; ID	Sinha, 1965; Reddy and Black, 1966; Gamez et al., 1967	
				Giri and Chessin, 1975	

"Descriptions of Plant Viruses" published by the Commonwealth Mycological Institute, Kew, England.

More than 20 years ago, the Committee on Virus Type Culture Collection of the American Phytopathological Society investigated the possibility of commercial production of plant virus antisera (see their report in *Phytopathology* 50, 428-431, 1960). It was found that the commercial exploitation of the production of plant virus antisera was unlikely to be very profitable, mainly because of the limited demand for individual sera, and the resulting prohibitive price that would have to be charged. At the present time, a number of reference antisera are available from the American Type Culture Collection (ATCC), who charge a nominal fee for small samples of sera. This organization, being the repository of type cultures, distributes only small quantities of reference materials, and does not supply large volumes of antisera for diagnostic work. A list of plant virus antisera available from the ATCC, Rockville, Maryland, United States, is presented in Table 8.2. Recently, immunological reagents for the diagnosis of potato virus diseases by ELISA have become available commercially. Conjugated immunoglobulins (labeled with alkaline phosphatase) specific for PVX, PVA, PVM, PVS, PVY, and PLRV can be purchased from Inotech, Wohlen, CH-5610, Switzerland. According to the manufacturers, these conjugates can be used in ELISA at a dilution of 1:1000.

It is clear that the need of many plant virologists, to have a large collection of diagnostic antisera readily available, is not being met at present. A few research laboratories dispose of large collections of virus antisera, but understandably, the scientists in these institutions are somewhat cautious in distributing this precious material all too readily. In general, a definitive identification requires that the homologous antigen be included for comparison with the unidentified isolate,

TABLE 8.2
Plant Virus Antisera Available from the ATCC, Rockville, Maryland^a

Antiserum to	ATCC code number
Apple mosaic virus	PVAS 32 and 32a
Arabis mosaic virus	PVAS 43 and PVAS 130
Barley stripe mosaic virus	PVAS 192
Bearded iris mosaic virus	PVAS 124
Belladonna mottle virus	PVAS 183
Bidens mottle virus	PVAS 165
Broad bean mottle virus	PVAS 111a and 111b
Broad bean wilt virus	PVAS 252
Brome mosaic virus	PVAS 178 and 180
Cactus virus X	PVAS 245, 246, 247

TABLE 8.2—Continued

Antiserum to	ATCC code number
Carnation mottle virus	PVAS 108a and 108b
Carnation ringspot virus	PVAS 21a and 21b
Carnation yellow fleck virus	PVAS 241
Cauliflower mosaic virus	PVAS 147
Cherry leaf roll virus	PVAS 142
Citrus leaf rugose virus	PVAS 195
Clover yellow mosaic virus	PVAS 200
Cowpea mosaic virus	PVAS 248 and 258
Cucumber mosaic virus	PVAS 242, 242a, 30, 260
Desmodium yellow mottle virus	PVAS 155
Dioscorea latent virus	PVAS 212
Elm mosaic virus	PVAS 253
Grapevine fanleaf virus	PVAS 238
Maize chlorotic mottle virus	PVAS 262
Maize colombian stripe virus	PVAS 262
Maize streak virus	PVAS 244
Myrobalan latent ringspot virus	PVAS 279
Peanut stunt virus	PVAS 187 and 249
Plantago mottle virus	PVAS 214
Potato virus S	PVAS 103
Potato virus X	PVAS 54a
Potato virus Y	PVAS 50
Potato yellow dwarf virus	PVAS 233 and 234
Prune dwarf virus	PVAS 33
Prunus necrotic ringspot virus	PVAS 22 and 259
Rose mosaic virus	PVAS 254
Saguaro cactus virus	PVAS 277
Scrophularia mottle virus	PVAS 269
Soil-borne wheat mosaic virus	PVAS 65
Southern bean mosaic virus	PVAS 114 and 37
Sowbane mosaic virus	PVAS 109a and 109b
Soybean mosaic virus	PVAS 94
Sugarcane mosaic virus	PVAS 51, 55a, 55b, 181, 186
Tobacco etch virus	PVAS 69
Tobacco mosaic virus	PVAS 135a, 135b, 257
Tobacco ringspot virus	PVAS 157
Tobacco streak virus	PVAS 49
Tomato bushy stunt virus	PVAS 163
Tomato ringspot virus	PVAS 174 and 239
Tulare apple mosaic virus	PVAS 80
Turnip yellow mosaic virus	PVAS 255 and 256
White clover mosaic virus	PVAS 190
Wound tumor virus	PVAS 235

^a From the ATCC catalog, Rockville, Maryland 20852, which should be consulted for further details. This information is concurrent with the 1981 catalog.

and this may present difficulties in view of existing quarantine regulations. The use of formalinized antigens may possibly be one way of surmounting this particular problem. It seems unrealistic, however, to expect that the exchange of virus antisera on a much wider basis than practiced at present, will occur on a voluntary basis, i.e., without the provision of special funds for this purpose. In view of the important contribution that antisera could make to the diagnosis of virus diseases, especially in developing countries, the sponsoring of a collaborative program in maintaining a serum bank and providing identification services would seem to be a rewarding venture for one of the international agencies committed to improving the world's food situation.

D. SENSITIVITY OF VIRUS DETECTION

The relative sensitivity of antigen detection by different serological techniques represents a vexed question to which it is difficult to give a precise answer (Wright and Stace-Smith, 1966; Sinha and Thottappilly, 1974). The main reason for this is that sensitivity can be influenced by many small variations in experimental procedure. Furthermore, sensitivity also depends on the avidity of the antibodies present in the serum as well as on the stability and size of the antigen under study. For comparison purposes, the best that can be done is to provide a range of antigen concentrations at which different techniques have been found to work satisfactorily (Table 8.3). The difficulty in defining precise limits of sensitivity may be illustrated in the case of double diffusion tests (Van Regenmortel and Engelbrecht, 1962; Shepard and Secor, 1969; Casper *et al.*, 1971). In this assay, the ultimate visibility of a precipitin line depends on the distance between antigen and antiserum wells, on the relative concentration of the two reactants,

TABLE 8.3
Sensitivity of Antigen Detection by Different Serological Techniques

Technique	Minimum range of antigen detection	
Double immunodiffusion	2-20	µg/ml
Liquid precipitin tests	1-10	µg/ml
Radial immunodiffusion	0.5-10	µg/ml
Rocket electrophoresis	0.2-1.0	µg/ml
Complement fixation	50-500	ng/ml
Immuno-osmophoresis	50-100	ng/ml
Passive hemagglutination	20-50	ng/ml
Latex test	5-20	ng/ml
ELISA	1-10	ng/ml
Immunoelectron microscopy	1-10	ng/ml

on the gel medium and buffer system used, on the time limit set for the experiment, on the quality of illumination used for examining the plate, and last, but not least, on the acuteness of vision of the observer. Many authors have reported that Ouchterlony tests will not detect viruses below a concentration of 0.5–1.0 mg/ml. However, by using serial dilutions of the reactants maintained at equivalence (instead of serially diluting only one of the reactants) it is possible to obtain very sharp precipitin lines with small icosahedral virus particles used at 5–10 $\mu\text{g}/\text{ml}$, provided evaporation is prevented by a layer of mineral oil and the results are scored after 5 days.



Virus Classification

A. USE OF SEROLOGICAL TESTS FOR DETERMINING RELATIONSHIPS BETWEEN VIRUSES

Some investigators have expressed doubts concerning the validity of serological criteria for measuring the degree of relationship between viruses. These reservations usually stem from the realization that the coat protein cistron represents only a small percentage of the total coding capacity of the viral genome, and that the number of amino acid residues of the coat protein that participate directly in the formation of antigenic determinants at the outer surface of virions is rather small. Furthermore, the fact that a single amino acid exchange, when it occurs in a region accessible to antibody binding, is able to significantly alter the serological properties of the virus, has been considered to make antigenic specificity very sensitive to mutational events. In practice, however, it is found that most single residue exchanges in the coat protein do not alter the antigenicity (Sengbusch, 1965; Wang and Knight, 1967; Van Regenmortel, 1967). Furthermore, it seems that the extent of changes in the coat protein cistron is paralleled by the importance of variations elsewhere in the viral genome. Viruses

that are serologically related always share most of their other properties, and there are very few exceptions to the rule that no members of the 26 recognized plant virus groups (See Table 9.2) cross-react serologically. The significance of the few reported exceptions, for instance the cross-reaction between TMV and the isometric cocksfoot mild mosaic virus (CMMV) (Bercks and Querfurth, 1971b; Bercks *et al.*, 1974; Querfurth and Bercks, 1976) and the cross-reaction between PVX and a carlavirus (Maat *et al.*, 1978) or a potyvirus (Koenig and Lesemann, 1974) is still unknown.

Viruses that cannot be distinguished serologically but differ even extensively in biological properties are usually considered to belong to the same virus species. Criteria for differentiating between strains vary considerably, and in principle any major difference in host range, symptoms, mode of transmission, or chemical composition could be used for this purpose (Knight, 1955; Bos, 1970). Kassanis (1961) suggested that when two isolates are only distantly related serologically, they should no longer be considered strains, but should be called serotypes. The ability to make such a distinction would depend on the feasibility of differentiating between close and distant serological relationships. Although it has been suggested (Babos and Kassanis, 1963) that such a distinction could be achieved by means of cross-absorption experiments, an analysis of the cross-absorption data published by different authors (Van Regenmortel and Von Wechmar, 1970) showed that this was not feasible in practice (see also Chapter 6, Section A,2).

