IMMUNOCHEMICAL STUDIES WITH TRYPTIC PEPTIDES OF TOBACCO MOSAIC VIRUS PROTEIN

BY

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CERTIFICATION OF SUPERVISOR

In terms of paragraph 8 of "Regulations for the Degree of Ph.D." I, as supervisor of the candidate, R.C. de Lisle Milton, certify that I approve of the incorporation in this thesis of material that has already been published or submitted for publication.

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SUMMARY

The antigenic determinants of the protein subunit of tobacco mosaic virus (TMV) have been studied by inhibition of complement fixation, inhibition of micro-precipitin and direct binding experiments.

The viral subunit has been found to possess six antigenic determinants. Two of these, situated in tryptic peptide I (residues 1-41), were found to be a cryptotope (i.e. an antigenic determinant absent from the outer surface of the assembled viral capsid) and a viral antigenic determinant. Tryptic peptides 4 (residues 62-68) and 8 (residues 93-112) also contained cryptotopes which were situated in the region of residues 63-65 and 108-112 respectively. Tryptic peptide 12 (residues 142-158) contained both a cryptotope and a neotope (i.e. an antigenic determinant which is only expressed on the outer surface of the viral capsid), which are situated in the C-terminal region of the polypeptide chain of the TMV protein, residue 156 being associated with the cryptotope and residue 158 with the neotope. No antigenic activity could be demonstrated in tryptic peptide 11 (residues 135-141).

When the results are analyzed in terms of the three-dimensional structure of the viral subunit, it appears that all the antigenic reactive regions occupy highly accessible locations on the surface of the protein.

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MATERIALS AND METHODS

PART I. REVIEW OF LITERATURE AND INTRODUCTION

I.1 PERSPECTIVE

From the outset of the pioneering work that established the complete antigenic structure of the protein myoglobin, Atassi did not believe that any single chemical approach would be adequate to the task, as each approach had its own limitations and shortcomings. Accordingly a strategy of five approaches, designed to minimize the drawbacks of any single approach, was evolved: (a) determination of the conformational influence on the immunochemical properties; (b) immunochemistry of specific derivatives of the intact protein; (c) preparation and immunochemistry of a large number of peptides with a variety of overlaps; (d) immunochemistry of specific derivatives of immunochemically reactive peptides; and (e) having narrowed down the reactive regions to a conveniently small size by the foregoing approaches, final delineation is achieved by studying the immunochemistry of synthetic peptides representing various parts of each region.

The sequential application of these approaches allows the information obtained to be assembled in such a way that the findings from any one approach can be confirmed or corrected by the results of the next approach in the strategy. Thus the application of a multifrontal attack minimizes labour and increases effectiveness in the solution of a complex and time-consuming problem.(Atassi, 1975; 1977 a; 1977 b.)

In contrast to the concerted labour that the immunochemistry of myoglobin and lysozyme received, the efforts to elucidate the antigenic structure of tobacco mosaic virus (TMV) and its protein subunit have continued sporadically for two decades (Harris and Knight, 1955;

van Regenmortel and Lelarge, 1973) with several independant groups each contributing a limited number of approaches out of the context of a guiding strategy. This, as can be expected, has led to misinterpretation of specific features and confusion over the operation of the system as a whole.

For this reason it is proposed that the literature as well as the present work be organised into the framework of Atassi's strategy for the sake of clarity, even if the historical perspective is lost.

1.2 CONFORMATION AND ITS INFLUENCE ON IMMUNOCHEMICAL PROPERTIES

1.2.1 RATIONALE

The role played by the tertiary conformation in the immunochemistry of a protein antigen must be assessed before comparison between the activity of modified protein derivatives and the native protein can be considered valid. In this way it can be ascertained whether any change in immunochemical activity is directly due to the modified residue(s) or to a conformational change associated with the modification. (Atassi, 1977 b.)

Over and above this important consideration, the matter becomes more complicated when dealing with antigens which have a quaternary protein structure. This is because two new kinds of antigenic sites, in addition to those sites which remain unaltered in the transition from the individual subunits to the quarternary complex, become possible. The first of these new antigenic sites has been called a cryptotope (Jerne, 1960; van Regenmortel, 1966) and is defined as an antigenic determinant of the subunit which is no longer present

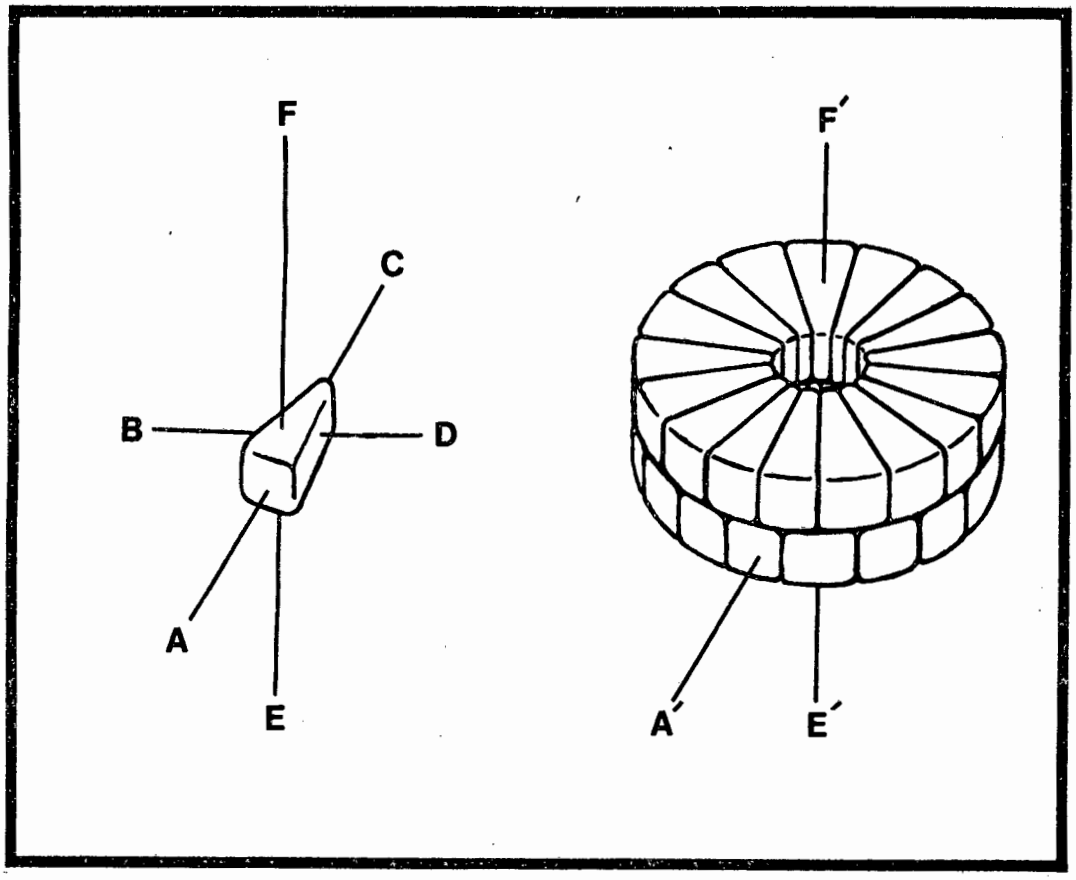

fig. 1.1 : A schematic representation of a TMV protein monomer and the corresponding polymer, showing the outside surfaces available for antibody binding. In a helical polymer like TMV, surfaces B, C, D, E and F would be hidden and could harbour cryptotopes. Surface A could also possess cryptotopes if it differed from surface A' because of conformational change in the polypeptide chain (adapted from van Regenmortel and Lelarge, 1973).

in the quaternary complex. This may be due to conformational change induced in the subunit during the formation of the quaternary complex or merely due to the fact that portions of the subunit's surface area are hidden in the quaternary complex (fig. 1.1). Similarly, the second kind of new antigenic site, called a neotope (van Regenmortel, 1966), is defined as an antigenic determinant which exists only in the quaternary complex, and may be due to the unique conformation of the quaternary complex or to the juxtapositioning of amino acid residues from neighbouring subunits (van Regenmortel and Lelarge, 1973).

As all three kinds of antigenic sites are possible in TMV and its protein subunits, it is necessary to devote attention to the structural and conformational features of the virus and protein subunit before an understanding of its immunochemistry is possible.

1.2.2 THE STRUCTURE AND CONFORMATION OF TMV AND ITS PROTEIN SUBUNIT

The TMV virion is a rod-like particle with the dimensions 300x18 nm and a molecular weight of 40×10^6 daltons. Each particle is a protein helix with a pitch of 23 Å and 130 turns, and each turn contains $16\frac{1}{3}$ protein subunits (there are 49 subunits in every three turns - the repeat unit). At the centre of the helix there is a hole with a diameter of 20 Å. The total number of protein subunits per virion is 2130 and each subunit has a molecular weight of 17500 daltons (fig. 1.2). The RNA content of each virion is 5% and three nucleotides are bound to each protein subunit, probably by hydrophobic interactions between the bases and aliphatic amino acids as well as by ionic interactions between the phosphate groups of the RNA and three arginine residues (Stubbs et al., 1977).

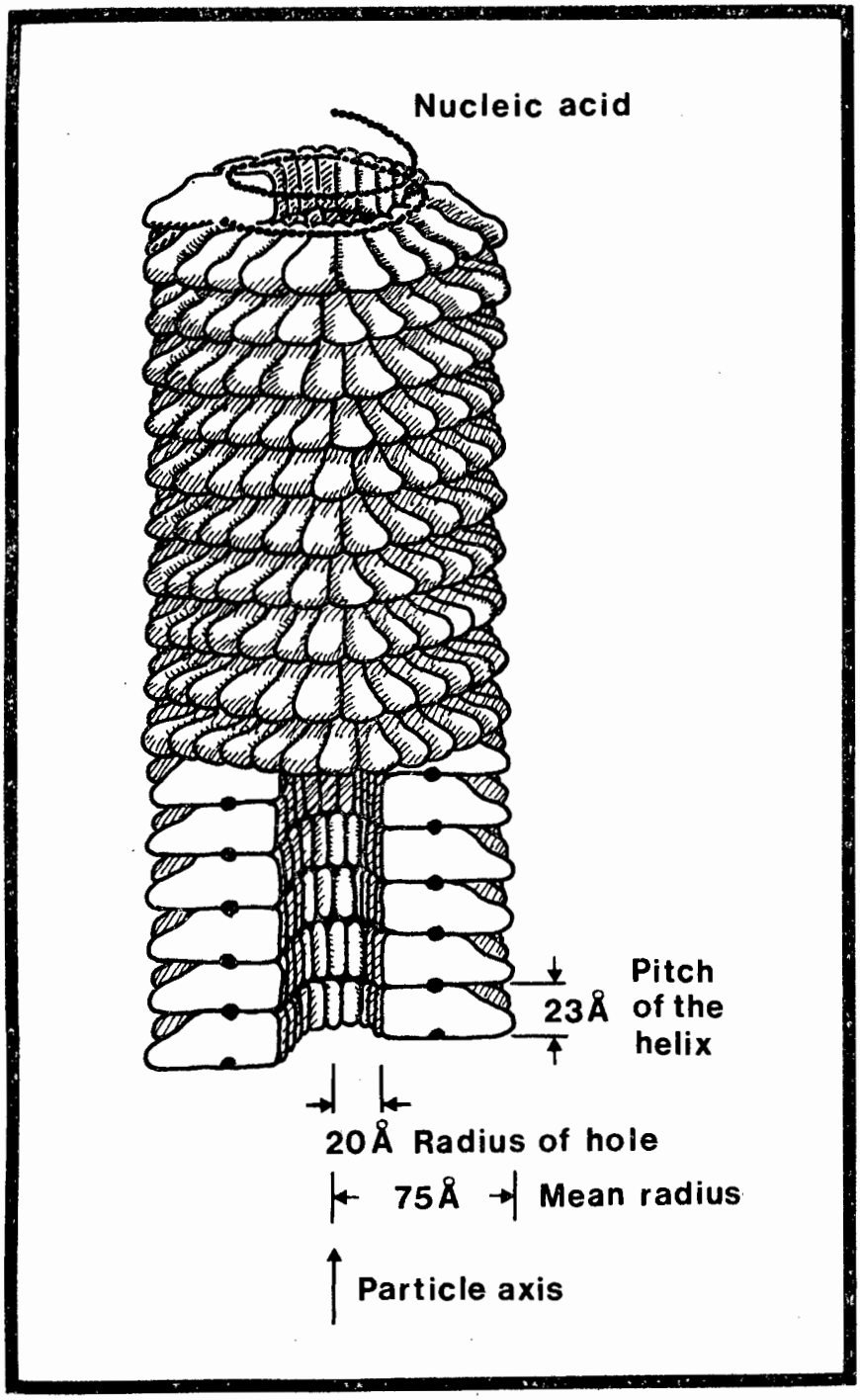

fig. I.2 : A schematic representation of a short length of a virion of TMV showing the helical structure. The lower portion of the virion is cut in half to show the dimensions of the helix, the central hole and the location of the strand of RNA at 40 Å. (Adapted from Rappaport, 1965).

In addition to these two components, each virion also contains a 26500 dalton protein of host origin, the H-protein, which is not associated with a specific site in the virion, but may be intercalated between the protein subunits, or more probably, lodged in the central hole of the virion (Asselin and Zaitlin, 1978).
The primary structure of the TMV protein subunit was initially determined by two groups working separately, but simultaneously, by the use of classical sequencing methods (Anderer et al., 1960; Tsugita et al., 1960). Minor disagreements about parts of the sequence (particularly the I-peptide, residues 1 - 41 - so named by Gish (Ramachandran and Gish, 1959) because of its insolubility at pH4,5) and the placement of amide groups were resolved (Anderer and Handschuh, 1962; Funatsu et al., 1964) into the sequence presented in figure 1.3.

The TMV protein is a single chain of 158 amino acids containing one cysteine (residue 27), but not cystine, nor methionine or histidine. The advantage conferred by this lack is not readily apparent except in as far as the virus would make an inadequate diet for most microorganisms. In fact the HR strain which has both histidine and methionine is more susceptible to bacterial spoilage than the other strains. (Fraenkel-Conrat, 1962.)

The C-terminal threonine is readily released by carboxypeptidase, even from the intact virus, while no further amino acids are split off by enzyme action (Harris and Knight, 1952). This is due to the location of proline third from the end of the chain, which prevents the enzymatic release of alanine. The protective role that proline plays

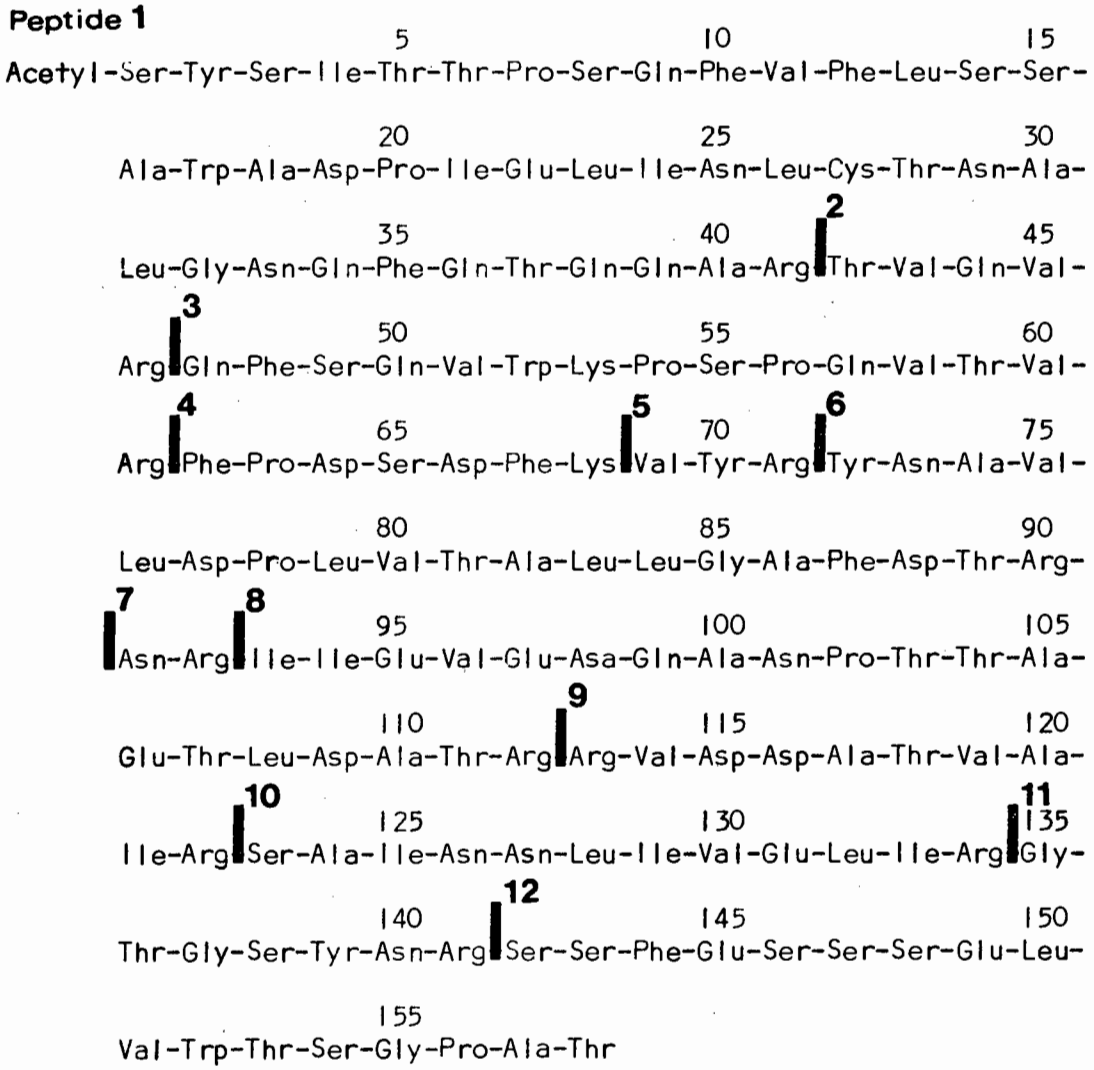

fig. I.3 : The primary structure of the protein of the common strain of TMV. Tryptic cleavage sites are indicated with a bar (|) and the peptide number is written to the right of it.

against exopeptidases is illustrated by the properties of certain mutants of TMV where the proline in position 156 is replaced by leucine: in contrast to TMV vulgare, these mutants are extensively degraded by carboxypeptidase (Tsugita and Fraenkel-Conrat, 1962). Similarly the N-terminal serine is acetylated (Narita, 1958) or replaced by proline in some strains (Wittmann, 1965) and this, with the proline near the C-terminus, appears to represent an evolutionary advantage in giving the protein additional stability against exopeptidases (Fraenkel-Conrat, 1969). Protection of the viral rod against endopeptidases,which degrade the isolated protein, would be afforded by the tight packing of the subunits in the helix (Fraenkel-Conrat, 1962).