When a sufficiently large number of virus strains are examined serologically, it is invariably found that they can be arranged in a continuous series of increasingly distantly related entities (Uyemoto *et al.*, 1968; Koenig and Givord, 1974; Van Regenmortel, 1975). The distinction between strain and serotype is thus arbitrary, and it seems preferable to call serotype any virus strain that is serologically distinguishable from a type strain, regardless of whether the serological differences are large or small. Serotypes would then represent a subclass of strains with distinguishable antigenic specificities. According to this definition, *serotype* does not apply to a cross-reacting entity that is considered to belong to another virus species on the basis of properties other than serology.

It has been found expedient to express the degree of serological cross-reactivity between two viruses by the number of twofold dilution steps separating homologous from heterologous precipitin titers. This number, which is called the serological differentiation index or SDI, is only reliable if it represents the average value of several measurements (Van Regenmortel and Von Wechmar, 1970; Van Regenmortel, 1975). It cannot be overemphasized that individual antisera, obtained from different animals immunized with the same antigen, or from the same animal at different times, often show considerable variation in the amount of cross-reacting antibodies they contain (Wetter, 1961; Bercks, 1966; Koenig and Bercks, 1968). A progressive rise in antibody cross-reactivity is

often observed with increasing time of immunization (Tremaine and Wright, 1967; Kassanis and Philipps, 1970; Crumpton, 1974), although the reverse situation can also occur. Unfortunately, in many studies, the number of animals used for immunization and the number of bleedings obtained from each animal are too small to allow reliable conclusions to be drawn. The importance of using a large number of antisera for any detailed serological study was clearly demonstrated by the results of Bercks (1963). This author examined the ability of 55 PVX antisera to cross-react with four potexviruses and found considerable variation in the degree of serological relationships demonstrated by individual antisera (Table 9.1).

A similar degree of variability was observed in an extensive study of the cross-reactivity between two tobamoviruses. Calculated SDI values showed considerable variation and not much reliance could be placed on data obtained from only a few bleedings (Van Regenmortel and Von Wechmar, 1970). Out of 22 antisera, 10 showed a SDI of 2-3 and nine a SDI of 5-7. Such differences explain the discrepancies in the results reported by individual investigators (Bawden and Kassanis, 1968) as well as the difference in cross-reactivity that is often observed in reciprocal tests. In a subsequent study of the cross-reactivity between several tobamoviruses, it was found that meaningful serological comparisons were nevertheless possible, provided a sufficient number of antisera were analyzed and data pertaining to the first 3 weeks of immunization were not taken into account (Van Regenmortel, 1975). Average SDI values between two

TABLE 9.1
Variation in Cross-Reactivity of PVX Antisera with Four Other Potexviruses
(HyRSV, CVX, WCIMV, CIYMV)^a

Reciprocal of homologous precipitin titer	128	256	512	1024	2048	4096	16,384	Total number of sera
Sera showing no cross-reactivity	1	2	1	3	1	1	—	9
Sera cross-reacting with one virus	—	1	—	3	1	—	—	5
Sera cross-reacting with two viruses	—	—	2	3	3	—	1	9
Sera cross-reacting with three viruses	—	1	4	7	2	1	—	15
Sera cross-reacting with four viruses	—	1	4	3	5	4	—	17
Total number of sera examined	1	5	11	19	12	6	1	55

^a Adapted from Bercks (1963).

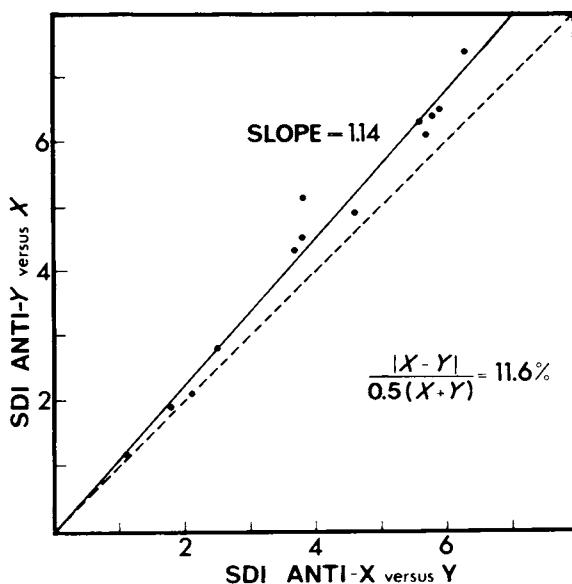


Fig. 9.1. Serological relationship between 13 pairs of tobamoviruses expressed as serological differentiation indices and determined in reciprocal tests. Perfect agreement in the reciprocal tests would have produced a slope of 1 (dashed line) (based on data of Van Regenmortel, 1975).

strains obtained in reciprocal tests, when antisera were used against each of the two strains, were found to agree closely (Fig. 9.1). Since the intraclass correlation coefficient between two sets of reciprocal SDI values was $r' = 0.95$, it seems safe to generalize that a lack of reciprocity in any individual test is more likely to be due to animal variability than to intrinsic differences in immunogenicity or antigenic reactivity of the two strains. The excellent agreement found in reciprocal tests means that it is justified to use the average of two sets of reciprocal SDI values as an index of the serological cross-reactivity between virus strains.

It is clear that the distinction between a close and a distant serological relationship is an arbitrary one, and that there is no clear-cut borderline between them. The label distant is commonly used when the SDI between two viruses is about 4 or greater.

B. AIMS, METHODS, AND USES OF VIRUS CLASSIFICATION

A classification is a conceptual system of order which groups together entities that present certain analogies to a human observer. The perception of an analogy

between two viruses presupposes that a specific property is deemed relevant for characterizing the object under study. Human judgment is needed both for distinguishing between trivial and relevant properties and for evaluating if the extent of similarity that is found to exist is significant.

In recent years an interesting controversy opposed the classical taxonomists who believe that characters are of graded importance and allow a hierarchy (Lwoff and Tournier, 1966, 1971) to the computer taxonomists who maintain (some would say arbitrarily) that no character should be considered to be more important than others (Gibbs *et al.*, 1966; Gibbs, 1969). It seems that most virologists are of the opinion that the various properties used for evaluating the similarity between viruses should not be given equal weight. Stable characters, for instance, genome size, that are not modified by mutation are generally considered more important than properties that are genetically unstable such as pathogenicity. The implicit hierarchy in the value of different characters is reflected in the existing virus groups (Table 9.2), which are based on properties such as the kind of nucleic acid of the viral genome and the morphology of the virion (Brandes and Bercks, 1965; Matthews, 1979). The main contribution of computer taxonomy lies in its ability to produce a series of very precise similarity coefficients (Gibbs and Harrison, 1976). The claim that computer taxonomy is more "objective" than classical taxonomy is unfounded, since the derivation of similarity coefficients does not obviate the need for someone to make a subjective decision on how to utilize these coefficients for building coherent groups. All classifications are conceptual constructions, and the categories used for building them are not found in nature but arise in human minds. Until now, plant virologists have been reluctant to concede that a cluster of virus strains could correspond to the category *species* used by classical taxonomists, or that a cluster of similar virus species could correspond to a *genus*. At present, viruses with similar properties are said to form a *group*, whereas a collection of strains is said to constitute a *virus*. The absence of a classical terminology for the classification categories, unfortunately, does not make it any easier to decide on how to delineate a strain, a virus, or a group. From the preceding discussion, it is clear that attempts to devise "objective" criteria for considering two related viruses as strains rather than as separate viruses are doomed to fail. Such distinctions are inherently arbitrary and arise only by virtue of a human decision. The need for such decision-making was emphasized again recently by Matthews (1979) who also pleaded for the creation of official species names for different viruses, in addition to English vernacular names.

The development of a sound system of virus classification is of considerable practical importance mainly because of its predictive value. As soon as a new virus isolate has been placed in the correct group, many of its properties can be predicted by analogy with the better studied members of the group. As discussed in Chapter 8, the single most useful property for assigning a virus to a particular

group is its antigenic reactivity. No other single property allows the identification to be carried out also at the species and serotype (or strain) level.

Another important use of a classification system is to serve as the basis for a sound nomenclature. The present system of vernacular names derived from hosts and symptoms is chaotic, and encourages the tendency of some plant pathologists to add new disease "labels" to the list without trying to ascertain if the causative agent is a new virus, a strain of a known virus, or identical to a previously described virus. A further increase in the number of synonyms could be prevented if all new descriptions of virus isolates included the results of exhaustive serological comparisons with known viruses of similar morphology.

C. SEROLOGICAL RELATIONSHIPS AMONG MEMBERS OF RECOGNIZED VIRUS GROUPS

In 1980, a total of 26 plant virus groups are officially recognized by the International Committee on Taxonomy of Viruses (ICTV). A list of these groups appears in Table 9.2 (Matthews, 1979).

1. Tobamovirus Group

This group consists of viruses with rod-shaped particles about 300 nm long (Van Regenmortel, 1981b). The type member is TMV. Serological relationships have been established between the following members of this group.

Broad bean necrosis virus	Sammons <i>Opuntia</i> virus
Cucumber green mottle mosaic virus	Soil-borne wheat mosaic virus
Cucumber virus 4	Sunn-hemp mosaic virus
Frangipani mosaic virus	Tobacco mosaic virus
Odontoglossum ringspot virus	Tomato mosaic virus
Potato mop top virus	U2-strain of TMV
Ribgrass mosaic virus	

The tobacco mosaic virus species comprises *inter alia* the common strain (also known as *vulgare*) and various strains that differ from common TMV by only a few amino acid exchanges in the coat protein (Hennig and Wittmann, 1972). Some of these strains such as 01 and 06 are serologically indistinguishable from common TMV (Kado *et al.*, 1968), whereas others such as YA are serologically distinct (Malkiel, 1948; Aach, 1957; Van Regenmortel, 1967). The minor serological differences that exist between these strains and TMV *vulgare* are often more easily distinguished by means of antisera specific for some other more distant tobamovirus such as U2 than by antisera to TMV *vulgare* (Van Regenmortel, 1967).