The elucidation of the secondary and tertiary structure of the TMV protein subunit proved to be more problematic than the solution of the primary structure, because TMV protein presents a difficult problem for X-ray crystallographic structure determination. It can be crystallized as disks consisting of two layers of rings (each 17 subunits in circumference) without RNA, but the asymmetric unit is very large (600 000 daltons) and this posed a formidable technical problem (Durham and Butler, 1975; Champness et al., 1976). This encouraged structural predictions based on sequence data, low resolution X-ray crystallography and the availability of specific residues for enzymic, immunological or chemical reaction (Schiffer and Edmundson, 1967; Fraenkel-Conrat, 1968; Leberman, 1971; Durham and Butler, 1975).

Leberman (1971) modified the rules formulated by Kotelchuck and Scheraga (1969) and using these, roughly predicted three of the α-helices in the

protein subunit, while Durham and Butler (1975), utilizing the Chou and Fasman rules for predicting protein conformation (Chou and Fasman, 1974 a, b) suggested a wedge-shaped molecule with the N- and C-terminal residues on the surface of the virion and a secondary structure of α-helices and β-pleated sheets. Although they anticipated α-helical structure below the 40 Å radius, they suggested that the backbone of the protein, above the 40 Å radius was β-structure. This has been shown to be unlikely by Holmes et al. (1975) who were able to produce an electron density map of the protein at a resolution of 6,7 Å by analysis of the X-ray diffraction pattern given by orientated gels of the virus. They concluded that, apart from very short sequences, the only place where a β-sheet could be accommodated was above the 70 Å radius (Holmes et al., 1975).

Champness et al. (1976) used the method of isomorphous replacement to solve the structure of TMV protein in crystals of the disk to a resolution of 5 Å. They used only two heavy atom derivatives (Hg, Au), but these nevertheless gave good phases using the 17-fold rotational symmetry of the disk which gave rise to redundant information in the X-ray data that could be exploited in phase determination.

With consideration for sequence data relating to the locations of the heavy atom labelled residues, the accessibility of certain residues to reactants and regions of conserved sequence in the strains and mutants, the polypeptide chain could be traced for the 5 Å model (Champness et al., 1976) with little ambiguity. The main structural feature of this model was a super-secondary structure (Rao and Rossman, 1973), which determines the shape and rigidity of the molecule and consists of four

approximately radial "rods" of α-helix between the 40 Å and 70 Å radii (fig. I.4). If the course of the polypeptide chain is viewed in plan, the upper two helices were called the left and right slewed rods (LS and RS respectively) while the lower helices were called the left and right radial rods (LR and RR). The order of these helices from the N-terminus to the C-terminus on the polypeptide chain is LS, RS, RR and LR.

The spatial similarity of this arrangement of α-helices in TMV protein to one of the three domains in tyrosyl-tRNA synthetase has led to the speculation that this structure is associated with the functional property of RNA binding and is conserved during evolution for this reason (Argos et al., 1977).

The 5 Å map is, however, deficient in two respects. Firstly, the lack of density below the 40 Å radius, where it is expected as judged from the 6,7 Å map of the protein in the viral helix (Holmes et al., 1975), suggests that this region is very disordered in the disk (Champness et al., 1976). Secondly, at this low resolution the chain tracing is ambiguous at the level of the 70 Å radius and the direction of the sequence through the "loop" from the RS helix to the RR helix was chosen as the best fit for the electron density map (Champness et al., 1976).

The extension of the map to a resolution of 2,8 Å by the same group of workers provided more detail of the conformation and interactions of the subunits in the disk (Bloomer et al., 1978). The higher resolution of the electron density map confirmed that all the subunits in the disk are practically identical in conformation.

It also showed that the wrong alternative for the connectivity of the polypeptide chain at the 70 Å radius had been chosen (Bloomer et al., 1978). This did not affect the sequence of the four α-helices, but it did mean that the course of the polypeptide chain above the 70 Å radius had to be rearranged in the form depicted in figure 1.4.

This revealed that the pair of slewed helices was tied to the lower pair of radial helices by four strands of anti-parallel β-sheet at the distal ends, and perpendicular to the axis of the helices. Beyond this a group of aromatic residues (tryptophan 17, tyrosine 70, tyrosine 139, phenylalanine 12 and phenylalanine 144) together with proline 56 and proline 63 formed a hydrophobic cluster in the neighbourhood of the 75 Å radius. A further hydrophobic cluster on both sides of the molecular interface (phenylalanine 67 and proline 20 with phenylalanine 10 and proline 7 on the adjacent subunit) and several aliphatic side chains at the same radius make up a continuous belt of hydrophobic interactions which circles the disk from subunit to subunit. Distal to this are the residues that make up the surface of the virion - the projecting N- and C-terminal amino acids. (Bloomer et al., 1978.)

The higher resolution of the 2,8 Å map did not clarify the path of the polypeptide chain below the 40 Å radius, instead it emphasized the disorder in this region (Bloomer et al., 1978). High resolution proton nuclear magnetic resonance (NMR) studies by Jardetzky et al. (1978) confirmed that the lack of definition in the electron density maps is due to thermal disorder, which indicates flexibility

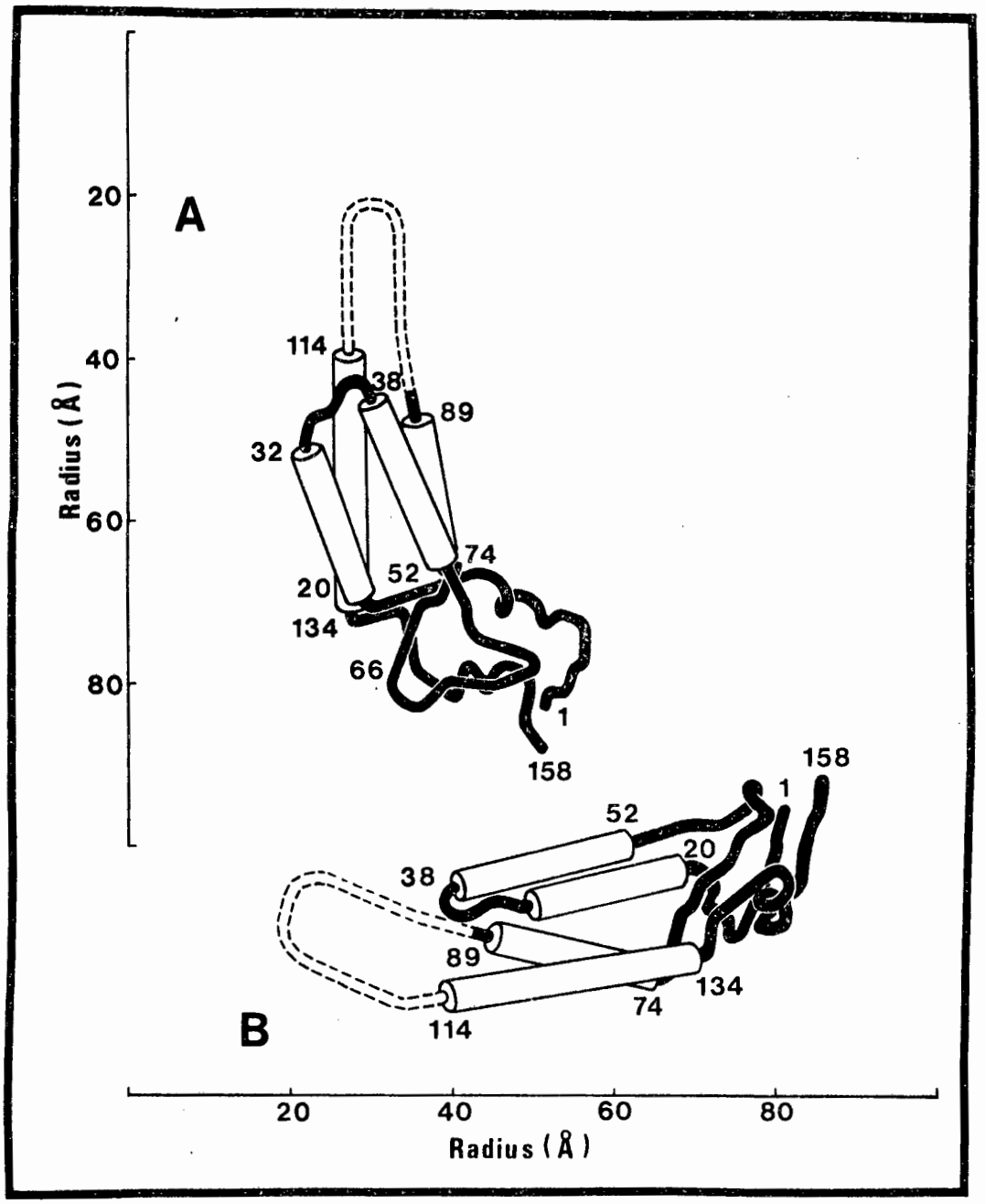

fig. 1.4 : A diagramatic representation of the secondary and tertiary structure of the TMV protein in the 20 S disk. The four approximately parallel helices are represented as cylinders (based on Bloomer et al., 1978; Argos et al., 1977). Residues 1 and 158 are located at the surface of the subunit which is exposed at the outer periphery of the capsid. Residues 90-114 are hidden inside the assembled capsid. (A) The molecule viewed in plan. (B) The molecule viewed from a side elevation.

in the region rather than irregular packing of this segment of the polypeptide chain in the crystal.

The 4 Å electron density map of Stubbs et al. (1977), which was obtained from X-ray fibre diffraction patterns of orientated gels of the virus, shows a vertical α-helix (the V-helix) extending from residues 103-112 at the 20 Å radius (fig. 1.5). This helix, which constitutes the major difference between the conformation of the virus and the disk, is also present in reconstituted TMV protein subunits as judged by the radial electron density distribution (Franklin, 1956). This suggests that the conformational change that gives rise to this helix is more directly caused by protein-protein interactions rather than by protein-RNA interactions when the helix is formed. Other differences between the two models concern the exact location of, and the residues comprising the main α-helices (Bloomer et al., 1978).

The 4 Å electron density map is deficient in that it yields very little information about the outer region of the protein subunit in the virion (Stubbs et al., 1977). The resolution falls off rapidly above the 65 Å radius and is as low as 5,5 Å at the 80 Å radius. As a result of this, the authors did not attempt a detailed fit for their model above the 60 Å radius (Stubbs et al., 1977). This deficiency leaves the question of the similarity between the conformation of the surface residues in the viral helix and the disk unanswered. Graham and Butler (1978), however, compared the locations of labelled cysteine 27 and tyrosine 139 in the disk and the viral helix by X-ray crystallography. They concluded that, during the transformation from the disk

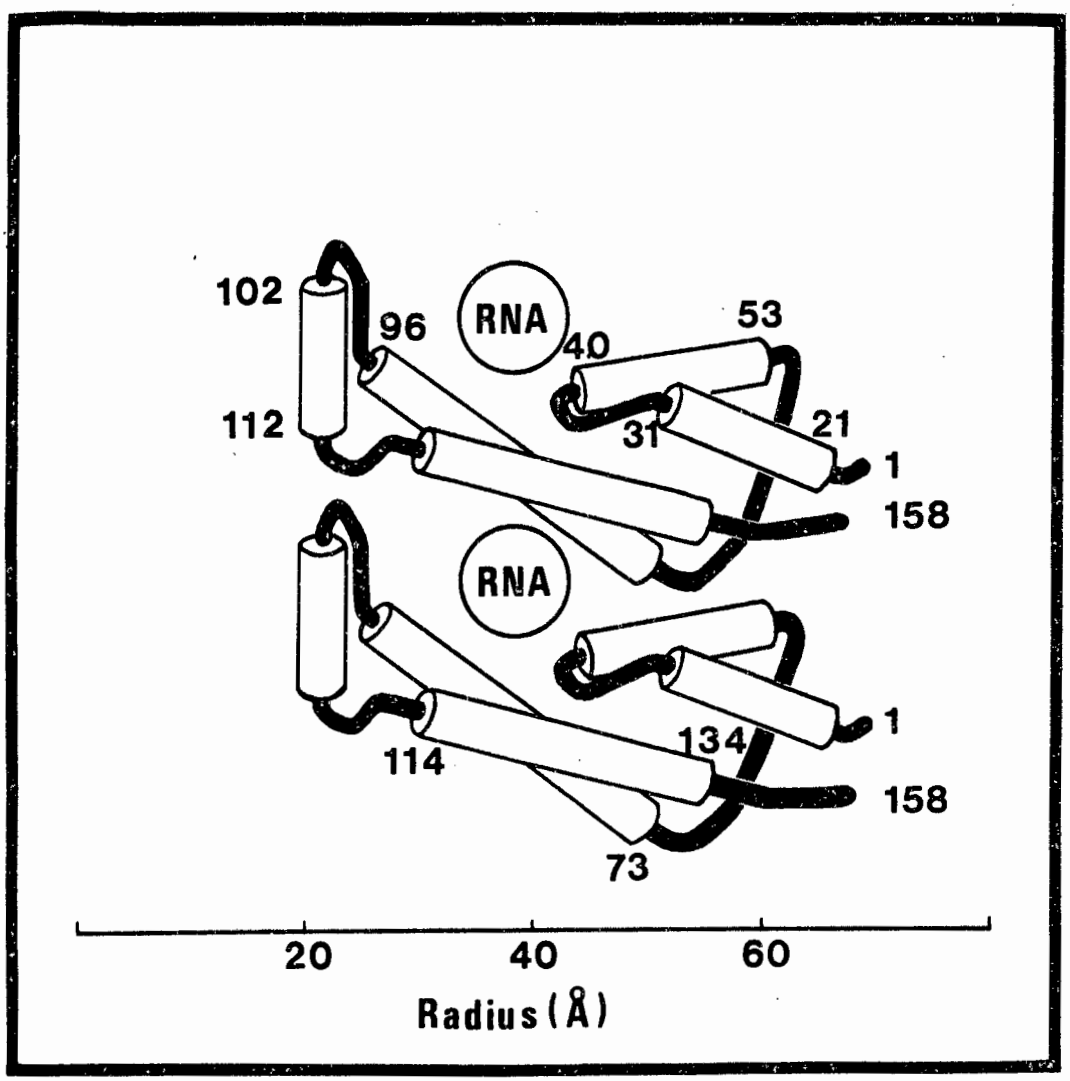

fig. 1.5 : A diagramatic representation of the secondary and tertiary structure of the TMV protein in the viral helix. The five helices are represented as cylinders (based on Stubbs et al., 1977; Argos et al., 1977).
Residues 1 and 158 are located at the surface of the subunit which is exposed at the outer periphery of the capsid. The helix consisting of residues 103-112 is hidden inside the assembled capsid. The location of the RNA relative to the protein subunits is shown in side elevation.

to the helix, the subunit undergoes a rigid body downward tilt of at least 10°, but that there is little distortion of the conformation of the subunit during this process, at least between the 60 Å and 75 Å radii (Graham and Butler, 1978). This being so, the surface conformation of the subunit in the disk and the viral helix would be practically identical.

Less is known about the conformation of the TMV protein monomer which exists only in conditions of low temperature, low ionic strength and high pH. In milder conditions it is found in the form of the A-protein, a small aggregate of three or more subunits with a sedimentation coefficient of 4S, but, as conditions become more favourable for aggregation, a series of larger discrete aggregates with sedimentation coefficients of 7-8S, 18-22S (the disk), 28-33S and larger, appear as is illustrated in fig. 1.6 (Klug and Durham, 1971). This tendency to aggregate can readily be understood in terms of the alternating patches of hydrophobic residues and salt interactions on the inter-surfaces between the subunits in the disk (Bloomer et al., 1978). Circular dichroism (CD) studies of the transition of the A-protein to the disk indicate that the gross conformation is the same for both of these species, and that the structural change which occurs is localized (Vogel, 1973). Considering that the greatest dichroic absorption change occurs between 250 nm and 295 nm and that tyrosine 139, which is near the C-terminus, participates in the formation of the disk (Ohno et al., 1972), it is likely that the C-terminal end of the TMV protein is involved in the conformational change leading to the formation of the disk (Vogel and Jaenicke, 1974). This is confirmed by the observation that the exchange of proline 156 to leucine

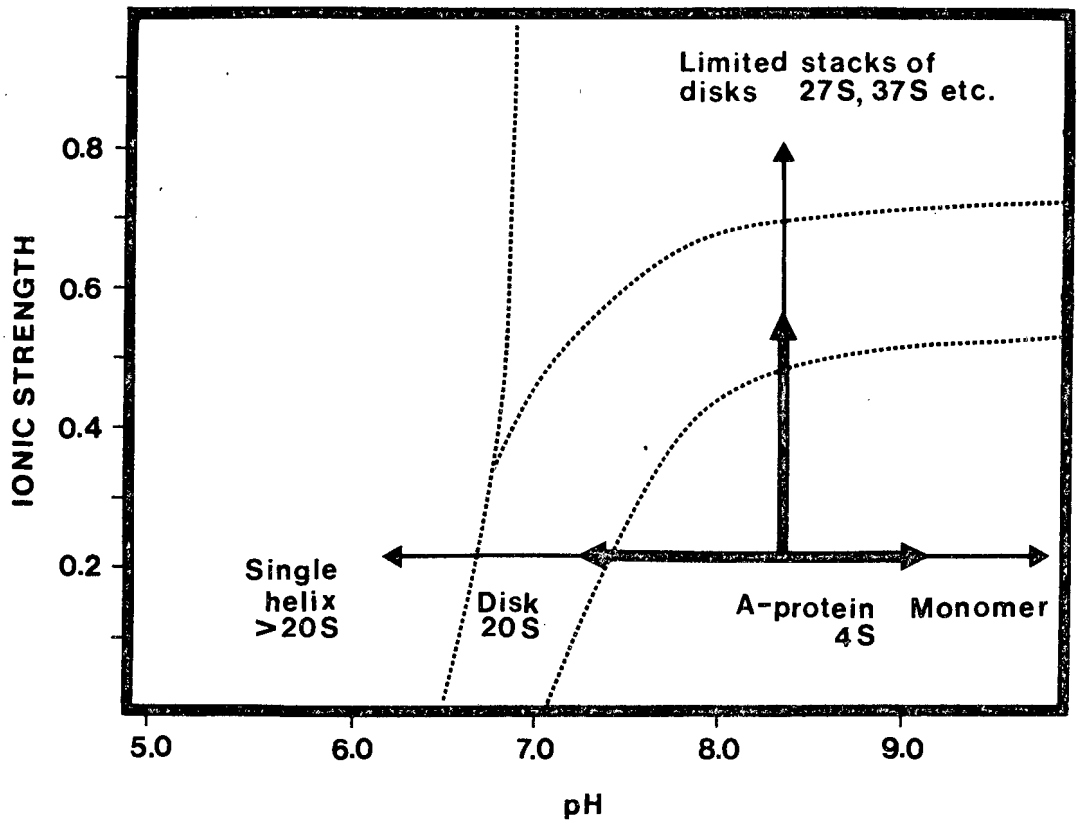

fig. 1.6 : A diagram showing the ranges over which the different modes of aggregation of TMV protein predominate in the equilibrium. The conditions were a concentration of 5mg/ml at 20° C. (A) pH-dependant aggregation to disks (thick arrows) and helices (thin arrows) at constant ionic strength. (B) Ionic strength-dependant aggregation to disks (thick arrows) and stacked disks (thin arrows) at constant pH. (Adapted from Klug and Durham, 1971; Vogel and Jaenicke, 1974.)

in the mutant Ni 1927 causes comparable dichroic absorption changes (Vogel and Jaenicke, 1974).