Some of the tobamovirus species such as cucumber green mottle mosaic virus

TABLE 9.2

List of Plant Virus Groups Defined by the International Committee on Taxonomy of Viruses

Plant virus group	Approximate size (nm)
A. Viruses with a monopartite genome of single-stranded (ss) RNA	
(I) Rod-shaped virions	
1 Tobamovirus group	300
2 Potexvirus group	470-580
3 Carlavirus group	620-700
4 Polyvirus group	680-900
5 Closterovirus group	600-2000
B. Viruses with a monopartite genome of ssRNA	
(II) Isometric virions	
6 Tymovirus group	30
7 Luteovirus group	25
8 Tombusvirus group	30
9 Southern bean mosaic virus group	30
10 Tobacco necrosis virus group	28
11 Maize chlorotic dwarf virus group	30
C. Viruses with a bipartite genome of ssRNA	
(I) Isometric virions	
12 Nepovirus group	28
13 Pea enation mosaic virus group	28
14 Comovirus group	28
15 Tobravirus group	50-110, 180-215
D. Viruses with tripartite genome of ssRNA	
(I) Isometric virions	
16 Cucumovirus group	28
17 Bromovirus group	26
18 Ilovirus group	26-35
19 Alfalfa mosaic virus group	58, 48, 36, 28 × 18
20 Hordeivirus group	100-150
21 Tomato spotted wilt virus group	80
22 Plant rhabdovirus group (in family Rhabdoviridae)	(160-380 nm) × (50-95 nm)
23 Phytoporeovirus genus	70
24 Fijivirus genus	70
25 Geminivirus group	(18 nm) × 2
26 Caulimovirus group	50

(CGMMV) and ribgrass mosaic virus (RMV) comprise strains that are readily distinguishable serologically (Juretic and Wetter, 1973).

In the past, most tobamoviruses were considered to be strains of TMV (Siegel and Wildman, 1954; Hennig and Wittmann, 1972). According to the current list of the ICTV, 12 different species are now recognized. The appreciation of the degree of difference between these viruses is of course subjective and depends on which properties are singled out for attention. As discussed in Sections A and B the distinction between virus strain and virus species is arbitrary. It seems perfectly reasonable, for instance, to subscribe to the view that the different isolates that infect tomato crops in the field and are serologically indistinguishable (Wang and Knight, 1967) from a homogeneous cluster that justifies the status of a virus species and a separate name.

The extent of serological relationship that exists between different tobamoviruses has been studied in detail (Van Regenmortel, 1975). The results,

TABLE 9.3
Extent of Serological Relationship between Tobamoviruses Expressed as Serological Differentiation Indices (SDI)^a

Virus pairs	Average SDI	Sequence difference (%)
TMV-D	1.2	18
D-U2	1.9	30
TMV-RMV	2.1	56
TMV-U2	2.7	26
D-RMV	4.0	53
TMV-CV4	4.2	
U2-RMV	4.5	54
RMV-CV4	4.8	
CGMMV-RMV	5.0	
SHMV-U2	5.3	62
TMV-SHMV	5.7	61
D-CV4	5.9	
CGMMV-U2	6.0	
U2-CV4	6.1	
CV4-CGMMV	6.2	
TMV-CGMMV	6.8	
TMV-FMV	3.0-6.0 ^b	
TMV-PMTV	7.0-8.0 ^c	
TMV-SBWMV	4.0 ^d	

^a From Van Regenmortel, 1975, and unpublished results.

^b From Francki et al., 1971.

^c From Kassanis et al., 1972.

^d From Powell, 1976.

summarized in Table 9.3, showed that these relationships ranged from very close to very distant. Such data illustrate that serology is unable to provide clear-cut borderlines for distinguishing between closely related virus strains and more distantly related virus species.

The estimates of serological relatedness among tobamoviruses are in close agreement with the degree of similarity revealed by chemical analysis of the various coat proteins (Gibbs and Harrison, 1976).

2. Potexvirus Group

This group consists of viruses with flexuous filamentous particles of a length varying between 470 and 580 nm (Koenig and Lesemann, 1978). The type member of the group is potato virus X. Serological relationships have been established between the following members of the group.

Cactus virus X	Parsley virus 5
Clover yellow mosaic virus	Parsnip virus 3
Cymbidium mosaic virus	Pepino mosaic virus
Hydrangea ringspot virus	Potato virus X
Narcissus mosaic virus	Viola mottle virus
Nerine virus X	White clover mosaic virus
Papaya mosaic virus	

The extent of serological relationship between the various potexviruses is not known with the same degree of precision as in the case of the tobamovirus group. In many cases, the number of antisera used in the tests was small and in other cases the variations between individual antisera were considerable (Bercks, 1963; Koenig and Bercks, 1968). Average SDI values between different potexviruses range from 2 to 9. These values tend to be somewhat unreliable because of the tendency of the virions to aggregate and because the viral coat proteins are sometimes degraded by host enzymes (Koenig, 1975; Koenig *et al.*, 1978). This latter phenomenon for instance, may explain the finding that the host plant on which NaMV was propagated had some influence on the strength of the heterologous precipitin reactions of narcissus mosaic virus (NaMV) antisera (Koenig, 1975). When NaMV was propagated in *Gomphrena globosa* instead of *Nicotiana clevelandii*, the average SDI between NaMV and six potexviruses was considerably reduced, i.e., antisera obtained with NaMV from *G. globosa* had lower homologous and higher heterologous titers.

Relationships between potexviruses have also been studied in immunodiffusion tests, using sonicated virions (Koenig, 1969a). No cross-reactions between the degraded protein subunits of several potexviruses could be demonstrated when low-titered antisera were used in immundiffusion tests.

3. **Carlavirus Group**

This group consists of viruses with slightly flexuous rods of a length varying between 620 and 700 nm. The type member of the group is carnation latent virus. Distant serological relationships have been established between the following members of the group.

Carnation latent virus	Passiflora latent virus
Chrysanthemum virus B	Pea streak virus
Cowpea mild mottle virus	Poplar mosaic virus
Lily symptomless virus	Potato virus M
Lonicera latent virus	Potato virus S
Mulberry latent virus	Red clover vein mosaic virus
Narcissus latent virus	Shallot latent virus

4. **Potyvirus Group**

This group consists of viruses with flexuous filamentous particles that are 680–900 nm long. The type member is potato virus Y. Serological relationships, mostly of a distant nature, have been demonstrated between the following members of the group.

Bean common mosaic virus	Pea seed-borne mosaic virus
Bean yellow mosaic virus	Pepper mottle virus
Sugar beet mosaic virus	Pepper veinal mottle virus
Bidens mottle virus	Peru tomato virus
Blackeye cowpea mosaic virus	Plum pox virus
Carnation vein mottle virus	Pokeweed mosaic virus
Celery mosaic virus	Potato virus A
Clover yellow vein virus	Potato virus Y
Columbia datura virus	Soybean mosaic virus
Cowpea aphid-borne mosaic virus	Statice virus Y
Dasheen mosaic virus	Sugarcane mosaic virus
Guinea grass mosaic virus	Tobacco etch virus
Henbane mosaic virus	Tulip breaking virus
Lettuce mosaic virus	Turnip mosaic virus
Maize dwarf mosaic virus	Watermelon mosaic virus
Onion yellow dwarf virus	Wild potato mosaic virus
Papaya ringspot virus	

Because of similar morphological and biological properties many other viruses have been tentatively assigned to the potyvirus group (Matthews, 1979), although they have not yet been shown to be serologically related to any of the established members of the group listed above.

It has been suggested that a number of potyviruses that infect the Gramineae and are not transmitted by aphids should be viewed as a separate subgroup (Gill,

1976). Serological cross-reactions have been observed only between some members of this subgroup, although this may have been caused by the use of antisera of too low titer. Relationships have been demonstrated between oat necrotic mottle virus (ONMV) and WSMV (Gill, 1976) and between agropyron mosaic virus (AgMV) and WSMV (Slykhuys and Bell, 1966; Langenberg, 1974).

5. Closterovirus Group

This group consists of viruses with very flexuous filamentous particles with a length varying from 600 to 2000 nm. The type member is sugar beet yellows virus, of which several serologically distinct strains have been described (Polak, 1971).

Three of the established members of the group have been found to be serologically related (Bar-Joseph *et al.*, 1979c), namely:

Sugar beet yellow virus
Carnation necrotic fleck virus

Wheat yellow leaf virus

Carnation necrotic fleck virus (CNFV) and carnation yellow fleck virus (CYFV) are closely related serologically and should be considered as strains of the same virus (Bar-Joseph *et al.*, 1976). No cross-reaction between CNFV and any of the four viruses CTV, lilac chlorotic leafspot virus (LCLV), ACLSV, and heracleum latent virus (HLV) could be demonstrated. On the other hand, two possible members of the group, PVT and ACLSV, were found to be serologically related. At the present time, insufficient information is available to build a coherent group out of all the viruses that could be conceived to be closteroviruses on morphological grounds (Brunt, 1978; Bar-Joseph *et al.*, 1979c).

6. Tymovirus Group

Tymoviruses are one of the largest groups of isometric plant viruses with a diameter of 30 nm. The following 16 members have been shown to be serologically related (Peters and Derkx, 1974; Koenig, 1976; Shukla *et al.*, 1980).