The chief conformational difference between the surface residues of the subunit in the viral helix and those same residues in the A-protein is therefore expected to be the configuration of the C-terminal amino acids.

1.2.3 IMMUNOLOGICAL CROSS-REACTIONS BETWEEN TMV AND THE AGGREGATES OF ITS PROTEIN MONOMER

The manner in which TMV protein changes its state of aggregation with differing physical conditions (Klug and Durham, 1971) and the evidence that the various aggregates express different conformational features of the protein subunit (Vogel, 1973; Vogel and Jaenicke, 1974) means that immunization with a single immunogen is practically impossible. Injected virus,which has partially dissociated into protein monomers or A-protein in the animal,will give rise to a heterogenous population of antibodies which will be specific for the conformations of the subunit in the virion as well as in the free protein (van Regenmortel and Lelarge, 1973). Similarly, injected A-protein can aggregate to give rise to antibodies which will recognize the viral subunit conformation (Loor, 1967; van Regenmortel and Lelarge, 1973). This serological multispecificity can be limited by the use of cross-absorbed sera, but still the results of cross-reaction experiments may be ambiguous.

RNA-free reaggregated protein rods have been shown to be serologically identical to the virus (Aach, 1959) and the 20S disk reacts with anti-

TMV sera containing only antibodies specific for the virus (van Regenmortel and Lelarge, 1973). This is consistent with the crystallographic data (Graham and Butler, 1978) that the surface residues of the subunits in the virus and in the aggregates, 20S and larger, are practically identical in conformation. They would be expected to share common antigenic determinants. The rest of the subunit is increasingly hidden in the larger aggregates and would be unavailable to react with antibody except at the extremities of the rods. In the disk these "rod extremities" would account for the reaction with the antibodies remaining in anti-TMV sera absorbed with formalinized TMV (van Regenmortel and Lelarge, 1973), but they would not be numerically significant in the case of the larger aggregates and would so escape detection.

Although the A-protein and the virus are known to show a weak cross-reaction indicative of related antigenic determinants (van Regenmortel and Lelarge, 1973), they possess several distinct antigenic determinants as well. Following absorption of anti-TMV serum with A-protein, antibodies specific for the virus still remain (Starlinger, 1955; Takahashi and Gold, 1960; van Regenmortel, 1967). Similarly absorption of anti-protein serum with virus does not remove all the antibodies that react with the A-protien (Aach, 1959; van Regenmortel, 1967; van Regenmortel and Lelarge, 1973). This phenomenon has been explained in two ways. Firstly, Rappaport et al. (1965) proposed that the TMV protein subunit had a single antigenic determinant which assumed a variety of slightly altered configurations as the result of polymerization, so giving rise to an antibody population of heterogenous specificity. This explanation is no longer tenable in view of the monomer's

calculated antigenic valence of 5-6 in the precipitin reaction (van Regenmortel and Lelarge, 1973). The alternative multideterminant model (van Regenmortel, 1967) proposes that the virus specific antibodies remaining after A-protein absorption, are directed against neotopes and that the protein specific antibodies remaining after virus absorption are directed against cryptotopes.

The phenomena described in the preceding paragraph have not been universally observed. Kleczkowski (1961) found that no detectable anti-virus activity remained when a considerable excess of A-protein (50 mg/ml) was used to absorb the anti-TMV serum. However, the use of such concentrated A-protien preparations could have resulted in considerable aggregation with the concomitant production of neotopes (van Regenmortel, 1966). Du Plessis and van Regenmortel (1977) completely inhibited the protein-anti-protein reaction in a modified phage assay with an approximately ten fold excess of intact virus. This would seem to contradict the presence of cryptotopes on the protein subunit, but the explanation may lie in the possible partial dissociation of the virus or in the presence of sufficient cryptotopes on the extremities of the virus rods.

TMV and its protein can therefore be expected to express all three types of antigenic determinants: neotopes, cryptotopes and determinants common to both the virus and the dissociated protein subunit.

1.3 IMMUNOCHEMISTRY OF SPECIFIC DERIVATIVES

1.3.1 EFFECTIVENESS OF THE APPROACH

Immunochemical studies which compare the reactivities of a protein

antigen and its derivatives which,have specifically modified residues, are able to indicate the rough locations of antigenic determinants as well as yielding information about the particular residues that make up the determinant. For this approach to be truly effective, however, the specificity of the modification and the homogeneity of the derivative are important. The modification reaction should be chosen to be specific for a single, or a limited number of amino acid side chains, and the particular derivative must be rigorously purified to homogeneity. Only results obtained with a single molecular species can be meaningful. Another consideration is whether conformational change is induced by a particular modification. The resulting change in the immunochemical reactivity of the derivative could well be independant of the specific residue that was modified and could lead to invalid conclusions in respect of the locations of antigenic determinants. Only derivatives that have suffered no conformational change can yield meaningful results. On the other hand, the absence of a change in immunochemical reactivity after a particular modification does not exclude the possibility of that residue being part of an antigenic determinant. The nature of the modification may be chemically or sterically insufficient to affect the role played by the side chain in the antigenic activity of the molecule. This problem can be overcome by modifying each amino acid side chain in more ways than one before assessing its role in the immunochemistry of the protein. The number of available modification reactions and the steric availability of the side chain to the reagent does constitute a limitation. (Atassi, 1977 b.)

Apart from the possible chemical derivatives of TMV and its protein,

there also exists a set of well characterized strains and mutants (Hennig and Wittmann, 1972), which may be regarded as derivatives for the purposes of this approach. Here modifications of the viral RNA are expressed as a limited number of amino acid exchanges in the sequence of the coat protein. The limitation is imposed by the necessity for the conservation of certain amino acid residues and sequences which are essential for biological function. Alteration of these latter amino acids results in the inactivation of the virus molecule.

Virus variants that are isolated from the field are distinguished as strains, whereas variants arising under experimental conditions are termed mutants. The spontaneous mutation rate is relatively high ($\sim 1 \times 10^{-2}$) (Sehgal and Krause, 1968), and can still be enhanced up to 30 times (Mundry and Gierer, 1958) by the use of chemical or physical means. Nitrous acid (HNO_2) which deaminates the nucleotide bases without the RNA chain breaking, is the most effective tool for inducing mutations and has been used almost exclusively to produce large amounts of mutants (Hennig and Wittmann, 1972). Other reagents which have a mutagenic effect are hydroxylamine, alkylating agents such as dimethyl suplhate (DMS), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and substances which cause the halogenation of the nucleotide bases such as N-bromosuccinimide (NBSI). The physical induction of mutants can be accomplished by X- or U.V. irradiation or by increased temperature. (Hennig and Wittmann, 1972.)

More than 300 spontaneous and chemically induced mutants of TMV have been examined for their amino acid composition and it was found that more than two-thirds of these have only one amino acid replacement

each, and that the rest have two or, quite seldomly, three exchanges each (Hennig and Wittmann, 1972). This makes them ideal for use in immunochemical comparisons with the common strain since their viability attests to the retention of biological function, and hence, to a larger extent, the natural conformation of the wild type protein subunits. The only remaining consideration is whether a particular amino acid exchange is sufficient to affect the immunochemical role played by a specific residue occupying an identical position in the sequence of the wild type protein.

The same criteria apply to the strains, although they usually have more amino acid exchanges, probably due to a number of single mutation steps that have accumulated with time (Hennig and Wittmann, 1972).

1.3.2 SEROLOGICAL STUDIES WITH CHEMICAL DERIVATIVES AND POINT MUTANTS OF TMV

Specific derivatives of TMV have been obtained in two ways. Some workers have chemically modified the side chains of certain amino acids of the proteins of the virus (Malkiel, 1952; Price, 1954; Anderer and Handschuh, 1963; and Staab and Anderer, 1976), or released amino acids from the C-termini of the protein in the viral helix (Harris and Knight, 1952 and 1955; von Sengbusch and Wittmann, 1965). Others have exploited the usefulness of the mutants of TMV as a probe for the quaternary and antigenic structure of the protein subunit in the intact virus (von Sengbusch and Wittmann, 1965; von Sengbusch, 1965; and van Regenmortel, 1967).

The interpretation of the results of the serological studies with

chemically derivatized TMV requires that three criteria be considered: the number of different residues in the TMV protein modified simultaneously, the extent of the modification reaction in as far as the number of protein subunits modified per virus particle is concerned and the effect of a particular modification on the native conformation of the protein subunit in the virus. For example, Malkiel (1952) concluded that derivatives with phenol groups modified by the attachment of an azo-arsanilic group, exhibited serological behaviour identical to the untreated TMV, while Price (1954) found that the diiodo-tyrosine derivative of TMV had undergone a significant change in serological specificity. In view of the finding that none of the tyrosine or lysine residues of the intact virus could be substituted by dinitrophenyl groups (Anderer and Handschuh, 1963), it was suggested (von Sengbusch, 1965; van Regenmortel, 1966) that tyrosine residues near the surface of the subunit in the viral helix could be available to the small iodine molecule, but not to the larger dinitrophenyl group or the azo-arsanilic group. A more simple explanation would be that the modification reaction at 56°C to produce the diiodo-tyrosine derivative (Price, 1954) caused a certain amount of denaturation and conformational change which made normally hidden residues available and caused the change in serological specificity. The modification reaction of Malkiel (1952) may not have affected sufficient tyrosine residues per virus particle to change serological behaviour.

Similarly, Staab and Anderer (1976) found that when dimethyl sulphate was used to methylate the residues: cysteine 27; tyrosine 2,70 (?), 72, 139; lysine 68; and the surface carboxyl groups of TMV, it still showed a strong cross-reaction with untreated TMV so long as the

degree of methylation did not affect the conformation of the protein subunits. It is not clear whether this modification was inadequate to affect the immunochemical role played by any of the above mentioned residues, or whether an insufficient number of residues of the intact virus were modified before conformational change caused the disintegration of the virus particles.

There are fewer variables to consider in the serological studies with point mutants of TMV. Each mutant has a limited number of characterized amino acid exchanges and each exchange is expressed in every subunit of the virus. Nevertheless, the possibility of localized conformational modulations due to the presence of the new amino acids can not be ruled out (von Sengbusch, 1965).

Von Sengbusch (1965) compared the serological reactivity of twenty-four mutants of TMV with known amino acid exchanges (Wittmann, 1962 and 1964; von Sengbusch, 1965) with that of TMV. He used the technique of cross-absorption of anti-mutant and anti-TMV sera in conjugation with quantitative precipitin assays. This work was extended by van Regenmortel (1967), who used the intragel cross-absorption technique to compare a further sixteen chemically induced mutants (Funatsu and Fraenkel-Conrat, 1964) with TMV vulgare. Some of these mutants had the same amino acid exchanges as those used by von Sengbusch (1965) while others had different amino acid exchanges in the same position or amino acid exchanges in different positions on the polypeptide chain. With the exception of the mutant 171 (proline in position 156 replaced by leucine), van Regenmortel (1967) found that, while a serum against the U2 strain was able to differentiate between some of the mutants and TMV, a TMV antiserum and a

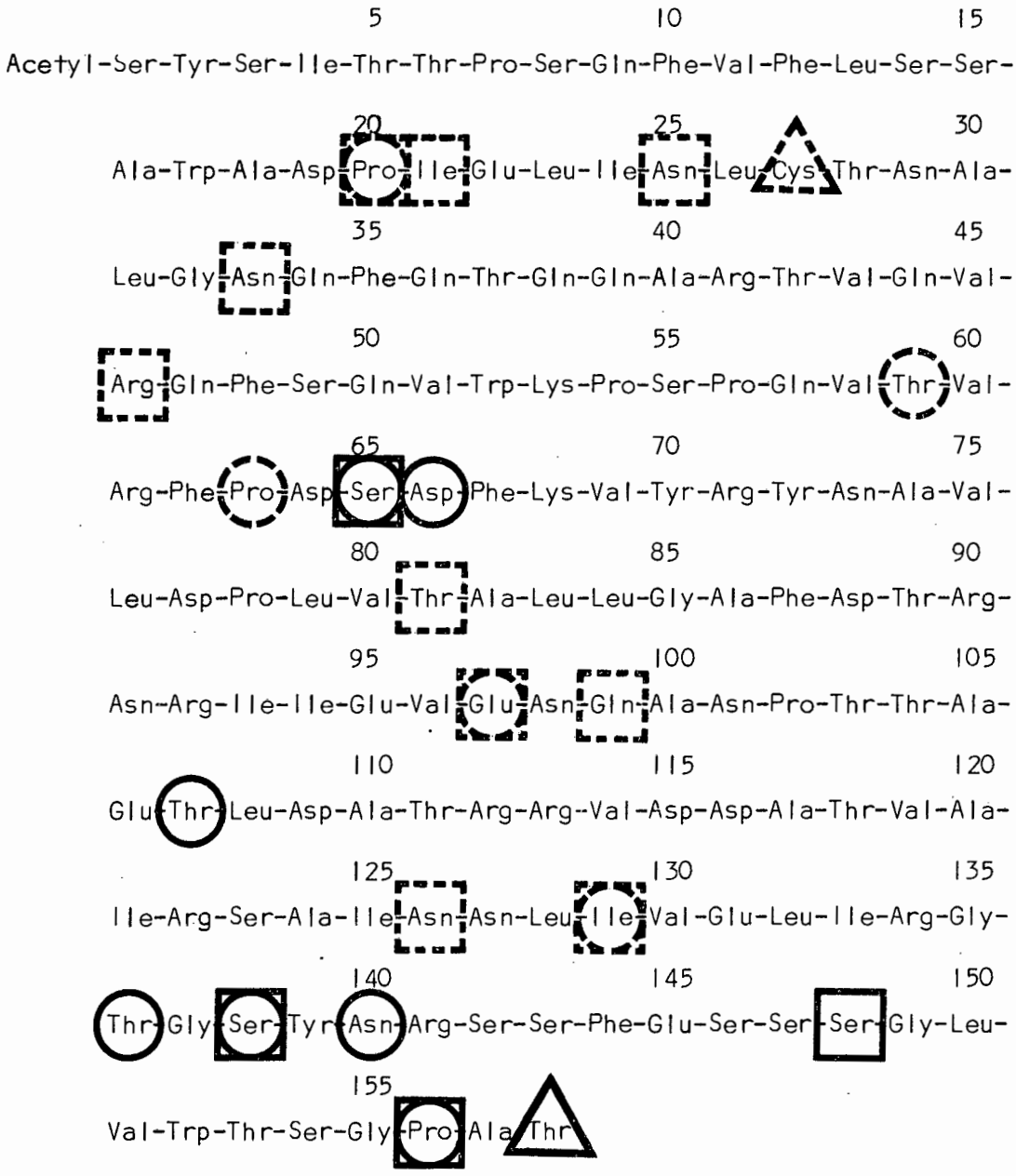

fig. 1.7 : The polypeptide chain of TMV protein. Amino acid exchanges studied by von Sengbusch (1965) and van Regenmortel (1967) are indicated by circles and squares respectively. Chemically altered residues in derivatives studied by Price (1954), Harris and Knight (1955) and Anderer and Handschuh (1963) are indicated by triangles. Serologically distinguishable derivatives are indicated by solid line symbols, while those derivatives that are serologically indistinguishable are indicated by dashed symbols. (Adapted from van Regenmortel, 1967.)

number of anti-mutant sera could not. Mutants that could not be differentiated from TMV by the U2 antiserum were regarded as being serologically identical to TMV vulgare.

The significant results and conclusions from the serological comparison of both chemical derivatives and point mutants with TMV vulgare are summarized in fig. I.7 and table I.I. Serologically indistinguishable modifications or amino acid exchanges are associated with those residues which are unlikely to be involved in antigenic determinants. Modifications or amino acid exchanges that are serologically distinguishable indicate residues that are, either directly involved in, or, have a conformational modulating affect on an antigenic determinant.