Andean potato latent virus
Belladonna mottle virus
Cacao yellow mosaic virus
Clitoria yellow vein virus
Desmodium yellow mottle virus
Dulcamara mottle virus
Eggplant mosaic virus
Erysimum latent virus

Kennedy yellow mosaic virus
Okra mosaic virus
Ononis yellow mosaic virus
Physalis mosaic virus
Plantago mottle virus
Scrophularia mottle virus
Turnip yellow mosaic virus
Wild cucumber mosaic virus

Some of these viruses are very closely related serologically; SDI values of 1 were found, for instance, for the pairs eggplant mosaic virus-Andean potato

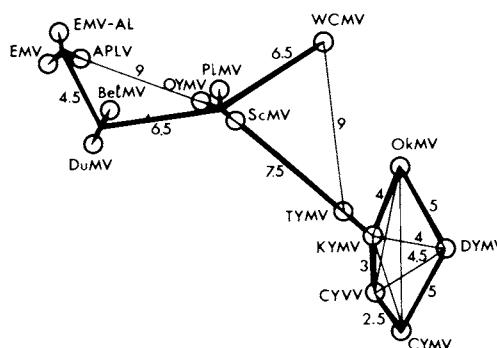


Fig. 9.2. Serological classification of tymoviruses based on average SDI values in reciprocal tests. The numbers correspond to the SDI values depicted as length units (adapted from Koenig, 1976).

latent virus (EMV-APLV), Belladonna mottle virus-Dulcamara mottle virus (BelMV-DuMV), and Ononis yellow mosaic virus-Scrophularia mottle virus (OYMV-ScMV) (Koenig, 1976).

With the exception of erysimum latent virus (EryLV) and Physalis mosaic virus (PhMV), the remaining 14 viruses were fitted into a serological classification scheme in which SDI values were depicted diagrammatically as length units. The resulting diagram, which took the form of a loop structure, is shown in Fig. 9.2. It was found that several viruses that were either unrelated or only distantly related to one another, became interconnected stepwise via others that were more closely related.

The relationships of 11 of the 14 viruses represented in Fig. 9.2 were also assessed from the amino acid composition of their coat proteins (Paul *et al.*, 1980b). Although some of the viruses that are closely related serologically were also found to be chemically similar, in general there was a very poor correlation between similarity in protein composition and serological relatedness. This contrasts with the good correlation found in an earlier comparison of chemical and antigenic similarities within the tobamovirus group (Gibbs and Harrison, 1976). It seems likely that the lack of correlation observed in the case of the tymoviruses will find an explanation only when more data on coat protein sequences and location of epitopes become available. When viral proteins show increasingly large differences in amino acid composition, there is of course less reason to expect that the degree of antigenic similarity will mirror compositional data.

7. Luteovirus Group

The luteovirus group consists of viruses with isometric particles of 25 nm diameter that are confined to the phloem tissue of their host plants and are not

transmitted by mechanical inoculation (Rochow and Israel, 1977). The type member is BYDV, of which several serologically distinct strains have been described (Aapola and Rochow, 1971; Paliwal, 1979). Serological cross-reactions have been found between the following members of the group (Duffus and Russell, 1975; Rochow and Duffus, 1978; Roberts *et al.*, 1980).

Barley yellow dwarf virus	Potato yellow leafroll virus
Beet mild yellowing virus	Soybean dwarf virus
Beet western yellows virus	Turnip yellows virus

8. Tombusvirus Group

This group consists of the following five viruses with isometric particles of 30 nm diameter.

Artichoke mottle crinkle virus	Petunia asteroid mosaic virus
Carnation Italian ringspot virus	Tomato bushy stunt virus
Pelargonium leaf-curl virus	

Various degrees of serological relationship exist between all five members of the group (Hollings and Stone, 1965b, 1975; Bercks and Lovisolo, 1965; Wetter and Luisoni, 1969). The serological distance between some strains within one virus species is greater than between some isolates belonging to different species. The separate names AMCV, CIRSV, PLCV, and PAMV were coined before it was realized that these viruses were serologically fairly closely related to TBSV. The division of the group into the above five species does not reflect the degree of antigenic similarity between many tombusvirus isolates.

9. Southern Bean Mosaic Virus Group

No serological data that could be used for showing affinities within this group are available.

10. Tobacco Necrosis Virus Group

Viruses belonging to this group have isometric particles of 28 nm diameter. Although the serological relationship between some members of the group is fairly distant (SDI of 3-5), they are all considered to be strains of one virus species. Eight TNV strains were divided into two serotypes on the basis of serological affinity (Babos and Kassanis, 1963; Kassanis and Phillips, 1970). Strains belonging to each serotype were more closely related to each other than to members of the other serotype. The validity of this distinction was questioned by Uyemoto *et al.* (1968) who were unable to confirm the existence of clear-cut boundaries between the two serotypes.

11. Maize Chlorotic Dwarf Virus Group

No serological relationship between maize chlorotic dwarf virus (MCDV) and any other virus has been demonstrated.

12. Nepovirus Group

This group consists of nematode-transmitted viruses with isometric particles of 28 nm diameter. According to the ICTV (Matthews, 1979), 14 nepovirus species can be recognized as distinct. However, only five of these viruses [i.e., CNV, grapevine chrome mosaic virus (GCMV), GFLV, Myrobalan latent ringspot virus (MLRSV), and potato black ringspot virus (PBRSV)] are serologically related to any other members of the group (Taylor and Hewitt, 1964; Kenten, 1972; Salazar and Harrison, 1978b). This means that the nepovirus group can be divided into 9 serologically distinct subgroups in the following way (Francki and Hatta, 1977; Harrison and Murant, 1977).

- Arabis mosaic virus (including grapevine fanleaf virus)
- Artichoke Italian latent virus
- Cherry leaf roll virus
- Mulberry ringspot virus
- Peach rosette mosaic virus
- Raspberry ringspot virus
- Tobacco ringspot virus, type member (including potato black ringspot virus)
- Tomato black ring virus (including cacao necrosis virus, grapevine chrome mosaic virus, and Myrobalan latent ringspot virus)
- Tomato ringspot virus

13. Pea Enation Mosaic Virus Group

The only member of this group is PEMV.

14. Comovirus Group

This group consists of multicomponent viruses with isometric particles of 28 nm diameter. Ten members have been recognized which are all serologically related.

- | | |
|-----------------------------------|------------------------------|
| Andean potato mottle virus | Quail pea mosaic virus |
| Bean pod mottle virus | Radish mosaic virus |
| Bean rugose mosaic virus | Red clover mottle virus |
| Broad bean stain virus | Squash mosaic virus |
| Cowpea mosaic virus (type member) | True broad bean mosaic virus |

The usual difficulty of distinguishing between separate strains and separate species is also demonstrated with this group. According to some authors (Bruen-

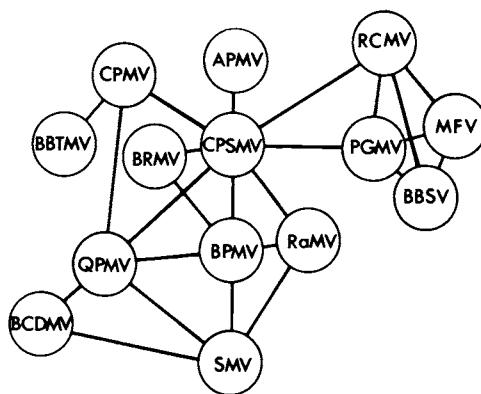


Fig. 9.3. Serological relationship between comoviruses (adapted from Bruening, 1978). The extent of cross-reactivity corresponded to SDI values varying from 1 to 6.

ing, 1978; Fulton and Scott, 1979), cowpea severe mosaic virus (CPSVM) and CPMV should be considered as distinct viruses, although this would introduce some further complications in the nomenclature (Swaans and Van Kammen, 1973). The serological relationships that have been established between comoviruses are presented in Fig. 9.3. All the relationships included in the diagram correspond to SDI values smaller than 6 (Bruening, 1978).

15. Tobravirus Group

This group consists of two viruses with multicomponent rod-shaped particles, TRV and pea early-browning virus (PEBV). The serological distance between some serotypes of TRV appears to be of the same order of magnitude (SDI of about 7) as between TRV and PEBV (Maat, 1963; Harrison and Woods, 1966).

16. Cucumovirus Group

The cucumovirus group consists of three members, CMV, TAV, and peanut stunt viruses (PSV). Most CMV strains have unstable capsids, and in double immunodiffusion tests, it is common to observe only subunit precipitin lines (Francki *et al.*, 1966; Scott, 1968; Devergne *et al.*, 1972). Meaningful antigenic comparisons are only possible if virions stabilized with formaldehyde (Fig. 3.1) are used for immunization (Devergne and Cardin, 1970, 1973). Two groups of CMV strains, labeled ToRS and DTL, have been distinguished on the basis of their antigenic properties (Devergne and Cardin, 1973, 1975) and this division

parallels the grouping of strains established on the basis of symptomatology and nucleic acid homology (Piazzolla *et al.*, 1979). The two groups of CMV strains could be recognized in immunodiffusion tests by the formation of spurs between precipitin lines caused by intact capsids. By contrast, the lines caused by protein subunits of different strains showed reactions of complete fusion (Lawson, 1967a; Mink, 1969; Devergne and Cardin, 1970).

The existence of a serological relationship between CMV and TAV has given rise to much controversy (Govier, 1957; Grogan *et al.*, 1963; Van Regenmortel, 1966b; Lawson, 1967a; Habil and Francki, 1975). The inability of some authors to detect a relationship between the two viruses may be due to virion instability and to the fact that the cross-reaction is stronger with certain serotypes than with others. In this type of situation, it is of course the positive results that outweigh the negative findings (Mink, 1969; Waterworth *et al.*, 1973; Devergne and Cardin, 1975; Mink *et al.*, 1975).

Different PSV serotypes have also been recognized (Devergne and Cardin, 1976; Douine and Devergne, 1978). PSV has been shown to represent an antigenic intermediate between CMV and TAV (Mink, 1969; Mink *et al.*, 1975). In TAV antisera, all the antibodies responsible for the cross-reaction between TAV and CMV were found to react with PSV (Devergne and Cardin, 1975). A serological classification scheme of the four cucumoviruses is presented in Fig. 9.4. In Fig. 9.4 the average SDI values between the different viruses were calculated from the data of Devergne and Cardin (1975).

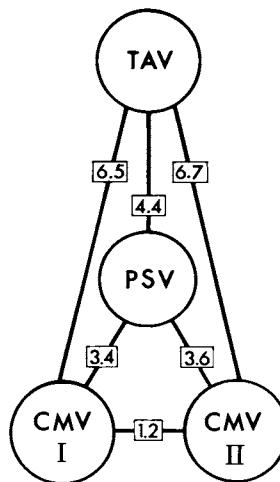


Fig. 9.4. Serological classification of cucumoviruses based on the data of Devergne and Cardin (1975). CMV I and CMV-II correspond to the ToRS and DTL groups of strains. The numbers represent the average SDI values between the different virus groups.

17. Bromovirus Group

This group consists of three viruses with isometric particles of 26 nm diameter: broad bean mottle virus (BBMV), BMV, and CCMV. The antigenic specificity of BMV protein subunits is markedly different from that of BMV capsids (Von Wechmar and Van Regenmortel, 1968). The virions of bromoviruses are unstable and swell above pH 6.5 in the absence of divalent cations. Capsid swelling affects the antigenicity of BMV and CCMV, and leads to the exposure of epitopes specific of the protein monomers (Rybicki and Von Wechmar, 1981). All three bromoviruses were found to be serologically related when tested by ELISA, although BBMV could not be shown to be related to either BMV or CCMV by immunodiffusion tests (Scott and Slack, 1971; Rybicki and Von Wechmar, 1981). The coat proteins of all three viruses are more closely related serologically than the parent viruses.

18. Ilarvirus Group

This group consists of multicomponent viruses with a divided genome, which were originally grouped together because of similarities in virion morphology and stability and in symptoms produced in infected plants. Two serologically distinct subgroups have been recognized. Subgroup A contains the following members:

Black raspberry latent virus	Elm mottle virus
Citrus leaf rugose virus	Tobacco streak virus (type member)
Citrus variegation	Tulare apple mosaic virus

CLRV and CVV have been shown to be serologically related (Garnsey, 1975). Subgroup B contains the following members:

Apple mosaic virus	Prunus necrotic ringspot virus
Plum line pattern virus	Rose mosaic virus
Prune dwarf virus	

ApMV, PLPV, PNRSV, and RosMV are serologically related (Fulton, 1968) and several distinct serotypes of ApMV and PNRSV have been described (Bock, 1967; Barbara *et al.*, 1978).

19. Alfalfa Mosaic Virus Group

AMV is the only member of this group. Although many isolates with different biological properties have been described, they are all considered to be strains of AMV. Most strains cannot be distinguished serologically.

It has been suggested that AMV should be grouped with the ilarviruses be-

cause of similarities in biological, chemical, and physical properties (Lister and Saksena, 1976). Preparations of several ilarviruses have been shown to contain oval or bacilliform particles (see Halk and Fulton, 1978) and both virus groups show the phenomenon of coat protein activation of the genome (Van Vloten-Doting, 1975).

20. Hordeivirus Group

This group consists of viruses with a tripartite genome and rod-shaped particles of a length varying from 100 to 150 nm. The following three members are distantly related serologically (Gibbs *et al.*, 1963; Polak and Slykhuis, 1972):

Barley stripe mosaic virus
Lychnis ringspot virus

Poa semilatent virus

21. Tomato Spotted Wilt Virus Group

TSWV is the only member of this group. No distinct serotypes have been described (Tas *et al.*, 1977).

22. Plant Rhabdovirus Group

This group consists of viruses with bacilliform particles that are 160–380 nm long and 50–95 nm wide. Only a few members [broccoli necrotic yellow virus (BNYV), lettuce necrotic yellows virus (LNYV), potato yellow dwarf virus (PYDV), sowthistle yellow vein virus (SYVV), wheat striate mosaic virus (WStMV)] have been studied by serological techniques, and no cross-reactions have been detected between them.

23. Phytoreovirus Group

The phytoreovirus and Fijivirus groups represent the two plant virus genera of the family Reoviridae. It has been suggested that plant and human reoviruses are serologically related (Streissle and Maramorosch, 1963), but subsequent work showed that this was not the case (Gamez *et al.*, 1967).

The two members of the phytovirus group, wound tumor virus and rice dwarf virus, have a double-stranded RNA genome consisting of 12 segments.

24. Fijivirus Group

Fijiviruses differ from the phytoreovirus group in the structure of the protein shell (presence of spikes), type of vector, and genome pattern (10 genome seg-

ments instead of 12). The group comprises the following members:

Cereal tillering disease virus	Oat sterile dwarf virus
Fiji disease virus	Pangola stunt virus
Maize rough dwarf virus	Rice black streaked dwarf virus

CTDV, MRDV, PSV, and RBSDV have been shown to be serologically related in both the inner capsid and the B spike (Luisoni *et al.*, 1973; Milne and Luisoni, 1977b). FDV is not serologically related to OSDV or MRDV. Two isolates known as *Arrhenatherum* blue dwarf virus and *Lolium* enation virus are serologically indistinguishable from OSDV and should not be considered as separate virus species.

25. Geminivirus Group

This group consists of DNA-containing viruses with isometric particles of 18 nm in diameter that occur predominantly in pairs. No serological relationships have been detected between the following four members of the group (Francki *et al.*, 1979):

Bean golden mosaic virus	Chloris striate mosaic virus
Cassava latent virus	Maize streak virus (type member)

26. Caulimovirus Group

This group consists of DNA-containing viruses with isometric particles of 50 nm diameter. The following members have been shown to be serologically related (Mortis *et al.*, 1980):

Cauliflower mosaic virus (type member)	Strawberry vein banding virus
Dahlia mosaic virus	

Immunochemical Studies

Plant viruses represent an interesting experimental material for a variety of immunological investigations. Many viruses can be obtained in large quantities in a highly purified form, and they are usually very immunogenic. Viruses such as TMV or TYMV are some of the best-studied entities in the whole of molecular biology, and the wealth of available information on their structural and physicochemical properties facilitates the interpretation of their behavior as antigens. Particular features of multivalent antigens such as the multiplicity of identical epitopes at the virion surface, or the conformational changes induced in the subunits by their polymerization can be studied fairly easily in the case of viruses.

In this chapter, the antigenic structure of TMV will be analyzed in detail and various studies that have utilized plant viruses as model antigens will be described.

A. ELUCIDATION OF THE ANTIGENIC STRUCTURE OF PLANT VIRUSES

1. Tobacco Mosaic Virus

TMV is probably the best-known virological entity. TMV was the first virus to be purified and shown to contain RNA as genetic material, and was also the first one to be reassembled *in vitro* from its constituent parts. Many fundamental investigations into the molecular structure of viruses have used TMV as a model, and a wealth of information has been obtained, which contributed significantly to the development of molecular biology (Hennig and Wittmann, 1972; Lauffer, 1975; Butler and Durham, 1977).

TMV is a rod-shaped particle, 300 nm long, which consists of 2130 identical protein subunits arranged as a helix around an RNA molecule of molecular weight 2×10^6 . The TMV protein subunit contains 158 amino acid residues, and its primary structure was elucidated more than 20 years ago (Fig. 10.1). The three-dimensional structure of TMV protein has been established by X-ray crystallography (Champness *et al.*, 1976; Bloomer *et al.*, 1978). The folding of the polypeptide chain was determined from electron density maps obtained at a resolution of 2.8 Å. The central part of the subunit consists of two pairs of α -helices, which comprise about 60 residues. In the assembled virus, the RNA fits between the protein subunits in a groove at a radius of 4 nm (Stubbs *et al.*, 1977). In view of the extensive knowledge of its chemical structure, TMV represents an excellent model for studying the antigenicity of a viral capsid at the molecular level.

Several experimental approaches have been used to locate the antigenic determinants of TMV. The first approach used by Anderer (1963a) consisted of testing the ability of different peptides of TMV protein (TMVP) to inhibit the precipitin reaction between virus and antibody. The peptides were obtained by the action of various enzymes. Regions corresponding to residues 18–23, 62–68, 123–134, and 153–158 in TMVP were found to possess the highest inhibitory capacity. In subsequent studies, Anderer and co-workers focused their attention on the carboxy-terminal hexapeptide of TMVP (residues 153–158). This region had been known for a long time to participate in the serological specificity of TMV (Harris and Knight, 1952, 1955).