The antigenic role of the N-terminal amino acids, which are on the surface of the TMV protein subunit in the viral helix, cannot be assessed in this approach due to the lack of chemical derivatives and amino acid exchanges in mutants in this region. However, neither the replacement of proline in position 20 by leucine (von Sengbusch, 1965; van Regenmortel, 1967) or threonine (van Regenmortel, 1967), nor the replacement of isoleucine in position 21 by threonine or valine (van Regenmortel, 1967), is serologically distinguishable as would be expected from the location of these amino acids at the beginning of the LS α-helix which lies approximately radially to the axis of the viral rod and is thus hidden between adjacent protein subunits (Champness et al., 1976; Stubbs et al., 1977; Bloomer et al., 1978). The replacement of asparagine in position 25 by serine (van Regenmortel, 1967) and the chemical modification of cysteine in position 27 by, either the abolition of its sulphydryl group with

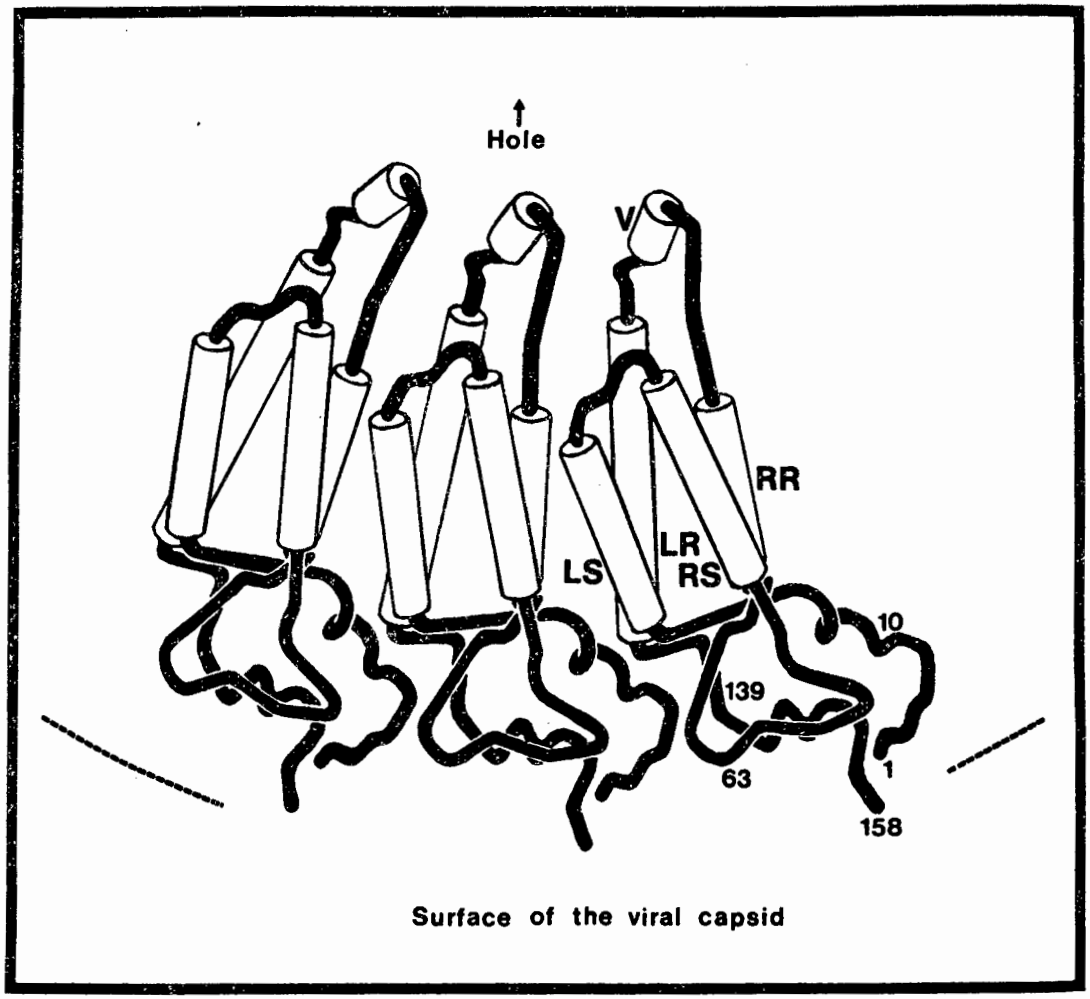

fig. I.8 : A diagramatic representation of three protein subunits in the TMV helix viewed in plan. The five α-helices are represented as cylinders and the N- and C-terminal residues are located at the surface of the subunits (Stubbs et al., 1977; Argos et al., 1977; and Bloomer et al., 1978). The nomenclature of the helices (right hand side) is that of Champness et al. (1976) and Stubbs et al. (1977).

iodine in potassium iodide at pH 7,0 (Price, 1954), or carbamidomethylation (Anderer and Handschuh, 1963), or reaction with mercuribenzoate (Anderer and Handschuh, 1963), are also serologically indistinguishable due to their location in the LS α-helix (Champness et al., 1976; Bloomer et al., 1978).

Mutants with the amino acid exchanges, serine in place of asparagine in position 33, and lysine or glycine in place of arginine in position 46, were also found to be serologically indistinguishable from TMV vulgare (van Regenmortel, 1967). These residues are located near the 50 Å radius in the protein subunit (Bloomer et al., 1978) and would be hidden in the viral helix.

The amino acids between residues 59 and 66 form part of an extended chain across the surface of the protein subunit in the viral helix (Bloomer et al., 1978; Stubbs et al., 1977) and so are available to antibodies. Of these amino acids, the replacements of threonine in position 59 by isoleucine, and proline in position 63 by serine, are not serologically distinguishable (von Sengbusch, 1965), while the replacements of both serine in position 65 (von Sengbusch, 1965; van Regenmortel, 1967) and aspartic acid in position 66 (von Sengbusch, 1965) by glycine, are serologically distinguishable. In view of their surface locations, these latter two residues are likely to be part of an antigenic determinant.

After residue 66 the polypeptide chain continues to turn away from the viral surface, inwards towards the centre of the viral helix and develops into the RR α-helix (Stubbs et al., 1977; Bloomer et al., 1978). The replacement of a residue in this α-helix, threonine in position 81,

by alanine, is not serologically distinguishable (van Regenmortel, 1967).

The next two amino acid replacements, glycine for glutamic acid in position 97 (von Sengbusch, 1965; van Regenmortel, 1967) and arginine for glutamine in position 99 (van Regenmortel, 1967), are found near the central hole in the virus rod at approximately 25 Å radius (Stubbs et al., 1977), and they too are serologically indistinguishable.

A short length of α-helix, from approximately residue 103-112, is found parallel to the viral axis and lining the central hole of the virus rod (Stubbs et al., 1977). Surprisingly, an amino acid exchange in this V α-helix, that of methionine for threonine in position 107, is serologically distinguishable (von Sengbusch, 1965). As this residue is too deeply hidden to be part of any viral antigenic determinant, the amino acid exchange at position 107 must be assumed to cause a conformational change in the protein subunit that affects those residues on the surface of the viral rod.

Subsequent to the V α-helix, as the polypeptide chain changes direction back to the viral surface (Stubbs et al., 1977), two amino acid replacements, serine for asparagine in position 126 (van Regenmortel, 1967) and valine for isoleucine in position 129 (von Sengbusch, 1965; van Regenmortel, 1967), were found to be serologically indistinguishable.

The polypeptide chain continues along the LR α-helix, which extends from residues 114-134, and emerges on the viral surface (Stubbs et al.,

1977; Bloomer et al., 1978). Three amino acid replacements in this region have been found to be serologically distinguishable. They are the replacement of threonine by isoleucine in position 136 (von Sengbusch, 1965), the replacement of serine by phenylalanine in position 138 (von Sengbusch, 1965; van Regenmortel, 1967) and the replacement of asparagine by lysine in position 140 (von Sengbusch, 1965). In view of their closeness to the viral surface, these residues are likely to be part of an antigenic determinant in this region. However, the alternate possibility, that they exert their influence by modulating the conformation of the projecting C-terminus, cannot be excluded.

The same arguments can be used in the case of the serologically distinguishable amino acid exchange at position 148, where serine is replaced by threonine (van Regenmortel, 1967). This residue is on the viral surface, but may exert its influence by altering the conformation of the C-terminal amino acids.

Von Sengbusch (1965) found that two mutants, Ni 1927 and Ni 1688, which share a common amino acid exchange in position 156, where a proline residue is replaced by leucine, still reacted with absorbed TMV antisera after all the TMV specific antibodies had been removed. The mutant Ni 1927 also formed more precipitate with TMV antisera in quantitative precipitin tests than TMV vulgare itself did. Similar phenomena were noted by van Regenmortel (1967) with the mutant 171 which also has the proline residue in position 156 replaced by leucine.

The C-terminal threonine of the TMV protein is readily released, even from the intact virus, by the action of carboxypeptidase A (Harris and

Knight, 1952), but no further amino acids are liberated by the enzyme due to the location of a proline residue in position 156. The symptoms and infectivity of the carboxypeptidase treated virus (C T virus) in Turkish tobacco are unaltered (Harris and Knight, 1955). Antisera raised in rabbits to C T virus and to untreated TMV showed strong mutual cross-reactions in precipitin tests, but if the anti-TMV sera were absorbed with C T virus, they were still able to precipitate TMV although the heterologous reaction was prevented. Similarly C T virus antisera absorbed with TMV were able to precipitate C T virus, but not TMV. (Harris and Knight, 1955.) This change in specificity, that occured when the threonine was removed, implicated the C-terminal residue of the subunit in the virus as part of an antigenic determinant. When this residue was removed, antibodies specific for this determinant could no longer recognise it.

The treatment of intact virus of the TMV mutant Ni 1927, which has proline in position 156 replaced by leucine, with carboxypeptidase A results in the release of all three C-terminal amino acid residues (Leu-Ala-Thr) from each subunit of the virus (von Sengbusch and Wittmann, 1965). Similar to the change in specificity between C T virus and TMV, Ni 1927 antiserum absorbed with Ni 1927 virus still reacted with the carboxypeptidase treated Ni 1927 virus derivative (von Sengbusch and Wittmann, 1965). More suprisingly, this derivative reacted more strongly with TMV antiserum than either Ni 1927 virus, TMV or C T virus did (von Sengbusch, 1965). Also, a TMV antiserum absorbed with Ni 1927 virus still reacted with the carboxypeptidase treated Ni 1927 virus derivative (von Sengbusch and Wittmann, 1965).

It is arguable whether the removal of a single amino acid residue from

the C-terminus of the TMV protein would cause a drastic conformational change in the whole subunit as the biological properties of the C T virus are the same as those of TMV (Harris and Knight, 1955), but a localized conformational change affecting only the viral subunit surface cannot be excluded. However, when the observation that untreated Ni 1927 virus could still react with TMV antiserum after absorption with TMV (von Sengbusch and Wittmann, 1965) is taken into account, these results point to the existence of heterospecific antibodies in TMV antisera (van Regenmortel, 1978).

Van Regenmortel (1966, 1967) has proposed that in addition to the antigenic determinant at the C-terminus, there exists an immunogenic determinant that cannot react with its specific antibody because of steric hindrance by the proline residue in position 156. If this proline were replaced by the less rigid leucine, or if all three of the C-terminal amino acids were removed, the "non-reactive" antigenic determinant would become exposed and so be able to react with its antibody (von Sengbusch and Wittmann, 1965).

Loor (1971), however, suggested that the heterospecific antibodies in TMV antisera have affinities for the immunogen that are too low to measure, but, by chance, have higher affinities for cross-reacting antigens.

A third explanation can be formulated in terms of the antibody composition of the antisera as well as the structure of the viral subunit. It is well known that antisera obtained after intramuscular injection of TMV (or other relatively unstable viruses) contain both antibodies specific for cryptotopes of free protein subunits, which have arisen

by breakdown of the virus, as well as antibodies specific for viral antigenic determinants (van Regenmortel, 1966; van Regenmortel and Lelarge, 1973; von Wechmar and van Regenmortel, 1968). It is also known that free TMV protein can exist in two conformational forms with different C-terminal configurations (Vogel, 1973; Vogel and Jaenicke, 1974) and that one of these configurations is very similar to the C-terminal configuration of the 20S disk (Vogel, 1973), and hence the protein subunit in the virus (Graham and Butler, 1978). The virus-like C-terminal configuration of the free protein is likely to have the same antigenic determinant as the C-terminal region of the polypeptide chain in the intact virus and it is probably located at the C-terminus of each viral subunit (Harris and Knight, 1955; van Regenmortel, 1966). If the other C-terminal configurational form of the free protein had a cryptotope further from the C-terminus, say in the region of residues 150-155 or even overlapping the viral determinant at position 156, TMV protein antibodies (in TMV antisera) specific for this cryptotope would not be able to react properly with it on the intact virus, because of the steric hindrance of the C-terminal amino acids and especially the proline in position 156. These antibodies could, therefore, not be removed from the antiserum by absorption with intact TMV. Although low affinity antibodies specific for such a cryptotope could react with mutant viruses which have the proline in position 156 replaced by a more flexible amino acid, absorption with these mutants may still not accomplish the complete removal of the high affinity antibodies. Only derivatives lacking the C-terminal amino acids (such as carboxypeptidase A treated Ni 1927 virus) could react fully (von Sengbusch and Wittmann, 1965) with all the antibodies specific for such a cryptotope. This argument, based on the existence of two conformational forms of the subunit, will be further developed in the Discussion (3.2).

Table I.1: DERIVATIVES OF TMV : RESULTS AND CONCLUSIONS

	Derivative	Residue(s) modified	Conformational change	Serologically distinguishable	Residues in a determinant	Residues not in a determinant	Reference
1.	Acetyl TMV Benzal TMV Acetyl glycine treated TMV Carbenoxy TMV Reduced carbenoxy TMV p-chlorobenzoyl TMV	amino, phenol and indole groups	?	No	-	?	Maklei, 1952
2.	Diiodo-tyrosine TMV	Cys 27 Tyr	?	Yes	?	-	Price, 1954
3.	Completely dinitrophenylated TMVP	Tyr, lys	Yes	Yes		-	Anderer and Handschuh, 1963
4.	E-dinitrophenyl-lysine TMVP	Lys 53 68	Yes	?	-	-	
5.	Methylated TMV	Cys 27 Tyr 2,70(?), 72,139 Lys 68 surface carboxyl groups	viral helix structure	No	-	?	Staab and Anderer, 1976.
6.	SH abolished TMV	Cys 27	?	No	-	Cys 27	Price, 1954
7.	S-carbamidomethyl TMV	Cys 27	viral helix structure	No	-	Cys 27	Anderer and Handschuh, 1963
8.	S-mercuribenzoate TMV	Cys 27	viral helix structure(?)	No	-	Cys 27	
9.	Carboxypeptidase treated TMV	Thr 158	viral helix structure and infectivity	Yes	Thr 158	-	Harris and Knight, 1955
10.	Carboxypeptidase treated N11927	Thr 158 Ala 157 Leu 156	viral helix structure	Yes	Thr 158	-	von Sengbusch and Wittmann, 1965.
11.	Mutants - single						
	218 430 N1118	Pro 20	viral helix structure and infectivity	No	-	Pro 20	van Regenmortel, 1967 " von Sengbusch, 1965
	330 415	Ile 21	"	No	-	Ile 21	van Regenmortel, 1967
	357	Asn 25	"	No	-	Asn 25	" "
	403	Arg 46	"	No	-	Arg 46	" "
	414 Fu243	Ser 65	"	Yes	Ser 65	-	" " von Sengbusch, 1965
	N1102 N1116	Asp 66	"	Yes	Asp 66	-	" "
	N1109	Glu 97	"	No	-	Glu 97	" "
	427	Gln 99	"	No	-	Gln 99	van Regenmortel, 1967
	A 14	Ile 129	"	No	-	Ile 129	van Sengbusch, 1965
	N12032	Thr 136	"	Yes	Thr 136	-	" "
	E 66 CP415 N11151	Asn 140	"	Yes	Asn 140	-	" "
	419	Ser 148	"	Yes	Ser 148	-	van Regenmortel, 1967
	N11927	Pro 156	"	Yes	Pro 156	-	von Sengbusch, 1965
	- double						
	284	Arg 46, Glu 97	"	No	-	Arg 46, Glu 97	van Regenmortel, 1967
	344	Thr 81, Ser 148	"	Yes	Ser 148	Thr 81	" "
	369	Arg 46, Ile 129	"	No	-	Arg 46, Ile 129	" "
	371	Ser 138, Ser 148	"	Yes	Ser 138, Ser 148	-	" "
	384	Asn 25, Thr 81	"	No	-	Asn 25, Thr 81	" "
	N1445	Ile 129, Ser 138	"	Yes	Ser 138	Ile 129	von Sengbusch, 1965
	N1458 N1470	Thr 59, Ile 129	"	No	-	Thr 59, Ile 129	" "
	N1630 N1725	Thr 107, Ile 129	"	Yes	Thr 107	Ile 129	" "
	N11045 N11196 N11234	Pro 63, Ile 129	"	No	-	Pro 63, Ile 129	" "
	N11688	Pro 63, Pro 156	"	Yes	Pro 156	Pro 63	" "
	- triple						
	171	Asn 25, Thr 81, Pro 156	"	Yes	Pro 156	Asn 25, Thr 81	van Regenmortel, 1967
	378	Asn 25, Asn 33, Asn 126	"	No	-	Asn 25, Asn 33, Asn 126	" "

I.3.3 A SEROLOGICAL STUDY WITH THE PROTEINS OF TMV MUTANTS AND ANTISERA AGAINST TMV PROTEIN

Use of the sensitive modified phage technique, developed by Haimovich et al. (1970), was made by Du Plessis and van Regenmortel (1977), who attempted to inhibit the inactivation of TMV protein - T4 phage conjugates by TMV protein antiserum with protein derived from the mutants 371 (serine in positions 138 and 148 replaced by phenylalanine) and CP 415 (asparagine in position 140 replaced by lysine). They found no significant differences in the degree of inhibition caused by the mutants and TMV vulgare protein.

If the discriminating powers of the modified phage assay are comparable to those of precipitin tests, residues 138, 140, and 148 are unlikely to form part of any antigenic determinant of the free protein, as the same residues were shown to be serologically distinguishable in the intact virus (von Sengbusch, 1965; van Regenmortel, 1967). It must be borne in mind, however, that each virion consists of 2130 protein subunits, and this may cause an amplification of any change in serological specificity in the intact virus that may go undetected in the free protein.

I.3.4 VALIDITY OF THE RESULTS OF THIS APPROACH

Just as the absence of a change in the immunochemical behaviour of a derivative is not conclusive of the non-participation of the modified residue in an antigenic determinant, neither is a change in immunochemical reactivity proof positive of the involvement of that residue unless it is possible to completely exclude the change of a minor conformational change due to the modification (Atassi, 1977 b). Not all of the amino acid residues implicated to be in antigenic determinants

of the virus by this approach are likely to lie directly on the approximately 700 $\mathring{A}^2$ surface of the subunit in the viral helix (von Sengbusch, 1965). Indeed, in the 4,0 $\mathring{A}$ model (Stubbs et al., 1977), shown in figure 1.5, residue 107, which is serologically distinguishable when threonine is replaced by methionine (von Sengbusch, 1965), lies buried near the core of the viral rod. It is possible that some of the modifications or exchanges of particular residues affect the immunochemical behaviour of the derivative indirectly by a subtle modulation of the conformation of the whole molecule or its surface region (von Sengbusch, 1965). It is necessary to confirm or correct the findings of this approach with the results from the other approaches.

In any event, it is not possible to derive the complete antigenic structure of a protein from this approach alone, as not all the amino acids can be suitably modified. All that can be achieved is a broad type of delineation deduced from the involvement or otherwise of certain residues in antigenic determinants. (Atassi, 1977 b.)

1.4 IMMUNOCHEMISTRY OF PEPTIDES

1.4.1 THE VALUE OF THE APPROACH

This approach is based on the likely ability of an antigenic region to react with the antibody specific for it when it is isolated free from the intact antigen (Atassi, 1977 b). Atassi and Saplin (1968) have suggested that a peptide in solution will exist in a number of unfolded conformational states and that very few of these conformational states will approach the mode of folding of that region in the native protein. Thus the need for the large molar exesses of peptide in immunochemical reactions was explained.