Antisera were prepared by using as the immunogen, the C-terminal hexa-, penta-, tetra-, or tripeptides of TMVP coupled to bovine serum albumin. The resulting antibodies were found to precipitate the virus and to neutralize its infectivity (Anderer, 1963b; Anderer and Schlumberger, 1965a,b). The antigenic reactivity of the terminal amino acid and terminal dipeptide of the two TMV strains, *vulgare* and *dahlemense*, were also compared (Anderer and Schlumberger, 1966a,b). The two terminal residues of these strains are Ala-Thr and

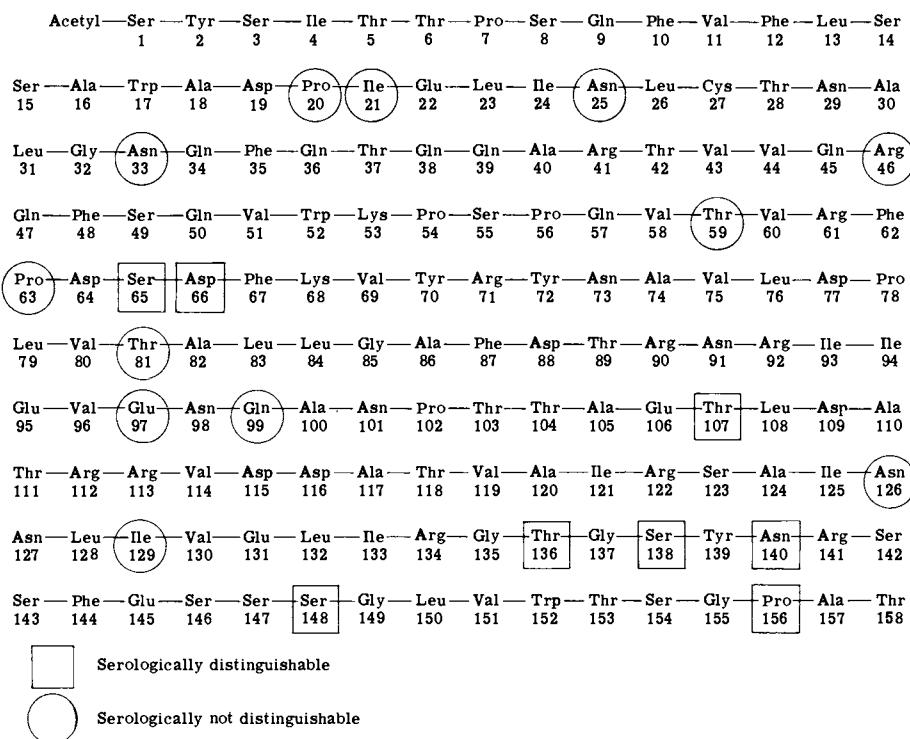


Fig. 10.1. Amino acid sequence of TMV protein. Mutants with residue exchanges at positions indicated by circles and squares were studied by Sengbusch (1965) and Van Regenmortel (1967). Circled residues correspond to exchanges that do not alter the antigenic properties of mutant virions; squares indicate residues that alter these properties.

Ala-Ser, respectively. Antisera were raised to the terminal residues conjugated to a protein carrier. Antibodies specific for Ala-Thr precipitated both strains, whereas antibodies to Thr precipitated only the homologous vulgare strain (Fig. 10.2). The specificity of the reaction was demonstrated by the fact that virus precipitation and neutralization could be inhibited by prior incubation of the antiserum with Ala-Thr and Thr. Similar results were obtained with the terminal residues of strain dahlemense. Furthermore, when the terminal Thr of strain vulgare was removed by carboxypeptidase treatment, the resulting de-threonylated virus, which now had a terminal Ala, could be precipitated by an anti-Ala serum (Anderer *et al.*, 1967). It may seem surprising that antibodies directed against one or two residues possess such a degree of specificity. In fact there is every reason to believe that this phenomenon represents an exceptional case linked to the highly exposed location of the C-terminus in TMVP (Anderer *et al.*, 1967).

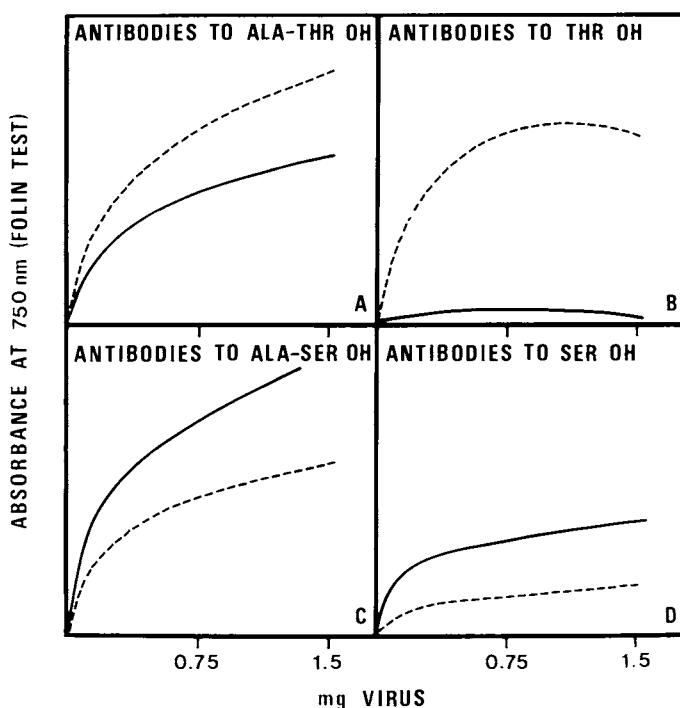


Fig. 10.2. Quantitative precipitin curves of antisera prepared against the conjugated C-terminal dipeptides and terminal residues of TMV strains vulgare (A and B) and dahlmense (C and D). Solid lines correspond to TMV dahlmense and dashed lines to TMV vulgare (from Anderer and Schlumberger 1966a,b).

When TMV antisera were tested for their ability to precipitate the C-terminal peptides of TMVP conjugated to a carrier, no reaction occurred. Only when the more sensitive passive hemagglutination test was used could the presence of antibodies reactive with the C-terminal peptides be detected (Anderer and Ströbel, 1972a,b). It was concluded from these results that the majority of antibodies present in TMV antisera are directed against conformational determinants and are unable to recognize the C-terminal protein sequence.

Another approach used for locating the epitopes of TMV consisted in studying the effect of modifying particular amino acid residues of the protein (see Chapter 1, Section D,2,a). Various amino acid side chains were chemically modified, and in some cases, the antigenic specificity was found to be altered (Malkiel, 1952; Price, 1954; Anderer and Handschuh, 1963; Slobin, 1970; Staab and Anderer, 1976). When the C-terminal threonine was removed by carboxypeptidase treatment, the antigenic properties of the virus were also altered (Harris and Knight, 1955; Vansanten *et al.*, 1964).

Modifications resulting from mutational events have also been studied. A large number of TMV mutants with 1–3 amino acid exchanges were compared with the wild strain by means of precipitin tests (Sengbusch, 1965; Van Regenmortel, 1967). Mutations that altered residues 65, 66, 107, 136, 138, 140, 148, and 156 were found to change the antigenic properties of the virus (Fig. 10.1). With the exception of the exchanges at residues 107, 136, and 140 all the modifications that were distinguishable serologically were located in peptide regions, which had shown inhibitory activity in the experiments of Anderer (1963a). However, some of the exchanges (i.e., residues 20, 21, 63, 126, and 129) which did not produce detectable antigenic changes in the virus were located in short peptides that possessed inhibitory activity. The significance of such apparent contradictions is unclear, since a mutated residue may be able to affect antigenicity not only locally at the site of the exchange, but also indirectly by altering the conformation of a distal epitope of the molecule. In a recent study, an attempt was made to distinguish between these two alternatives (Milton *et al.*, 1980). This was done by comparing the antigenic properties of tryptic peptides of TMV wild type with those of equivalent mutant peptides presenting a single amino acid exchange. Some of the results are summarized in Table 10.1.

It was found, for instance, that exchanges at positions 65 and 140 gave rise to capsids that were serologically distinguishable from TMV in precipitin tests, although the corresponding depolymerized subunits were not distinguishable in inhibition assays with a TMVP antiserum. On the other hand, the protein subunit of a mutant with an exchange at position 20 was distinguishable from TMVP, although the corresponding two capsids were not distinguishable in precipitin tests (Milton *et al.*, 1980). When the wild type and mutant tryptic peptides were compared in their ability to inhibit the precipitation reaction between radiolabeled TMVP and specific antibodies, it was found that the replacement in position 20 did not affect the antigenic properties; in contrast a replacement at position 63 could totally abolish the inhibitory activity of the heptapeptide 62–68. The results obtained in these studies with TMV mutants can be elucidated by referring to the three-dimensional folding of TMVP illustrated in Fig. 10.3. The position of residue 20 away from the virus surface readily explains the inability of the virions of mutant Ni 118 to be serologically distinguishable from wild type TMV. At the same time, the buried location of residue 20 throws doubt on the specificity of the inhibition of the TMV precipitin reaction by peptide 18–23, reported by Anderer (1963a). From the results of comparative inhibition tests with mutated and wild-type tryptic peptides, it seems unlikely that residue 20 is part of an antigenic determinant of TMVP. However, since the exchange Pro → Leu at position 20 alters the reactivity of the dissociated subunit, this probably occurs by a conformational effect that does not significantly affect the surface of the capsid. This example indicates that the interpretation of mutant studies is not at all straightforward, and that it is essential to distinguish between

TABLE 10.1

**Influence of Amino Acid Exchanges in the Coat Protein of TMV
on the Antigenic Properties of Assembled Capsids, Depolymerized Subunits,
and Tryptic Peptides^a**

Mutant	Location of exchange	Tryptic peptide	Antigenic difference measured in		
			Capsid ^b	Subunit ^c	Tryptic peptide ^d
414	65	4	+	-	+
Ni 118	20	1	-	+	-
Ni 1927	156	12	+	+	+
CP 415	140	11	+	-	0
Ni 568	5	1	+	+	-
	107	8			-
Ni 1688	63	4	+	+	+
	156	12			+

^a From Milton et al., 1980.

^b The presence (+) or absence (-) of serological differences between wild type and mutant capsids was determined by precipitin tests (Sengbusch, 1965; Van Regenmortel, 1967).