Circular dichroism (C D) and optical rotatory dispersion (ORD) studies on the conformation of peptides derived from Sperm Whale myoglobin by cleavage at the proline (Singhal and Atassi, 1970) and arginine residues (Atassi and Singhal, 1970) showed that, although these peptides contain a considerable helical content, it is much less than is expected for that region in the native protein. The addition of methanol to the solutions increased the helical content of the peptides, but it still remained only a fraction of the helical content of the equivalent region of the native protein. Peptides that were large enough to make long range interactions feasible were found to have the most stabilized helical structure. They also found that an increase of folded structure was accompanied by a decrease in the molar excess of peptide required for maximum immunochemical reactivity. Thus the greatest limitation of this approach lies in the unrelatedness of peptide structure to the structure of the same region in the native protein (Atassi, 1977 b) and in the necessity for the native mode of folding for the proper reaction of an antigenic determinant with antibody raised to that determinant in the intact protein antigen (Atassi, 1967; Atassi and Saplin, 1968; Habeeb and Atassi, 1971). On the other hand, the antibody may be able to induce a peptide, which contains an antigenic determinant, to assume the correct mode of folding necessary to fit the antibody combining site (Atassi, 1975).

The other limitations of this approach may be minimized with suitable precautions.

The use of a variety of cleavage procedures, which will produce a range of overlapping peptides, and reference to the results of the preceding

approach which utilized derivatives of the whole antigen, can reduce the possibility of scission of an antigenic determinant during the fragmentation of the protein (Atassi,1975,1977 a, 1977 b. Information gained by indiscriminate cleavage at multiple locations will be limited, confusing, and possibly erroneous (Atassi, 1977 b). Unfortunately, in the case of TMV and its protein subunit, peptide studies (Anderer, 1963 b; Benjamini et al., 1964) preceded the serological studies with the mutants (von Sengbusch, 1965; van Regenmortel, 1967).

A variety of overlapping peptides will also minimise the chance of bulky amino acid side chains on residues adjacent to an antigenic determinant sterically obstructing antibody binding (Atassi, 1977 b). This phenomenon has only been observed with synthetic peptides (Koketsu and Atassi, 1974 a, b; Atassi and Pai, 1975), but the principle applies to any cleavage site.

Care must be exercised in chemical cleavage operations that may be accompanied by other modifications, or cleavage side reactions, so that the resulting peptides are not internally modified (Atassi, 1977 b). Also, all peptides utilized in immunochemical work must be rigourously purified, as trace impurities that are immunologically active, will become significant in the large molar excesses of peptides used (Atassi, 1977 a, 1977 b).

From the foregoing, it can be appreciated that, although this approach can yield valuable information about the locations of antigenic determinants, the results with unreactive peptides must be confirmed by the other approaches before it can be certain that they do not contain antigenic regions (Atassi, 1977 b).

Unless the limitations of this approach are taken into account in both the design and analysis of experiments, and unless the results obtained are substantiated by other independant approaches, the conclusions that can be reached will be incomplete and less valuable to the understanding of the immunochemistry of the antigen as a whole. (Atassi, 1977 b.)

1.4.2 THE IMMUNOCHEMICAL RELATIONSHIP OF PEPTIDES OF TMV PROTEIN TO THE INTACT VIRUS

Anderer (1963 b) prepared twenty peptides of tryptic, chymotryptic and peptic origin (fig. 1.9) which accounted for the whole sequence of protein of TMV vulgare. The regions corresponding to the sequences 1-46, 113-122, 123-134 and 142-158 were covered by overlapping peptides. All the peptides were tested for their ability to inhibit the precipitin reaction between TMV and its antiserum in the equivalence zone close to the zone of antigen excess. He found that the inhibition of precipitation caused by a particular peptide was dependent on the concentration of the peptide and that a considerable molar excess of the peptide was required to achieve a significant inhibition effect.

Of the twenty peptides, nine failed to cause any inhibition and four peptides, residues 47-61, 69-71, 91-92 and 114-122 caused a slight inhibition effect (maximum 5-10%) which approximated the standard deviation of the experiment and was thought to result from temporary, non-specific reaction with the antibody (Anderer, 1963 b). Seven peptides, representing 15-25 % of the sequence of the TMV protein, caused a significant degree of inhibition of the TMV-anti-TMV reaction. These peptides corresponded to the following regions of the primary

Residues:					
	1 - 10	C,P.		93 - 112	T8.
	1 - 41	T1.		113 - 122	T9.
	17 - 23	P.		114 - 122	T.
	18 - 23	C.		123 - 134	T10.
	42 - 46	T2.		123 - 128	P.
	47 - 61	T3.		129 - 131	P.
	62 - 68	T4.		135 - 141	T11.
	69 - 71	T5.		142 - 158	T12.
	72 - 90	T6.		151 - 158	C,P.
	91 - 92	T7.		153 - 158	C.

Fig. 1.9: The twenty peptides used by Anderer (1963 b) in precipitation inhibition tests. Each peptide is identified by the corresponding sequence in TMV protein. The origin of the peptides is indicated as follows: P - obtained by hydrolysis with pepsin, C - obtained by hydrolysis with chymotrypsin and T - obtained by hydrolysis with trypsin. The tryptic peptides are numbered according to their position from the N-terminus of the protein.

sequence of TMV protein: residues 18-23, 62-68, 123-134, 129-131, 142-158, 151-158 and 153-158 (Anderer, 1963 b). All the possible two- and three-component mixtures of the reactive peptides were also tested. None of these mixtures caused inhibition equal to the sum of the inhibitions caused by the individual peptide components (Anderer, 1963 b). A selection of these results are presented in fig. 1.10. It was also found that some of the peptides formed aggregates as their concentration increased and this was suggested as the reason for the tailoff in inhibition caused by certain peptides (residues 142-158, 151-158 and 153-158) above an optimal concentration (Anderer, 1963 b).

The greatest inhibition of the reaction between TMV and its antiserum was caused by the peptide corresponding to the sequence 18-23 (just over 11 µmoles of the peptide caused approximately 40% inhibition),but two other peptides (residues 1-41 and 17-23) which overlap this region were found to be inactive (Anderer, 1963 b). Anderer (1963 b) suggested that aggregation of these latter peptides prevented their detection. On the basis of Braunitzer's findings that hot trichloroacetic acid and hydroxylamine preferentially split the aspartic acid-proline peptide bond between residues 19 and 20 (Schramm and Braunitzer, 1953; Braunitzer, 1955; Braunitzer, 1956; Ramachandran and Narita, 1958), Anderer (1963 b) argued that the region comprising residues 18-23 was on the surface of the viral subunit and thus available for reaction with antivirus antibodies. In actuality this region is almost 20 Å from the projecting C-terminus and forms the bend at the beginning of the LS α-helix (Bloomer et al., 1978).

The mutants Ni 118 (Wittmann, 1962) and 218 and 430 (Funatsu and Fraenkel-Conrat, 1964) which have the proline residue in position 20 replaced

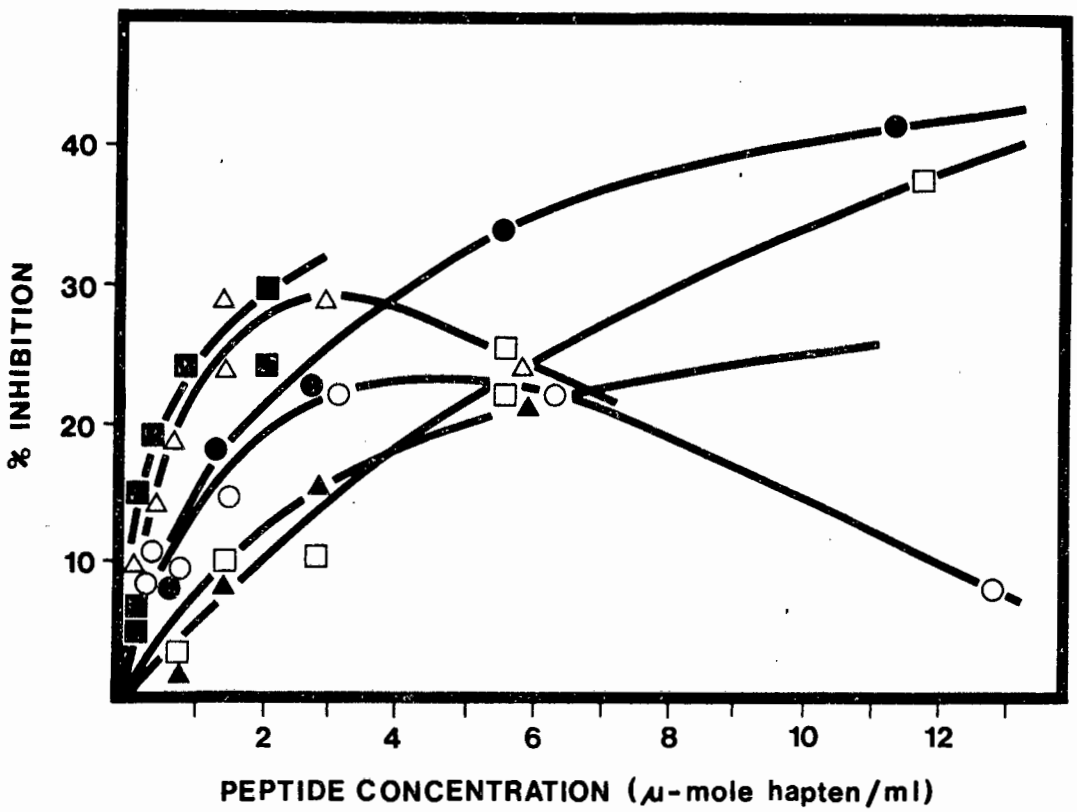

fig. I.10 : Hapten inhibition of the precipitin reaction of TMV and its antiserum by peptides derived from the TMV protein. 0,1 ml of a 1/64 dilution of TMV antiserum was added to 1 ml of peptide solution and incubated at 37°C for 2 hours before 0,1 ml of a TMV solution (1,5 mg/ml) was added. This was incubated at 37°C for an hour and then for 4 days at 4°C with intermittent shaking. After low speed centrifugation the precipitates were washed and quantitated by the Folin reaction. The amount of precipitate formed in the absence of inhibitor was compared to the amount of precipitate formed with increasing concentrations of each peptide. Peptides corresponding to the following regions in the protein were found to be immunologically active: residues 18-23(●); 62-68(□); 123-134(■); 129-131(▲); and 142-158(○). Mixtures of the active peptides did not cause inhibition equal to the sum of the individual peptides: residues 18-23 + 142-158(△). (Anderer, 1963 b.)

by leucine or threonine and the mutant 415 (Funatsu and Fraenkel-Conrat, 1964) which has the isoleucine residue in position 21 replaced by valine, were not serologically distinguishable from TMV vulgare either (von Sengbusch, 1965; van Regenmortel, 1967). These considerations diminish the likelihood of this region containing part of, or a whole antigenic determinant of the intact virus.

The inhibition of the TMV-anti-TMV reaction caused by the peptides corresponding to the sequences 123-134 and 129-131 (Anderer, 1963 b) is also in doubt as these residues make up part of the LR α-helix, which is not exposed on the surface of the viral subunit (Bloomer et al., 1978). Neither were the mutants 378 (Funatsu and Fraenkel-Conrat, 1964), which has the asparagine residue in position 126 replaced by serine, and Ni 458, Ni 470, Ni 1045, Ni 1196, Ni 1234 (Wittmann, 1962), A 14 (Melchers unpublished data - von Sengbusch, 1965) and 369 (Funatsu and Fraenkel-Conrat, 1964), which have the isoleucine residue in position 129 replaced by threonine or valine, serologically distinguishable from TMV vulgare (von Sengbusch, 1965; van Regenmortel, 1967). The discrepancies between the results of the derivative approach and the peptide approach concerning the immunological activity of the regions containing the residues 18-23 and 123-134 have led van Regenmortel (1967) to suggest that some of Anderer's effects (1963 b) were not specific.

The findings, that the mutants FU 243 (Wittmann, 1964) and 414 (Funatsu and Fraenkel-Conrat, 1964), which have the serine residue in position 65 replaced by glycine, and Ni 102 and Ni 116 (Wittmann, 1962), which have the aspartic acid residue in position 66 replaced

by glycine, are serologically distinguishable from TMV vulgare (von Sengbusch, 1965; van Regenmortel, 1967), confirmed that the inhibition of the TMV-anti-TMV reaction, that Anderer (1963 b) found with the peptide corresponding to the sequence 62-68, was likely to be specific. This region of the TMV protein sequence is also exposed on the surface of the viral subunit in the 2,8 Å model of Bloomer et al. (1978). In view of von Sengbusch's (1965) demonstration of the inability of the replacement of the proline residue in position 63 by serine, to affect the serological behaviour of the mutants Ni 1045, Ni 1196 and Ni 1234 (Wittmann, 1962) compared to TMV vulgare, it is likely that the residues comprising part of, or a whole antigenic determinant are accommodated in the latter half of the peptide.

The practically identical inhibition curves caused by the three peptides corresponding to the sequences 142-158, 151-158 and 153-158 (Anderer, 1963 b) encourage the conclusion that the entire immunological activity of these peptides in the TMV-anti-TMV system is expressed in the region containing the residues 153-158. This is supported by the change in serological behaviour resulting from the removal of the C-terminal residues as demonstrated by Harris and Knight (1955) and von Sengbusch and Wittmann (1965), and the replacement of the proline residue in position 156 by leucine in the mutants Ni 1688, Ni 1927 (Wittmann, 1964) and 171 (Funatsu and Fraenkel-Conrat, 1964) as shown by von Sengbusch (1965) and van Regenmortel (1967).

The antigenic determinant in the region 153-158 was investigated further by the immunization of rabbits with the N-(p-aminobenzoyl) derivatives of various C-terminal peptides of TMV protein coupled to cross-linked bovine serum albumin (BSA) by diazotization (Anderer, 1963 a; Anderer

and Schlumberger, 1965). Antibodies specific for the C-terminal hexa-, penta-, tetra- and tripeptides were able to precipitate and neutralize the infectivity of both TMV vulgare and dahlemense, which differs from vulgare in the C-terminal hexapeptide in that the glycine residue in position 155 is replaced by serine (Anderer and Schlumberger, 1965). Antisera prepared to threonine and serine (the C-terminal residues of TMV vulgare and dahlemense respectively) were only able to precipitate the virus with the homologous C-terminal amino acid in large amounts, but either antiserum readily and specifically neutralized the infectivity of both strains (Anderer and Schlumberger, 1966 a, b).

The C-terminal residues of the TMV protein project from the surface of the viral subunit (Bloomer et al., 1978) and, judging by its serological reactivity, the C-terminal threonine is exposed and probably kept in a favourable orientation by the proline residue at position 156 (van Regenmortel, 1978).

Although antibodies specific for the C-terminal hexapeptide of the TMV protein cross-react strongly with the intact virus (Anderer and Schlumberger, 1965), so few antibodies specific for the sequence of the six C-terminal residues exist in TMV antisera, that they cannot be detected by precipitin tests (Anderer, 1963 a; Anderer and Schlumberger, 1965; Anderer et al., 1971), but only by the more sensitive indirect haemagglutination test (Anderer and Ströbel, 1972). The projecting C-terminal residues are much more immunogenic in the surface conformation of the viral subunit than they are when coupled to a carrier protein (Anderer and Ströbel, 1972).

These findings, that the antigenic determinant at the C-terminus of the

polypeptide chain in the virus is dependant on the configuration of the terminal residues and hence is by definition a neotope (van Regenmortel, 1966) as the configuration of these residues differs in the free protein at pH 8,0 (Vogel, 1973; Vogel and Jaenicke, 1974), can be used to argue in support of the hypothesis (section 1.3.2) that the C-terminal region contains two antigenic determinants, one for the virus and the other for the protein. The antibodies that still react with the mutants, which have the proline in position 156 replaced by leucine, after absorption of the serum with TMV, were detected by precipitation (von Sengbusch and Wittmann, 1965; von Sengbusch, 1965; and van Regenmortel, 1967) and are unlikely to be specific for the sequence of the C-terminus as Anderer and co-workers (Anderer, 1963 a; Anderer and Schlumberger, 1965; and Anderer et al., 1971) found that sequence specific antibodies to this region were too scarce to be detected by precipitation tests. It is more likely that these non-absorbable antibodies recognize another part of the C-terminal region such as a cryptotope which is hidden by the surface conformation of the TMV subunit and exposed in the mutants.

1.4.3 THE IMMUNOCHEMICAL RELATIONSHIP OF THE TRYPTIC PEPTIDES OF TMV PROTEIN TO THE PROTEIN SUBUNIT

A tryptic digest of TMV protein, from which the 1-peptide (residues 1-41) had been removed, was chromatographed on Sephadex G50 and the fractions containing material with a molecular weight of less than 10000 daltons (G50 S_2) were pooled and dialysed against cold distilled water. Some of the material, thought to be partially digested fragments of TMV protein and those peptides that aggregate on standing, precipitated and was lyophilized as fraction D_1. The dialysable peptides were lyophilized as fraction D_2. These two fractions and whole

TMV protein, dissolved in phosphate buffered saline (PBS) containing Evans blue, were used to challenge guinea pigs which had been intradermally injected with TMV protein antiserum three hours previously. TMV protein and fraction D_1 caused cutaneous anaphylaxis, but fraction D_2 did not. Furthermore, when fraction D_2 was mixed with TMV protein antiserum prior to the initial injection, it completely inhibited the cutaneous anaphylaxis reaction resulting from the challenge with an excess of TMV protein. Fraction D_1 was able to significantly reduce the cutaneous anaphylaxis reaction when it was premixed with the TMV protein antiserum. (Young et al., 1963.)

The G50 S_2 fraction, which was incapable of fixing complement on its own, was able to specifically and completely inhibit the fixation of complement by TMV protein and different TMV protein antisera raised in three rabbits. The 1-peptide (residues 1-41) was also tested for its ability to either fix complement with TMV protein antisera, or to inhibit complement fixation by TMV protein-anti TMV protein complexes. It was found to be immunologically inactive by these criteria. (Benjamini et al., 1964.)

In an effort to locate the haptenic activity in the G50 S_2 fraction, it was subjected to ion exchange chromotography with Dowex 1-X2 resin and the isolated peptide fractions were tested for their ability to inhibit complement fixation by TMV protein and anti-TMV protein antibodies. Only the fraction containing tryptic peptides 4 (residues 62-68) and 8 (residues 93-112) and the fraction eluting immediately prior to it, were inhibitory, although the latter fraction was much less active than the former. When peptides 4 and 8 were separated by the isoelectric precipitation of peptide 8 at pH 3,8, and individually tested, only peptide

8 had activity. The less active fraction eluting prior to peptides 4 and 8 was found to contain trace amounts of peptide 8 which accounted for its activity. Results with two other antisera confirmed the immunological activity of peptide 8.(Benjamini et al., 1964.)

As peptide 8 was found to inhibit the complement fixation by TMV protein and individual antisera completely, and as this inhibition was specific for anti-TMV protein antibodies, it was concluded that this peptide was likely to contain the major part of, or a whole antigenic determinant of the protein (Benjamini et al., 1964). The possibility of other antigenic determinants on the protein was not excluded, but the total number of determinants was expected to be low (Benjamini, 1977).

1.5 THE NARROWING DOWN OF THE POSSIBLE LOCATIONS OF ANTIGENIC DETERMINANTS ON PEPTIDES

1.5.1 EVALUATION OF THE APPROACHES

The results obtained with immunochemically reactive overlapping peptides can be useful in deducing the locations of antigenic determinants on these peptides as long as the conclusions reached are not in conflict with the findings of any of the foregoing approaches (Atassi, 1975, 1977 b). An example of this process is the location of the antigenic determinant of the intact virus in the region 142-158 of the TMV protein. The similar reactivities of the peptides corresponding to the residues 142-158, 151-158 and 153-158 of the protein point to the C-terminal residues of these peptides being the immunochemically reactive region (Anderer, 1963 b). The results obtained with derivatives of the virus (Harris and Knight, 1955) and mutants with amino acid exchanges in this region (von Sengbusch and Wittmann, 1965; von Sengbusch, 1965; and

van Regenmortel, 1967) support this conclusion.

However, in some cases immunochemically reactive peptides which do not have overlaps, or where the degree of overlap is too great to be useful, will occur. In order to accurately locate the antigenic determinant in such peptides, additional approaches are required (Atassi, 1977 b).

The immunochemical reactivity of shortened peptides, corresponding to the regions of the reactive peptide, can be studied, but all the limitations that apply to peptide cleavage (discussed in section 1.4.1) are relevant to this approach. The antigenic determinant may be disrupted, or the results obtained with the shortened peptides may be misleading (Atassi and Saplin, 1968) as the mode of folding required for antibody binding of the peptide may be destroyed by the removal of an unrelated segment of the peptide (Atassi, 1977 b). Another limitation is the availability of cleavage reactions to give the desired shortened peptides (Atassi, 1977 b).

The approach of choice (Atassi, 1968, 1975 and 1977 b) for the location of antigenic determinants in immunochemically reactive peptides is the modification of specific amino acid side chains to form derivatives of the reactive peptide. This way the antigenic determinant cannot be disrupted and the conformation of the peptide derivative is more likely to resemble the unaltered peptide than is the case with shortened peptides (Atassi, 1968). The homogeneity and purity of the peptide derivatives will be of tantamount importance to the success of this approach (Atassi, 1977 b).

Once again, the mutants and strains of TMV are of potential use as a source of peptides with single amino acid exchanges which would serve as peptide derivatives of immunochemically active peptides in both the anti-virus and anti-TMV protein systems.

1.5.2 THE ROUGH DELINEATION OF THE ANTIGENIC DETERMINANT IN TRYPTIC PEPTIDE 8 (RESIDUES 93-112) OF TMV PROTEIN

The immunochemical activity of tryptic peptide 8 (residues 93-112) of TMV protein was confirmed by the direct binding of a N-(^{14}C) acetyl derivative of the peptide with TMV protein antibodies (Benjamini *et al.*, 1965) in a Farr assay (Farr, 1958). The marked difference between the binding of the peptide derivative by acetylcholinesterase antibodies and TMV protein antibodies, as well as the absence of binding with a TMV protein antiserum absorbed with TMV protein, proved the reaction to be specific (Benjamini *et al.*, 1965). The equivalent inhibition of complement fixation, by TMV protein and its antiserum, by the N-(^{14}C)acetyl derivative of peptide 8 and the native peptide (Young *et al.*, 1966) excluded the likelihood of the isoleucine residue in position 93 participating in an antigenic determinant.

In order to locate the antigenic region in tryptic peptide 8, degradation products of the peptide were prepared by digestion with pepsin, subtilisin and carboxypeptidases A and B, as well as by the stepwise degradation of the peptide from the N-terminal end using the phenyl isothiocyanate method (Crestfield *et al.*, 1963), and tested for their ability to inhibit the complement fixation of TMV protein and its antiserum or the direct binding of N-(^{14}C) acetyl peptide 8 by TMV protein antibodies (Young *et al.*, 1966). N-(^{14}C)acetyl derivatives

of some of the degradation products of peptide 8 were also tested for direct binding with TMV protein antibodies (Young et al., 1966). Shortened peptides, lacking either the five N-terminal residues or the two C-terminal residues, were immunochemically active (although the direct binding of their N-(^{14}C)acetyl derivatives was somewhat lower than that of N-(^{14}C)acetyl peptide 8) while the peptic and subtilisin digests were inactive. From this, Young and co-workers (1966) concluded that the sequence 98-110 was responsible for much of the antigenicity of tryptic peptide 8 in the TMV protein-anti TMV protein system.

1.6 THE ACCURATE DELINEATION OF ANTIGENIC DETERMINANTS

1.6.1 THE VALUE OF IMMUNOCHEMICAL STUDIES WITH SYNTHETIC PEPTIDES

Once the foregoing approaches have yielded the locations and rough delineations of the antigenic determinants of a protein antigen, a point is reached where further definition would require the modification of unreactive non-polar or hydrophobic amino acid side chains (Atassi, 1977b), or, in the case of virus antigens, the isolation of mutants with amino acid exchanges which would affect their viability. This impasse was broken by the pioneering work of the Berkely group who applied the technique of solid-phase peptide synthesis (Merrifield and Stewart, 1965) to the manufacture of a peptide corresponding to an antigenic region of the TMV protein (Stewart et al., 1966).

By careful consideration of the results of the foregoing approaches, which narrow down the antigenic regions, it is possible to deduce and synthesize a limited number of peptides corresponding to the various parts of each antigenic region. Immunochemical studies with these peptides can give the final definition of the antigenic determinants

and shed light on the roles played by the individual amino acids, which comprise each determinant, in antibody binding (Atassi, 1975, 1977 b; Benjamini et al., 1968 a,b, 1969; Young et al., 1967, 1968).

Care must however be exercised with regard to the purity and homogeneity of the synthetic peptides, as a drawback of the solid phase technique is product heterogeneity (especially with longer peptide sequences) and impure peptides can give misleading and erroneous results (Atassi, 1977 b). This necessitates the use of purification and monitoring procedures of high resolving power.

A special case is presented by proteins which have the amino acids comprising an antigenic determinant grouped in a cluster on the protein surface rather than sequentially on the polypeptide chain. An example of this phenomenon is the protein lysozyme which has each of its three antigenic determinants grouped around a disulphide bridge (Atassi and Habeeb, 1977). This problem has been solved by the development of the concept of the surface-simulation synthetic peptide (Atassi et al., 1976; Atassi and Habeeb, 1977; Atassi, 1978), which is constructed to duplicate the three dimensional surface structure of a region of the protein by having the active amino acids separated by glycine spacer residues. This requires a detailed knowledge of the three dimensional structure of the protein in question.

1.6.2 THE DEFINITION AND DESCRIPTION OF THE CRYPTOTOPE CORRESPONDING TO RESIDUES 108-112 OF THE TMV PROTEIN.

Having shown that the sequence 98-110 of the TMV protein was important for the antigenic reactivity of tryptic peptide 8 (residues 93-112) with antibodies to the whole protein (Young et al., 1966) Stewart

and co-workers (1966) tested the immunochemical activity of a synthetic decapeptide corresponding to residues 103-112, which was prepared by the method of Merrifield and Stewart (1965). After ion exchange chromatography on Dowex 1-X2 resin, the purity of the decapeptide was evaluated by electrophoresis and amino acid analysis before it was used to inhibit the direct binding of the N-(^{14}C)acetyl derivative of peptide 8 by TMV protein antibodies. The decapeptide was found to bind approximately 10 times less efficiently to TMV protein antibodies than peptide 8 did (Stewart et al., 1966). The binding of the N-(^{14}C)acetyl derivative of the decapeptide to TMV protein antibodies was specific as no binding with comparable amounts of acetylcholinesterase antibodies could be demonstrated (Stewart et al., 1966).

As a consequence of these findings, shorter N-(^{14}C)acetyl peptides corresponding to the C-terminal region of peptide 8 were synthesized and their direct binding with TMV protein antibodies was determined. The shortest peptide able to be bound by TMV protein specific antibodies (but not acetylcholinesterase antibodies) was the pentapeptide corresponding to the sequence Leu-Asp-Ala-Thr-Arg (residues 108-112), but the results indicated that the quantity of peptide bound by a given amount of antibodies increased with peptide length. (Young et al., 1967.)

The finding, that a N-(^{14}C)acetyl-$(Ala)_5$-pentapeptide conjugate was more efficiently bound by TMV protein antibodies than the pentapeptide, indicated that the increase in peptide size contributed in a non-specific manner to the binding of the pentapeptide. That this non-specific enhancement of binding was hydrophobic in origin, was demonstrated by the specific binding of a N-(^{14}C)acetyl-octanoyl-

tetrapeptide (residues 109-112) to TMV protein antibodies with an average association constant higher than that of the N-(^{14}C)acetyl pentapeptide, N-(^{14}C)acetyl-$(Ala)_5$-pentapeptide conjugate or the N-(^{14}C)acetyl decapeptide. The shortest octanoyl-peptide able to be specifically bound by the TMV protein antibodies was the C-terminal tripeptide, Ala-Thr-Arg (residues 110-112). (Benjamini *et al.*, 1968 b.)

It was also found (Benjamini *et al.*, 1968 a) that, although all animals (rabbits, guinea pigs, rats and mice), immunized with TMV protein, produced antibodies which bound N-(^{14}C) acetyl peptide 8, in only one out of four rabbits could this binding be inhibited by the decapeptide corresponding to residues 103-112. However, all the rabbit antisera were able to bind the N-(^{14}C)acetyl decapeptide and the binding of two of these antisera could be inhibited by the C-terminal pentapeptide or a $(Ala)_5$-pentapeptide conjugate. Some rabbits produced antibodies which bound the N-(^{14}C)acetyl pentapeptide, but others required the hexa- or heptapeptide for demonstrable binding. This suggested that the antibodies produced by different rabbits do not necessarily bind to the same region or regions of tryptic peptide 8, but, as all the rabbits do produce antibodies to peptides containing the C-terminal pentapeptide sequence, it was postulated that this sequence, or part of it, constitutes a portion of, or a region which is antigenic to all the rabbits tested (Benjamini *et al.*, 1968 a). This was confirmed by the specific binding of octanoyl-Ala-Thr-Arg by the antibodies of all rabbits tested, even though two of these rabbits produced antibodies which did not bind the N-(^{14}C)acetyl pentapeptide significantly (Benjamini *et al.*, 1969). This binding was in excess of that with the N-(^{14}C)acetyl decapeptide in all cases.

These results suggest that the tripeptide Ala-Thr-Arg dictates the antigenic specificity of the region, although, by itself, it has no capacity to bind with antibodies (Benjamini et al., 1968 a; Benjamini, 1977). The differences in binding between various antibody populations from different animals probably lie in the location of complementary hydrophobic areas in juxtaposition to the antibody combining site for Ala-Thr-Arg (Benjamini et al., 1969; Benjamini, 1977). Thus the antibody populations with inadequate or incorrectly located hydrophobic regions would not have a sufficiently high affinity to bind the pentapeptide irreversibly enough for measurement.

The roles of the amino acids which comprise the antigenic determinant in tryptic peptide 8 were studied by the use of synthetic homologues of the C-terminal pentapeptide (Young et al., 1968). Every one of the eleven peptide homologues,which differed from the pentapeptide by a single amino acid exchange, bound less strongly to the TMV protein antibodies than the pentapeptide, and, in general, the homologues with amino acid replacements similar to the original residue in the pentapeptide retained some immunochemical reactivity. A peptide with the reverse sequence of the pentapeptide was inactive.

When the N-terminal leucine of the pentapeptide was replaced by a hydrophobic amino acid such as D-leucine, isoleucine or tyrosine, the resultant peptide was still bound by TMV protein antibodies to a significant extent, but alanine in this position caused the peptide to retain only a marginal activity (Young et al., 1968). The substitution of this leucine residue by octanoic acid resulted in an octanoylated tetrapeptide which was more extensively bound by TMV protein antibodies than the pentapeptide (Benjamini et al., 1968 b). The

shape of this residue is therefore considered to be less important for antibody binding than its hydrophobic character (Young et al., 1968).

The shape of the aspartic acid residue corresponding to residue 109 of the TMV protein, is, on the other hand, more important than its negative charge, as the reactivity of the peptide is retained when aspartic acid is replaced by asparagine, and is lost when glutamic acid is substituted for it (Young et al., 1968).

Peptides in which the alanine (corresponding to position 110) was replaced by leucine or glycine, exhibited only marginal activity (Young et al., 1968), but an octanoylated tripeptide containing the α-aminobutyryl group instead of alanine was bound to a similar or greater extent than octanoyl-Ala-Thr-Arg by three out of four rabbit antisera, while the fourth antiserum barely recognized it (Benjamini et al., 1969). This latter observation was thought to reflect a structural difference in the antibody combining sites of different rabbits rather than a requirement of the particular amino acid residue, as leucine in this position of the octanoylated tripeptide did not exhibit a high association of significant specificity with any of the antisera (Benjamini et al., 1969).

Serine replacing threonine (corresponding to position 111) results in an active peptide, while glycine does not (Young et al., 1968). This points to the need of a hydroxyl group on the side chain of the amino acid in this position for antibody recognition, as octanoylated tripeptides with leucine or the α-aminobutyryl group replacing threonine exhibited low binding specificities (Benjamini et al., 1969).

Removal of the C-terminal arginine of the pentapeptide results in a loss of immunochemical activity even when a N-terminal threonine is added to maintain the size of the peptide. This shows that the arginine corresponding to position 112 is critical for binding by some antisera (Young et al., 1968), although it may be less important for others, as Young et al. (1966) have shown that the desarginated peptide 8 was still active. The addition of another arginine residue to the C-terminus of the pentapeptide and the removal of the N-terminal leucine to maintain peptide size, did not affect binding relative to the reference pentapeptide, while retention of the N-terminal leucine to form a hexapeptide, Leu-Asp-Ala-Thr-Arg-Arg, caused the binding almost to be doubled (Young et al., 1968). Another hexapeptide which had an N-terminal threonine and only one arginine was not bound significantly better than the pentapeptide (Young et al., 1968). Whether two populations of antibodies exist in a particular antiserum, one which recognizes the pentapeptide and another which is directed against an area of the protein composed of the C-terminal region of peptide 8 and the N-terminal region of peptide 9, or whether the arginine residue corresponding to position 113 merely enhances the binding of the pentapeptide by the antibodies specific for it, is not yet clear (Young et al., 1968).

The location of the antigenic determinant, Leu-Asp-Ala-Thr-Arg (residues 108-112), near the core of the virus rod (Champness et al., 1976; Stubbs et al., 1977; Bloomer et al., 1978) means that it would only be available to antibodies after the virus had dissociated and would be a cryptotope in terms of the definitions of Jerne (1960) and van Regenmortel (1966).

1.7 THE NEED FOR FURTHER INVESTIGATION OF THE ANTIGENICITY OF TMV AND ITS PROTEIN SUBUNIT.

Relevant aspects of the research to identify and delineate the antigenic determinants of TMV and its protein subunit have been described in the foregoing review. From this it is clear that discrepancies between the results of the various approaches exist, and that the overall picture is far from complete.

The results of two approaches, one utilizing specific derivatives and the other overlapping peptides, which have supplied the bulk of the information about the antigenic regions of the whole virus, are not completely in agreement. Amino acid exchanges in positions 20, 21, 63, 126 and 129, which are included in the active peptides corresponding to residues 18-23, 62-68 and 123-134 (Anderer, 1963 b) were not found to be serologically distinguishable (von Sengbusch, 1965; van Regenmortel, 1967) while amino acid exchanges in positions 107, 136, 138 and 140, which were serologically distinguishable (von Sengbusch, 1965; van Regenmortel, 1967) were part of peptides which were immunologically inactive (Anderer, 1963 b). In the 2,8 Å resolution model of the TMV protein subunit (Bloomer *et al.*, 1978) the peptides corresponding to residues 18-23 and 123-134 are not readily accessible from the viral surface, but neither is residue 107 where an amino acid exchange is serologically detectable. These conflicts can be rationalized in terms of the disruption of antigenic determinants during enzymatic hydrolysis and subtle conformational modulation effects, but no clear explanation is available.

In the system consisting of TMV protein and its antisera only one cryptotopic antigenic determinant, located in tryptic peptide 8

(residues 93-112), could be found (Benjamini et al., 1964). In view of the well known serological cross-reaction between whole TMV and TMV A-protein and the finding of van Regenmortel and Lelarge (1973) that the valence of the TMV protein subunit is at least 3, it is obvious that other antigenic determinants beside that one studied by Benjamini and co-workers must exist. Some of these regions will be identified in the present work.

Five tryptic peptides of TMV protein have been chosen on the basis of their positions in the conformation of the subunit and the results of earlier investigations. They are peptides 1 (residues 1-41), 4 (residues 62-68), 8 (residues 93-112), 11(residues 135-141) and 12 (residues 142-158). As it is now realised that TMV particles often spontaneously release protein subunits, and that TMV protein preparations, in addition to containing protein monomers, also contain disk aggregates which are antigenically similar to the whole virus (van Regenmortel and Lelarge, 1973), immunochemical studies with these peptides in which the state of aggregation of the antigen is controlled, should go some way to resolving the discrepancies of earlier investigations. In the present work, the contribution of virus-specific and protein-specific antibodies to the overall reactivity of the antisera was also tested. Some of the results here included have already been published (Milton et al., 1977; Milton and van Regenmortel, 1979).

PART 2. RESULTS

2.1 THE PREPARATION OF ANTIGENS AND PEPTIDES

2.1.1 EXTRACTION OF VIRUS FROM INFECTED LEAVES

TMV viruses were extracted from the leaves of infected Nicotiana tabacum cultivar soulouk plants by the method of von Wechmar and van Regenmortel (1970), followed by differential centrifugation until a clear pellet of virus was obtained (4.1.2).