^c Serological differences were determined in inhibition assays with radio-labeled TMVP using unlabeled mutant proteins.

^d Wild type and mutant peptides were compared in inhibition assays with labeled TMVP and specific TMVP antiserum. The same antiserum was used for comparing wild type and mutant subunits.

^e Not done.

exchanges that affect the antigenic reactivity of TMVP because they are located within an epitope and those that have an influence because they alter the conformation of the polypeptide chain.

The important role played by conformation is clearly illustrated in the case of the exchange Pro → Leu in position 156. This exchange leads to a conformational change, which allows the mutant virion to react with heterospecific antibodies present in TMV antisera (Sengbusch and Wittmann, 1965; Van Regenmortel, 1966b, 1967; Loor, 1971). Such heterospecific antibodies, which have been found in all animals immunized with TMV, are unable to react with the immunogen (Lelarge and Van Regenmortel, 1974). As shown in Fig. 10.4, a TMV antiserum that has been fully absorbed with the homologous TMV is still capable of reacting with a mutant showing an exchange at position 156. When the rigidity of the peptide chain caused by the presence of the proline residue is decreased by the exchange to leucine, it is probable that a particular epitope is rendered more accessible than in the wild type structure.

Another approach used to locate the epitopes of TMV consisted of studying the binding of peptides of TMVP to antibodies prepared against depolymerized

subunits instead of against the virus (Benjamini *et al.*, 1972a,b). Using the technique of inhibition of complement fixation with TMVP antisera, Benjamini *et al.* (1964) found inhibitory activity only in tryptic peptide 8 corresponding to residues 93–112. Subsequently, the antigenic activity of this region of the molecule was studied extensively with shorter peptides obtained by degradation and with synthetic peptides varying in length from dipeptide to decapeptide (Young *et al.*, 1966, 1967, 1968; Stewart *et al.*, 1966; Benjamini *et al.*, 1968a). The shortest peptide that possessed demonstrable binding activity was the pentapeptide 108–112 (Fig. 10.5). It was also found that the binding of this pentapeptide Leu-Asp-Ala-Thr-Arg to TMVP antibody was greatly enhanced by the addition of five alanine residues at its N-terminal end. Another interesting finding was that the inactive tripeptide 110–112 Ala-Thr-Arg acquired binding activity by N-octanoylation (Benjamini *et al.*, 1968b). This seemed to indicate that

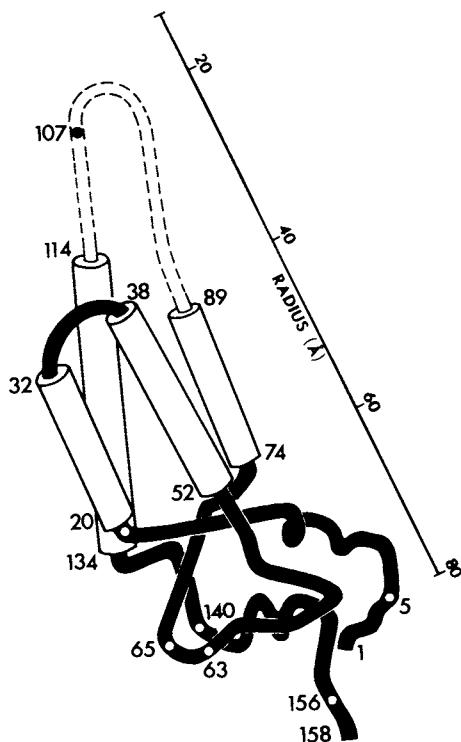


Fig. 10.3. Folding of the TMVP molecule, based on the X-ray crystallographic data of Bloomer *et al.* (1978). The radius scale starts at the center of the hole in the assembled virion. Mutant proteins with exchanges situated at positions 5, 20, 63, 65, 107, 140, and 156 were found to have altered antigenic properties when compared with wild type protein (from Milton *et al.*, 1980).

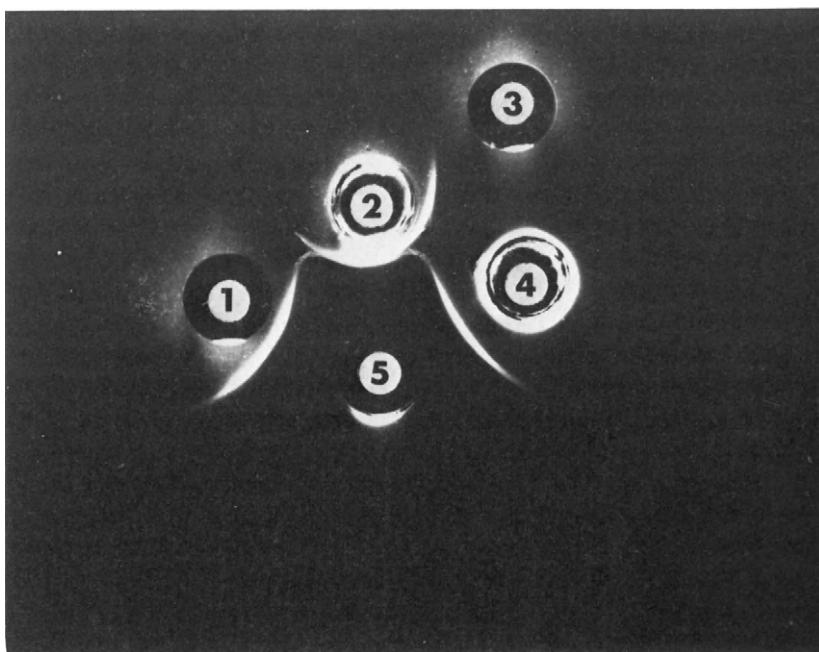


Fig. 10.4. Intragel absorption immunodiffusion experiment illustrating the presence of heterospecific (or heteroclitic) antibodies in TMV antisera. Wells 1 and 3 were filled with 15 mg/ml TMV; well 2 with 40 mg/ml of TMV mutant Ni 1927 which has an exchange Pro → Leu at position 156. Well 4 was initially filled with 15 mg/ml TMV and 24 hours later with TMV antiserum. After absorption this serum reacts with mutant Ni 1927 but not with the homologous TMV antigen diffusing from well 3. Well 5 was filled with TMV antiserum.

the increased hydrophobicity of the modified peptide enhanced its overall binding energy in a nonspecific manner. However, it is impossible to know if the increase in inhibitory activity observed when peptides of increasing size are tested is due to a direct participation of the additional residues in the structure of the epitope, or if it reflects the fact that larger peptides are better able to assume the native conformation present in the complete molecule.

It has been suggested that TMVP possesses one or at the most two antigenic determinants (Rappaport and Zaitlin, 1970; Benjamini, 1977), although such a view is contradicted by experimental evidence showing the presence of at least 3-5 epitopes on the surface of the TMV subunit (Van Regenmortel and Lelarge, 1973). The conclusion that the TMVP molecule does not contain more than two epitopes was based on the finding that tryptic peptide 8 completely inhibits the complement fixation between TMVP and anti-TMVP (Benjamini, 1977). Such an erroneous interpretation of the results of complement fixation inhibition tests is frequently encountered and it may be useful, therefore, to discuss this problem

at some length. In this type of inhibition assay, peptides obtained by chemical or enzymatic cleavage of the protein are tested for their ability to bind to specific antibodies and, in so doing, inhibit the subsequent reaction with the intact antigen. When the protein fragments are small and contain only one epitope, the binding of antibody to the peptide does not initiate complement fixation by itself but inhibits the subsequent reaction between antibody and the complete molecule and the resulting fixation of complement. It is important to realize that a single peptide capable of reacting for instance with only 10% of the total antibodies present in an antiserum will nevertheless cause complete inhibition of the complement fixation reaction. In other words, when the percentage of complement fixation passes from 80 to 0%, this does not mean that the entire antibody activity of the antiserum has been exhausted by the inhibitor. The decrease to 0% fixation only pertains to the particular antiserum dilution that was used in the test; indeed if the same antiserum had been used at a lower dilution, a considerable amount of complement fixation would have occurred again and no inhibitory effect

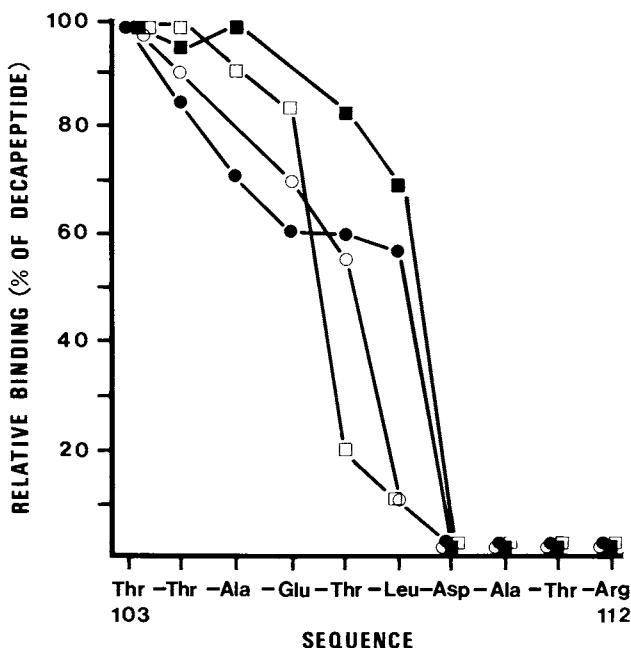


Fig. 10.5. The binding of N-[¹⁴C]acetyl peptides of increasing length (from dipeptide 111-112 to decapeptide 103-112) with equal amounts of globulins derived from four rabbits immunized with TMVP. Globulins were reacted with 7.5 nanomoles of each peptide, and the results are expressed as relative binding at each peptide compared to that of the decapeptide. The shortest peptide showing significant binding was the pentapeptide 108-112 (from Benjamini et al., 1968a).

would have been apparent. The degree of inhibitory activity of a peptide can only be expressed quantitatively by comparing the antiserum dilutions needed for obtaining the same percentage of complement fixation, in the presence and absence of the inhibitor.