In this method the virus is released from the leaf cells by homogenization and the particulate plant material is removed from the solution by filtration through cheese cloth and low speed centrifugation. The bulk of the dissolved plant material is removed by filtration through activated charcoal suspended in a matrix of celite. The virus, suspended in a large volume of filtrate, is then concentrated by an exclusion and salting out process when the filtrate is made 4% (w/v) with respect to both polyethylene glycol and NaCl, which causes the virus to precipitate so that it can be pelleted by low speed centrifugation. The virus, resuspended in 0,05M phosphate buffer pH 7,5, can either be further purified by a second polyethylene glycol/NaCl precipitation, or differential centrifugation, or both, and stored at $+4^{o}C$.

The approximate concentration of the virus solutions was determined by the use of an extinction coefficient of $E^{0,1\%}_{260\ nm} = 3,22$ (Takahashi, 1951), and yields of 2-3 g of virus per kg infected leaves were regularly obtained with this method. The purity of a virus preparation was judged by the profile of its ultraviolet absorbance spectrum in the wavelength range from 240-320 nm. Criteria of purity were the ratios of the extinctions at 250 nm and 260 nm as well as at 260 nm and 280 nm.

2.1.2 THE PREPARATION OF TMV PROTEIN

The proteins of TMV and the mutants were prepared from the virus by either the cold acetic acid method (4.1.3.1) of Fraenkel-Conrat (1957) or according to the ethanolamine-DEAE method (4.1.3.2) of Durham (1972).

In the cold acetic acid method, the virus is dissociated to protein and RNA in the non-polar environment of a solution of 66% (v/v) glacial acetic acid in water, which also causes the RNA to precipitate so that it can be removed by low speed centrifugation. The protein-containing supernatant is then dialysed against water until the pH is sufficiently high to allow the protein to precipitate at its isoelectric point. Centrifugation and resuspension in water effectively concentrates the protein and removes the remaining acetic acid from the protein solution.

In contrast, the method developed by Durham exploits the observation that the equilibrium between helices, disks and free protein subunits favours the dissociated protein at high pH. The virus dissociates in the 0,1M ethanolamine - HCl buffer pH 11,0, and the RNA is retained when the solution is passed through an anion exchanger such as DEAE-cellulose. If the dissociated virus is chromatographed through DEAE-cellulose a characteristic elution profile is obtained (fig. 2.1). The first peak consists of undissociated or reassociated virus and the first half of the second peak is pure protein. As the second peak tails off, however, the eluted protein is increasingly contaminated with RNA.

Of these two methods, the latter was found to be more advantageous in that the protein was generally more easily soluble and judicious pooling of the eluant from the DEAE-cellulose column could minimize the RNA contamination as judged by the A280/A250 ratio. Also, certain of the mutant

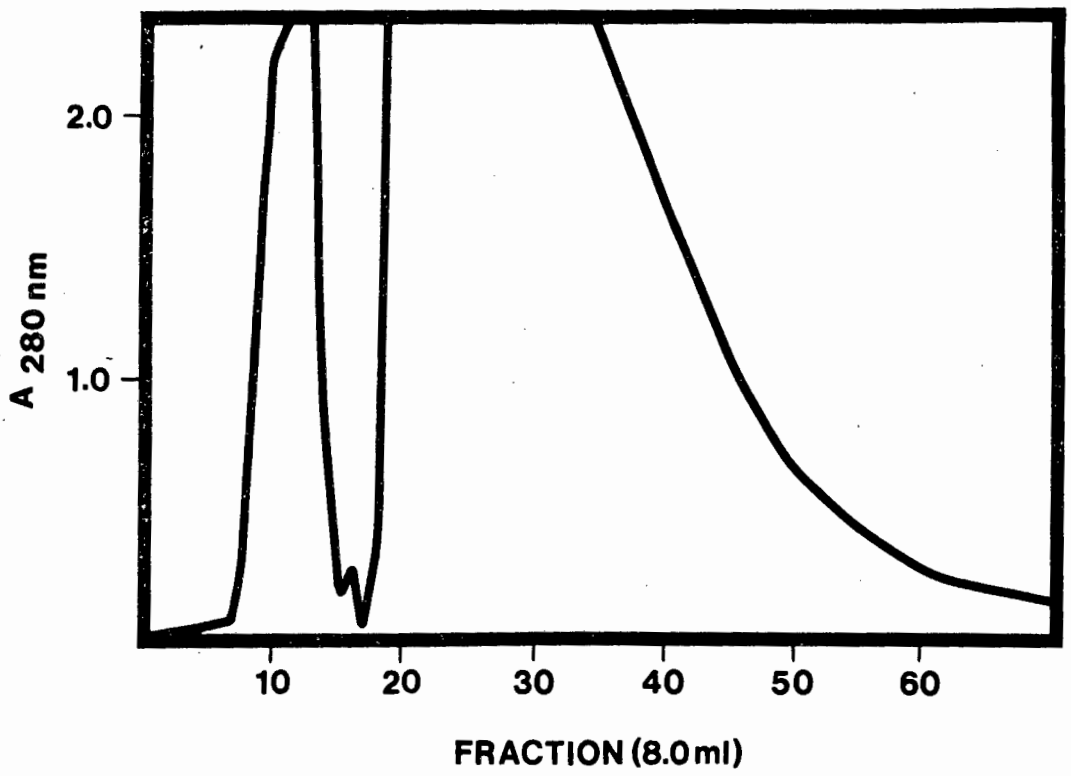

Fig. 2.1 : DEAE-cellulose chromatography of TMV disrupted in 0,1M ethanolamine -HCl buffer pH 11,0. The first peak consists of short rods of intact virus, while in the second peak pure TMV protein is initially eluted. From fraction 40 onwards RNA contamination may be encountered.

proteins (414, Ni 568 and Ni 1688) which were difficult to dissolve after isoelectric precipitation, were more easily prepared by substituting 0,1M NaOH for the ethanolamine buffer when disrupting the virus, and then, after the removal of the RNA, the protein was kept at a pH above its isoelectric point.

The concentrations of protein solutions were determined spectrophotometrically using an extinction coefficient of $E^{0,1\%}_{282\ nm} = 1,27$ (Fraenkel-Conrat, 1957). Both methods described gave yields greater than 90% of the virus present in the initial extract (2.1.1 and 4.1.2).

2.1.3 THE GENERATION AND PURIFICATION OF THE TRYPTIC PEPTIDES OF TMV PROTEIN

The proteins of TMV and the mutants were digested with trypsin (4.1.4.1) by the method utilized by Benjamini et al. (1964), except in as far as 1,0M ammonia solution, instead of 0,2M NaOH, was used to maintain the digest at pH 8,0. It was thought that this would decrease the salt content of the digest after lyophilization.

The 1-peptide (residues 1-41) was separated from the digest by isoelectric precipitation at pH 4,5, and purified by three further isoelectric precipitations followed by prolonged dialysis against frequent changes of water (4.1.4.2). Dialysis in cellophane tubing, boiled to reduce the pore size so that the 1-peptide was retained while the smaller peptides were not, gave a greater yield of pure peptide than an increased number of isoelectric precipitations would have done.

The differing electrophoretic mobilities of the eleven remaining pH 4,5 soluble tryptic peptides were exploited in an attempt to separate and

purify them by anion-exchange chromatography with the resin AGI-X2. Depending on the shape of the pH gradient used, characteristic elution profiles are obtained with each peak or shoulder representing one or two of the peptides (Funatsu, 1964; Fraenkel-Conrat, 1965; Wittmann, 1965). Two different pH gradients were used (4.1.4.3.1), one recommended by Funatsu (1964) - depicted in fig. 2.2 and the other by Wittmann (1965) - depicted in fig. 2.3. In both cases the pooled peaks contained a number of components as judged by peptide mapping (4.2.1). It has been suggested that a few fractions under each peak contain a pure peptide and the contamination found in pooled peaks is due to "overlap" or "tailoff" of the adjacent peaks (Fraenkel-Conrat, 1965). In any case, for preparative purposes, the pooled eluant from any peak must be further purified by an additional procedure, such as chromatography or electrophoresis (Funatsu, 1964; Fraenkel-Conrat, 1965).

In view of the requirement of this study for only four of the pH 4,5 soluble tryptic peptides, viz. peptide 4 (residues 62-68), peptide 8 (residues 93-112), peptide 11 (residues 135-141) and peptide 12 (residues 142-158), it was logical to omit the ion-exchange chromatography and to develop a preparative electrophoresis system that adequately separated these peptides. Increasing the time of the electrophoretic step of peptide mapping and applying 25 mg of the pH 4,5 soluble portion of the tryptic digest across the centre of the paper proved to be a good compromise (4.1.4.3.2). Yields of the individual peptides, from the paper, varied between 60-80% after a single electrophoretic run and in most cases amino acid analysis (4.2.2) proved the peptides to be sufficiently pure (table 2.2). An additional advantage of this system is that the individual peptides precipitate out of solution after a few weeks, and preparative electrophoresis allows a fresh supply to be made at short notice.

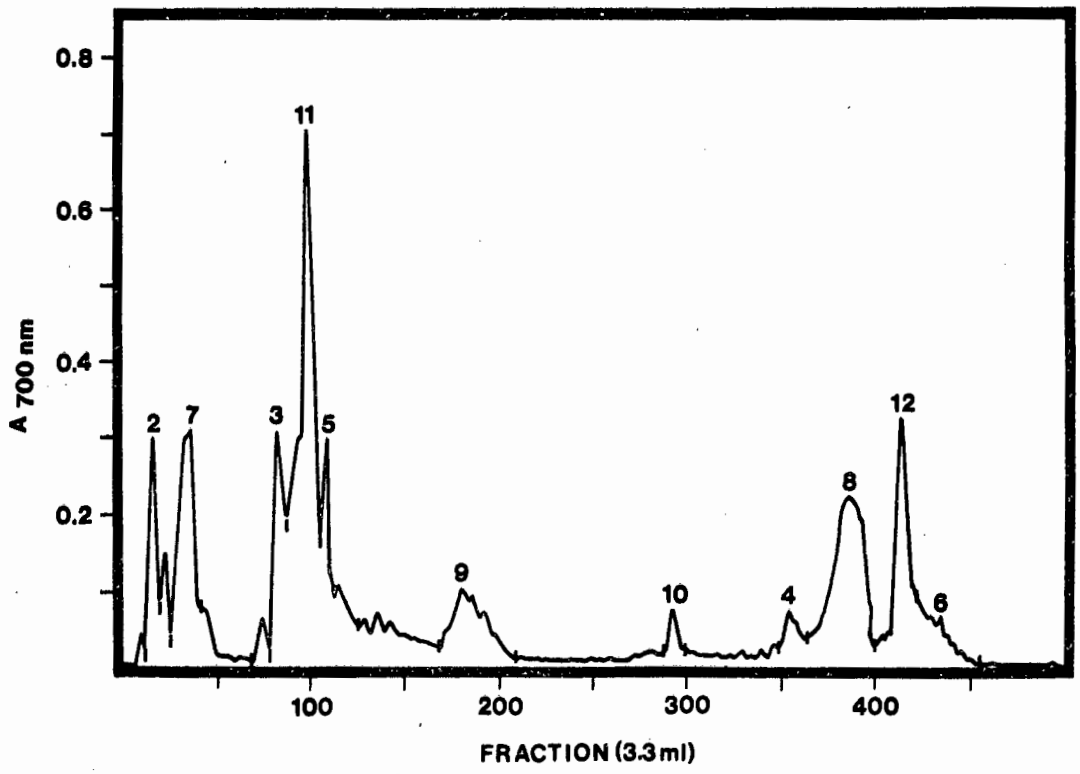

Fig. 2.2 : Ion-exchange chromatography on Dowex 1-X2 (150x0,9 cm column) of the pH 4,5 soluble tryptic peptides of TMV protein. The peptides were eluted by the following gradient (volume percentages): (1) 1% pyridine, 1% collidine and acetic acid pH 8,2 (250 ml) (2) 1% pyridine, 1% collidine and acetic acid pH 7,3 (360 ml) (3) 0,02M acetic acid (240 ml) (4) 0,2M acetic acid (120 ml) (5) 0,35M acetic acid (120 ml) (6) 0,5M acetic acid (120 ml) (7) 0,65M acetic acid (240 ml) (8) 1,7M acetic acid (120 ml) (9) 5,0M acetic acid (120 ml) (Funatsu, 1964). The flow rate was maintained at 40 ml/hr and the temperature at 37°C. The Folin-Lowry colour obtained with 0,1 ml of each 3,3 ml fraction was measured as the optical density at 700 nm. Tubes containing peptide 8 and peptide 10 showed a precipitate. The bars (▌) indicate the manner in which the fractions were pooled and the numbers refer to the tryptic peptide eluted under each peak.

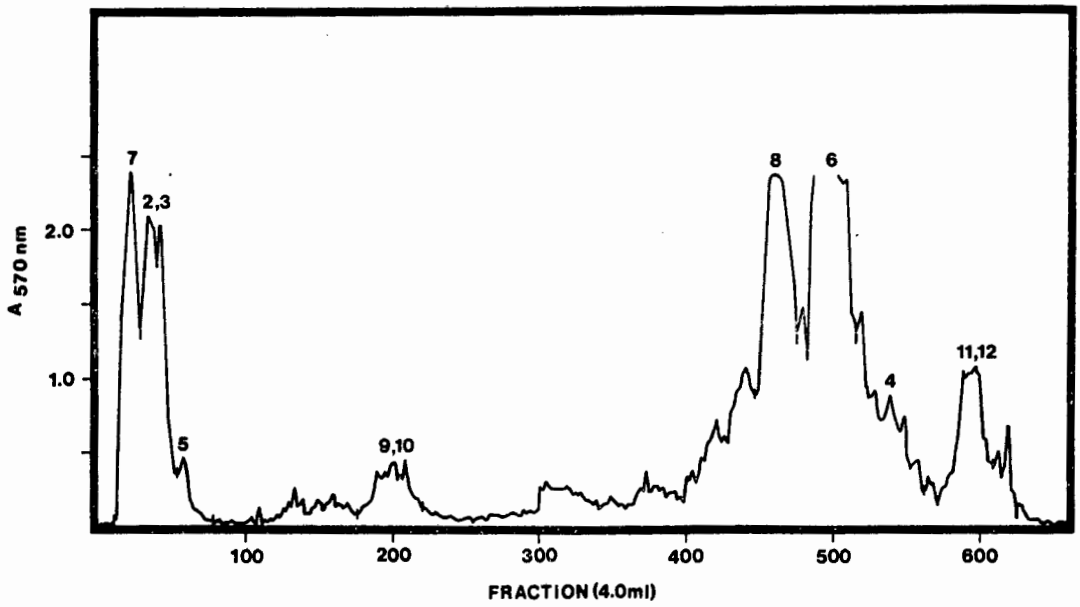

Fig. 2.3 : Ion-exchange chromatography on Dowex 1-X2 (150x0,9 cm column) of the pH 4,5 soluble tryptic peptides of TMV proteins. The peptides were eluted by the following gradient (volume percentages): (1) 1% pyridine, 1% collidine and acetic acid pH 8,4 (125 ml) (2) 2% pyridine, 1% collidine and acetic acid pH 7,5 (125 ml) (3) 1% pyridine, 1% picoline, 1% lutidine and acetic acid pH 7,2 (600 ml) (4) 1% pyridine, 1% picoline, 1% lutidine and acetic acid pH 6,0 (600 ml) (5) 2% pyridine, 2% picoline, 2% lutidine and acetic acid pH 5,0 (600 ml) (6) 3% pyridine, 3% lutidine and acetic acid pH 4,0 (600 ml) (7) 2,0M acetic acid (150 ml) (Wittmann, 1965).
The flow rate was maintained at 40ml/hr and the temperature at 37°C. The optical density at 570 nm was read after alkaline hydrolysis and the reaction with ninhydrin of 0,2 ml of each 4,0 ml fraction. The bars (▌) indicate the manner in which the fractions were pooled and the numbers refer to the tryptic peptides.

2.1.4 THE REMOVAL OF THREONINE FROM TRYPTIC PEPTIDE 12 (RESIDUES 142-158) BY CARBOXYPEPTIDASE A

TMV protein has a proline residue at position 156, third from the C-terminus (Anderer et al., 1960; Tsugita et al., 1960). The C-terminal threonine is therefore readily released by carboxypeptidase A, while no further amino acids are split off by the action of the enzyme (Harris and Knight, 1952). This was tested by subjecting tryptic peptide 12 (residues 142-158) to carboxypeptidase A digestion by a modification of a method described by Ambler (1972) and detailed in section 4.1.4.3.3.

The whole digest was chromatographed on a column of Sephadex G50 fine, equilibrated and run with distilled water (in which peptide 12 is soluble), and the elution profile shown in fig. 2.4 was obtained by plotting the absorbance at 280 nm and 210 nm against the fraction number. 20 µl of every second fraction was spotted onto Whatman 3 Chroma paper, dried and tested for colour development with 0,2% (w/v) ninhydrin in ethanol containing 5% (v/v) collidine. As only fractions 44-65 showed a blue colour, it was concluded that the first major peak consisted of undigested peptide 12, which does not stain with ninhydrin (2.2.1.1). This was confirmed by amino acid analysis of portions of each pool of the fractions under the final three peaks. The first major peak consisted of peptide 12, the second peak of peptide 12 minus threonine and the third peak of threonine. The yield of the carboxypeptidase A product (peptide 12 minus threonine) was only about 10% as judged by amino acid analysis (4.2.2).