Benjamini (1977) argued that since a tryptic digest of TMVP totally inhibited the complement fixation by TMVP and specific antiserum, the antibodies recognized only certain sequences in these peptides and not the original conformations, which must have been destroyed by the tryptic cleavage. From the above considerations, it is clear that such a conclusion is not valid. There is of course considerable experimental evidence which demonstrates the important role played by conformation on the antigenic structure of TMV (Anderer and Handschuh, 1963; Milton and Van Regenmortel, 1979).

From the three-dimensional structure of TMVP established by X-ray crystallography (Fig. 10.3), it is now apparent that the epitope studied by Benjamini and colleagues is located in the central hole of the assembled virus particle. Since this epitope is not expressed antigenically in the virus, it corresponds to a cryptotope of the assembled virion. Recently, the whole question of how many epitopes are expressed on the TMVP monomer was again investigated by means of complement fixation and direct binding assays (Milton and Van Regenmortel, 1979; Altschuh *et al.*, 1981). It was found that in addition to the epitope of tryptic peptide 8, several other epitopes were present in peptides 1, 4, and 12. In comparative tests, the regions 62–68 and 142–158 possessed a higher inhibitory activity, on a molar basis, than the peptide with the epitope corresponding to residues 108–112 (Milton *et al.*, 1977; Milton and Van Regenmortel, 1979). All the TMVP antisera that were examined produced similar results and it is not clear why Benjamini and colleagues found that only peptide 8 possessed inhibitory activity.

In summary, the different approaches used to elucidate the antigenic structure of TMV allow the following conclusions to be drawn:

1. The TMVP molecule possesses at least five epitopes. At least two epitopes situated in tryptic peptides 1 (residues 30–40) and 8 (residues 108–112) are cryptotopes that are not expressed at the surface of the virion.
2. Three epitopes of the viral subunit, situated in tryptic peptide 1 (residues 1–10), 4 (residues 62–68), and 12 (residues 142–158), are expressed in both the capsid and depolymerized subunit.
3. One epitope made up of residues of tryptic peptide 12 is a neotope that is not expressed at the surface of the dissociated subunit. This neotope is a product of the quaternary structure of the protein, and induces the formation of antibodies that do not react with TMVP monomer.

Further progress in the characterization of TMV epitopes will depend on the utilization of synthetic peptides. This approach is currently used in the author's

laboratory and it is hoped that these studies will also clarify the nature of the unusual cross-reaction that has been discovered between TMV and two isometric viruses (Bercks and Querfurth, 1971b; Bercks *et al.*, 1974; Querfurth and Bercks, 1976; Paul *et al.*, 1980a).

2. Turnip Yellow Mosaic Virus

The icosahedral capsid of TYMV consists of 180 protein subunits of molecular weight 20,000, arranged as 32 capsomeres. Although many biochemical studies of the virus and its RNA have been performed, only few data are available regarding the structure and topography of the protein subunit (Turano *et al.*, 1976; Hartman *et al.*, 1978). A limited amount of information on the folding of the peptide chain of TYMV protein (TYMVP) has been obtained by immunochemical methods.

A number of peptides of TYMVP were obtained by cleavage with trypsin or cyanogen bromide, and their activity was tested in inhibition of complement fixation experiments (Pratt *et al.*, 1980). The antisera used in these experiments were prepared by immunizing rabbits with TYMV or a decamer aggregate of TYMVP. Although no cross-reactions could be demonstrated in immunodiffusion tests between dissociated subunits and the intact virions, using either virus or subunit antiserum (Rappaport *et al.*, 1965), a weak cross-reaction was demonstrable by the complement fixation test. A TYMV antiserum at a dilution of 1:18,000 produced 87% complement fixation when tested against the virus, and 90% fixation when tested at a dilution of 1:200 against the protein decamer.

Four antigenic regions have been localized in the primary structure of the TYMV subunit. The reaction between TYMVP and its antiserum was inhibited most strongly by peptides corresponding to residues 1-12 and 46-67, whereas the reaction between the virus and its specific antibodies was inhibited only by the peptide corresponding to residues 46-67. These results suggest that at least a part of the region 46-67 of TYMVP lies at the surface of the subunit that is also exposed to the outside in the assembled capsid. Some of the epitopes of TYMVP appear to be located in regions of the polypeptide chain that are in close contact with the viral RNA in the assembled virion (Pratt *et al.*, 1980). The antigenicity of synthetic peptides corresponding to different regions of the TYMV subunit is at present being studied in the author's laboratory. Preliminary results indicate that the regions corresponding to residues 57-64 and 183-189 (C-terminal region) harbor epitopes common to both virion and protein decamer.

B. BINDING STUDIES

Virus particles represent an ideal material for performing quantitative measurements of antigen-antibody binding. Because of their size, the virus and

virus-antibody complexes can be easily separated from free antibody, either by ultracentrifugation or by equilibrium filtration (Fazekas de St Groth, 1979).

Since the virus surface is composed of a large number of repeating identical subunits, the possibility exists for a bivalent IgG molecule to have both binding sites attached to neighboring epitopes of the same virion (Chapter 5, Section C,1). This type of monogamous bivalent binding can occur because the IgG molecule possesses enough flexibility to adjust its two Fab arms to fit neighboring identical determinants on the antigen surface. It has been argued that monogamous bivalent binding of IgG is prevalent with all viruses, since the free energy change when such a complex is formed is much greater than when the antibody binds by only one site (Day, 1972). There is no evidence, however, that this type of bivalent binding occurs in the region of antibody excess of an antigen-antibody binding curve. This means that when the antigenic valence of a virus is determined from the maximum number of antibody molecules that can simultaneously bind to the virion surface, the calculation must be done on the basis of univalent IgG molecules (Van Regenmortel and Hardie, 1976).

Several authors (Mamet-Bratley, 1966; Anderer *et al.*, 1971b; Urbain *et al.*, 1972) have claimed that TMV antibodies and specific Fab fragments are homogeneous in their affinity for TMV. These claims were based on the fact that the binding data gave rise to straight line Scatchard plots and to Sips heterogeneity indices close to 1. It has been shown in Chapter 5 that unreliable results are obtained when binding data are analyzed by the type of Scatchard plot commonly used for monovalent haptens. When a more suitable type of plot is used, however, viral antibodies can be shown to have the normal extent of heterogeneity. Furthermore, depending on the relative concentration of the reactants, the proportion of antibody molecules that are bound in a monogamous fashion varies from practically none to practically all IgG molecules.

C. TMV AS AN EXPERIMENTAL ANTIGEN

It is well known that multivalent antigens that possess repeating identical determinants on their surface are not necessarily thymus-independent antigens (Sela, 1973). TMV is a good example of such an antigen. It has been demonstrated that TMV does not induce an antibody response in neonatally thymectomized mice, and that the immunological competence versus TMV can be restored in such animals by injections of low molecular weight substances extracted from mouse and calf thymus (Ströbel, 1972, 1974).

The relationship between antigenic structure and cell-mediated immunity has also been studied by using the TMVP epitope situated in tryptic peptide 8 as a model system. Guinea pigs that had been injected with different peptides bearing the TMVP cryptotope of peptide 8 failed to react with immediate or delayed skin

reactions when challenged with TMV (Spitler *et al.*, 1970). In contrast, immunization with the same peptide conjugated to albumin induced antibodies capable of reacting with TMV (Fearney *et al.*, 1971).

The antibody response to TMV has also been studied in order to gain insight into such questions as the increase of nonreactive immunoglobulins during immunological stimulation (Urbain-Vansanten, 1970; De Vos-Cloetens *et al.*, 1971; Urbain-Vansanten *et al.*, 1974), the mechanism of immunological memory (Hooghe *et al.*, 1975; Van Acker *et al.*, 1979), and the regulation of the immune response by the idiosyncratic specificities present on antibody molecules (Urbain *et al.*, 1975, 1979; Mariamé *et al.*, 1977; Urbain, 1977; Urbain-Vansanten *et al.*, 1979).

Results obtained with the TMV system confirmed the sharing of idiosyncratic specificities between antibodies and immunoglobulins without known antibody function, a phenomenon originally described by Oudin and Cazenave (1971). It was also shown that randomly chosen rabbits that had been preimmunized with anti-idiosyncratic antibodies (i.e., with anti-TMV antibodies), when given the original TMV antigen, synthesized antibodies bearing idiosyncratic specificities similar to those of the original idiosyncrasy (Wikler *et al.*, 1979; Urbain *et al.*, 1979). These findings demonstrated that idiosyncrasies are involved in clonal interactions and as such, they constitute a direct demonstration that the immune system functions as an idiosyncratic network (Jerne, 1974).

D. VIRUSES AS MARKERS IN ELECTRON MICROSCOPY

Plant viruses have been found useful as visual markers for identifying cell surface antigens in electron micrographs (Hämmerling *et al.*, 1969; Aoki *et al.*, 1971; Hämmerling, 1976). Bivalent hybrid antibodies with dual specificity for mouse IgG and for viruses such as SBMV or TMV have been used, for instance, for locating the H-2 alloantigen on the surface of mouse ascites leukemia cells, and the H-Y antigen on mouse spermatozoa (Koo *et al.*, 1973). TMV, because of its characteristic shape, was found very useful for labeling surface antigens in scanning electron microscopy (Hämmerling *et al.*, 1975; Kumon, 1976).

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