The chromatographic separation of the undigested peptide 12 and peptide 12 minus threonine (Fig. 2.4) was better than expected for the fraction-

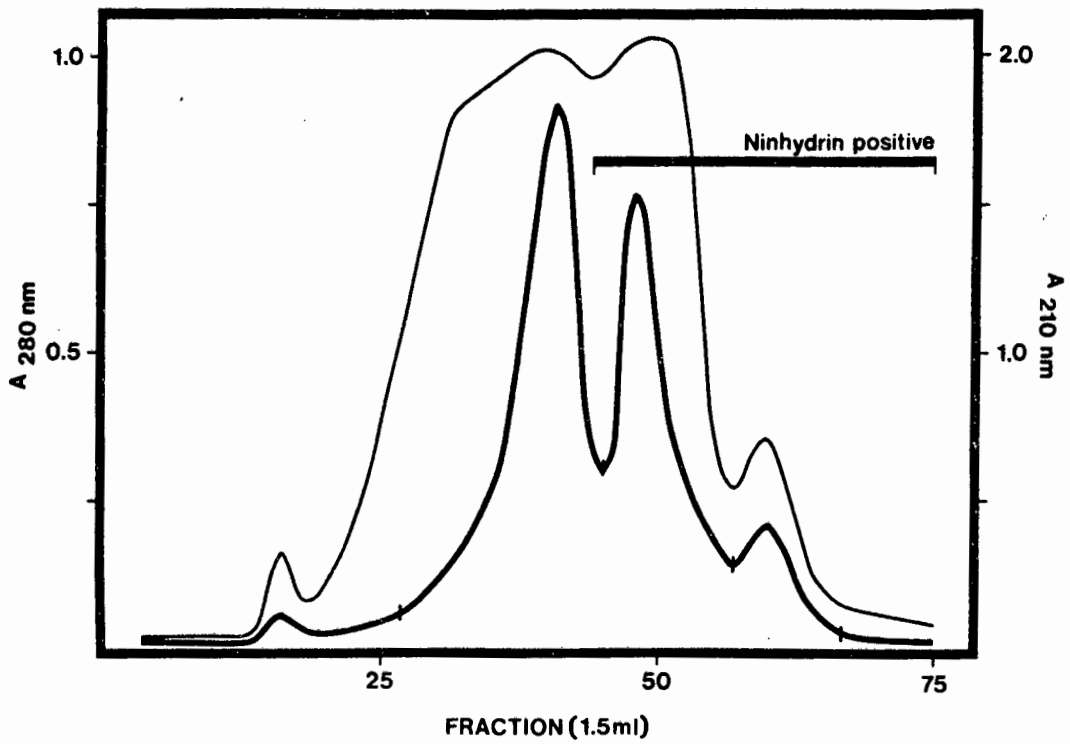

Fig. 2.4 : Gel exclusion chromatography of a carboxypeptidase A digest of peptide 12 (residues 142-158) on a 65x1,6 cm column of Sephadex G50 fine equilibrated and run with distilled water. 2,6 mg of digest, dissolved in N-ethylmorpholine buffer pH 8,1, was applied to the column at room temperature while the flow rate was maintained at 30 ml/hr. The absorbance at 280 nm (broad trace) and 210 nm (narrow trace) was plotted against the fraction (1,5 ml) number. The broad bracket indicates the fractions that were ninhydrin positive in a spot test on paper. The bars (▌) indicate the manner in which the fractions were pooled.

ation range of Sephadex G50, nevertheless the amino acid analysis (table 2.2) of the pooled peaks must be believed. It is possible that peptide 12 exists in solution in the form of small polymers (dimers or trimers), which cause it to be eluted from the column earlier.

2.1.5 THE GENERATION OF FORMIC ACID PEPTIDES FROM TMV PROTEIN

The TMV protein has two aspartyl-proline peptide linkages in its sequence that can be selectively cleaved by high concentrations of certain organic acids (Anderer, 1963 a; Baltz and van Regenmortel, 1974). The formic acid method of Miki and Knight (1965) was used to degrade TMV protein and the 1-peptide (residues 1-41) in an effort to prepare peptides corresponding to the first nineteen residues and the ensuing twenty-two residues of the protein's sequence (4.1.5.1).

TMV protein was incubated in a solution of 66,6% (v/v) formic acid in water for 20 hrs at 37°C and evaporated to dryness. On resuspension in water a precipitate was formed and this was separated from the supernatant by centrifugation. The supernatant was tested by the ninhydrin reaction after alkaline hydrolysis (Benson and Patterson, 1971) and found to contain proteinaceous material.

In an effort to discover the distribution of protein or peptides in the precipitate and supernatant, samples of both of these, as well as the whole digest, were submitted to SDS-polyacrylamide gel electrophoresis (4.1.5.3). Three proteins, viz. TMV protein (17500 daltons), lysozyme (14000 daltons) and insulin (6000 daltons), were used as markers. The whole digest was found to contain three components, the largest of which had a molecular weight just greater than that of insulin and was probably the peptide corresponding to the sequence 78-158. The other two

components had molecular weights similar to, and just greater than insulin and were probably the peptides corresponding to the sequences 20-77 and 1-19 respectively. The precipitate contained the largest and smallest of these peptides, while the supernatant contained the water soluble peptide corresponding to the sequence 20-77.

An attempt was made to separate the three formic acid peptides by exclusion chromatography with Sephadex G50 equilibrated and run with a solution of 50% (v/v) glacial acetic acid in water (4.1.5.2). An elution profile with three peaks was obtained (fig. 2.5), but SDS-polyacrylamide gel electrophoresis of samples from each pool of fractions corresponding to the individual peaks revealed that the first peak was composed of all three peptides while the second and third peaks contained the water soluble peptide. It is thought that the peaks in the elution profile correspond to different aggregation states of the peptides.

The 1-peptide resulting from tryptic digestion (4.1.4.1) contains a single aspartyl-proline peptide bond which should also be susceptible to formic acid cleavage, so it too was incubated in a solution of 66,6% (v/v) formic acid in water and dried. Resuspension in water again yielded an insoluble precipitate, but the supernatant was free of proteinaceous material as judged by the ninhydrin reaction (Benson and Patterson, 1971).

The precipitates of the formic acid degraded TMV protein and the 1-peptide were found to dissolve in 0,1M HCl, but when this was evaporated they still could not be dissolved in water. It was found, for both of the precipitates, that solubility in aqueous solutions could only be

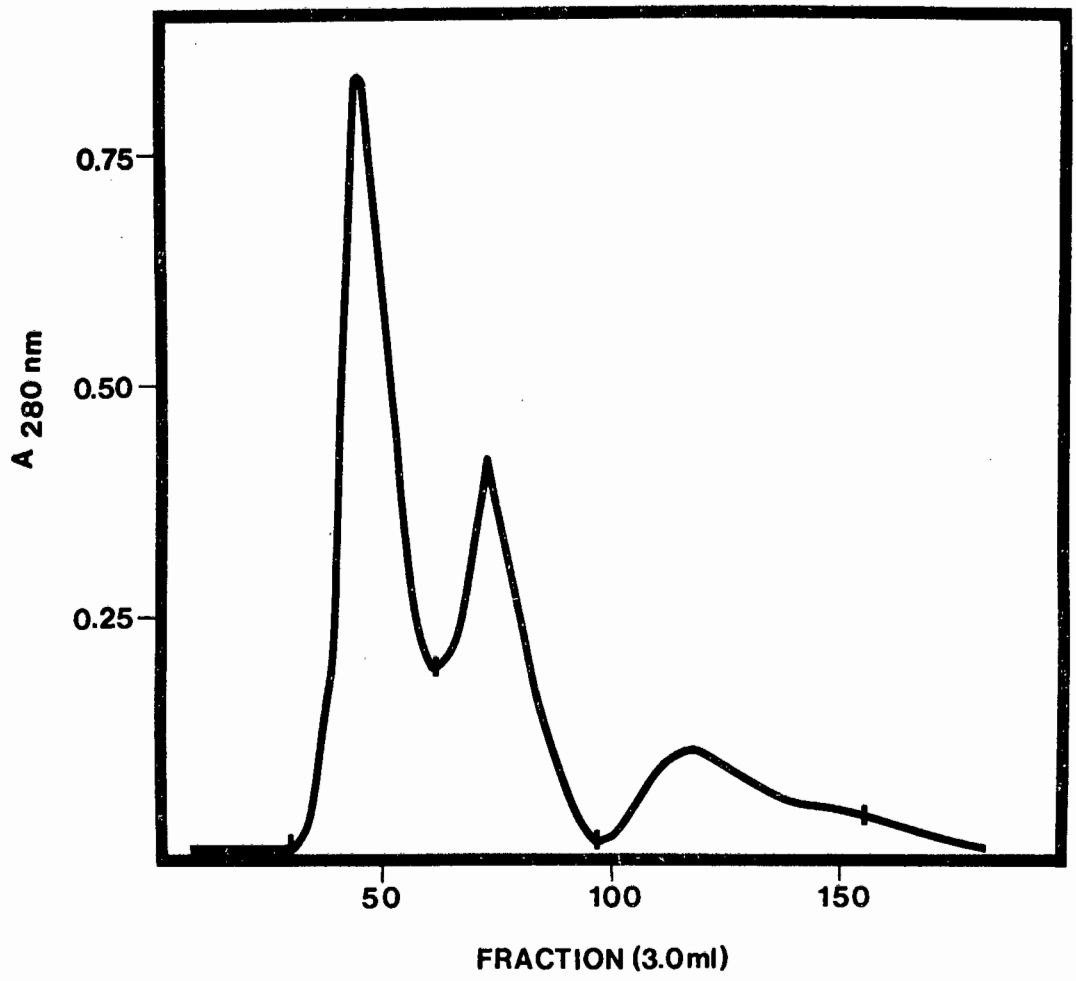

Fig. 2.5 : Gel exclusion chromatography of the formic acid peptides on a 80x1,6 cm column of G50 fine equilibrated and run with 50% (v/v) glacial acetic acid in water. 50 mg (5.0 ml) of the formic acid digest of TMV protein was dissolved in 50% (v/v) glacial acetic acid and applied to the column at room temperature. The flow rate was maintained at 30-40 ml/hr and the absorbance at 280 nm of each 3,0 ml fraction was read against a 50% (v/v) glacial acetic acid blank. The bars (▌) indicate the manner in which the fractions were pooled.

attained in the presence of 1,0% (w/v) SDS or when an equal volume of formic acid, glacial acetic acid or pyridine was added to the precipitates in water.

2.2 CHARACTERIZATION OF TMV PROTEIN, THE PROTEINS OF THE TMV MUTANTS AND THEIR PEPTIDES

2.2.1 PEPTIDE MAPPING

2.2.1.1 PEPTIDE MAPPING OF THE pH 4.5 SOLUBLE TRYPTIC PEPTIDES OF TMV PROTEIN

The pH 4.5 soluble portion of the tryptic digest of TMV protein (4.1.4.1) produces the characteristic pattern of peptide spots (depicted in fig. 2.6) when the prescribed conditions (4.2.1) are maintained during high voltage electrophoresis in the first dimension and ascending chromatography in the second. The pH, potential difference, and current were as suggested by Benjamini et al. (1964), except in as far as the electrophoretic buffer was made 20% (v/v) with acetone prior to use. The chromatographic system was suggested by Dr. B. Wittmann-Liebold (personal communication).

Using this system for peptide mapping, all the tryptic peptides, except peptides 6 (residues 72-90) and 10 (residues 123-134), which remain at the origin during electrophoresis, could be separated and identified by their differing electrophoretic and chromatographic mobilities and their differing staining properties. Peptide 2 (residues 42-46), 3 (residues 47-61), 4 (residues 62-68), 5 (residues 69-71), 8 (residues 93-112), and 9 (residues 113-122), as well as the spot consisting of peptides 6 and 10 stained purple with ninhydrin, while peptides 7 (residues 91-92) and 11 (residues 134-141) stained characteristically grey and yellow respectively (Woody and Knight, 1959; Benjamini et al., 1964). Peptide 12

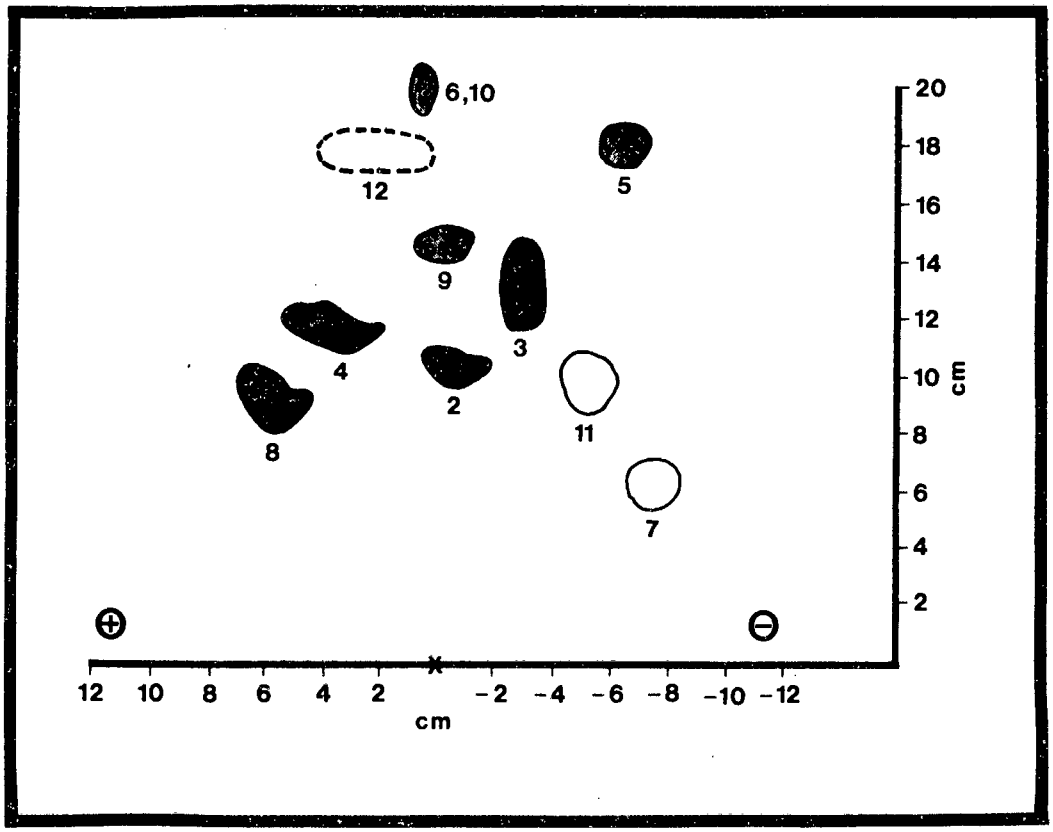

Fig. 2.6 : Peptide mapping of the tryptic peptides of TMV protein by high voltage electrophoresis and ascending chromatography on paper. 1 mg of the pH 4.5 soluble portion of a tryptic digest of TMV protein was applied to the origin on a 35x40 cm sheet of Whatman 3 Chroma paper, at the position marked x. After 2,5 hrs electrophoresis (1000 V, 45 mA) at pH 6,4 the paper was dried and subjected to ascending chromatography in pyridine, iso-amylalcohol and water (5:4:4). Numbers associated with the spots refer to the tryptic peptides. The staining colour of peptides 2,3,4,5,8 and 9, as well as 6 and 10, was purple, peptide 11 was yellow, peptide 7 was grey and peptide 12 did not stain with ninhydrin. Peptides 3 and 12 were stained with Erlichs reagent (Jepson, 1963) and only peptides 4 and 12 did not fluoresce under UV light after staining with pheneanthrenequinone (Yamada and Itano, 1966). The electrophoretic and chromatographic mobilities of the tryptic peptides from the TMV mutants, 414, CP 415, Ni 118, Ni 568, Ni 1688 and Ni 1927 did not differ significantly from the corresponding peptides of TMV and practically identical peptide maps were obtained with all these mutants except for peptide 11 of CP 415 which stained purple with ninhydrin instead of a characteristic yellow.

(residues 142-158) did not develop colour with the ninhydrin reagent. The two tryptophan containing peptides, 3 and 12, developed a violet colour with Ehrlichs reagent (Jepson, 1963) and all the peptides, except peptides 4 and 12 which do not contain arginine, could be made to fluoresce in ultraviolet light with a wavelength of 266 nm by treatment with pheneantrenequinone (Yamada and Itano, 1966)

2.2.1.2 PEPTIDE MAPPING OF THE pH 4,5 SOLUBLE TRYPTIC PEPTIDES OF THE PROTEINS OF THE TMV MUTANTS

The pH 4,5 soluble tryptic peptides of each of the six TMV mutants used in this work (viz. 414, CP 415, Ni 118, Ni 568, Ni 1688 and Ni 1927) were prepared (4.1.4.1) and subjected to peptide mapping (4.2.1).

None of the peptides, which contained amino acid exchanges, was found to have an electrophoretic or chromatographic mobility which was significantly different from that of the corresponding peptides of the wild type virus protein. Similarly, the mutant peptides stained identically with ninhydrin and Erlichs reagent (Jepson, 1963) to their wild type homologues, except for peptide 11 (residues 135-140) of CP 415 protein (asparagine in position 140 is replaced by lysine) which stained purple with ninhydrin rather than the yellow colour that is characteristic of the wild type peptide 11 (Woody and Knight, 1959; Benjamini et al., 1964).

2.2.1.3 PEPTIDE MAPPING OF INDIVIDUAL pH 4,5 SOLUBLE TRYPTIC PEPTIDES

Tryptic peptides isolated by the high voltage electrophoresis method (4.1.4.3.2) have been characterized as regards their electrophoretic mobility and purity during the preparative process. Only peptide bands with characteristic electrophoretic mobilities, which were sufficiently

separated from the adjacent bands, were extracted from the paper. Nevertheless, representative peptide preparations were subjected to peptide mapping to ascertain that single spots, with the correct electrophoretic and chromatographic mobilities and staining characteristics, resulted. Similarly, if the amino acid composition of an electrophoretically prepared peptide was doubtful, a peptide map was made to check the purity of the preparation.

2.2.2 AMINO ACID COMPOSITION

2.2.2.1 AMINO ACID COMPOSITION OF THE PROTEINS OF THE TMV MUTANTS

The amino acid composition after acid hydrolysis (4.2.2) of the proteins of the six TMV mutants used in this work is given in table 2.1. It can be seen that, although the general composition of the proteins is practically identical, the amino acid replacements characterizing each mutant are clearly demonstrated.

2.2.2.2 AMINO ACID COMPOSITION OF THE TRYPTIC PEPTIDES OF TMV PROTEIN AND THE PROTEINS OF THE TMV MUTANTS, AS WELL AS A DERIVATIVE OF PEPTIDE 12 (RESIDUES 142-157)

Typical amino acid compositions of each of the tryptic peptides from TMV protein, a carboxypeptidase A treated derivative of peptide 12 (residues 142-157) and the tryptic peptides of the TMV mutants, that were used in this work, are shown in tables 2.2 and 2.3.

No evidence of any significant cross-contamination between the various pH 4,5 soluble tryptic peptides could be found even when individual peptides were subjected to peptide mapping (4.2.1). The low level of contamination is thought to be the result of amino acids extracted from the paper simultaneously with the peptides.

Amino acid composition[a] of the proteins of TMV mutants

Amino Acids	414-Protein (Residue 65:Ser→Gly)		CP 415-Protein (Residue 140:Asn→Lys)		Ni 118-Protein (Residue 20:Pro→Leu)		Ni 568-Protein (Residues 5:Thr→Ile 107:Thr→Met)		Ni 1688-Protein (Residues 63:Pro→Ser 156:Pro→Leu)		Ni 1927-Protein (Residue 156:Pro→Leu)	
	Expected	Found	Expected	Found	Expected	Found	Expected	Found	Expected	Found	Expected	Found
Tryptophan[a]	3	0,61	3	0,42	3	0,70	3	0,56	3	0,50	3	0,56
Lysine	2	2,04	3	3,04	2	1,99	2	2,03	2	2,03	2	2,04
Arginine	11	10,61	11	10,67	11	10,58	11	10,62	11	10,61	11	10,78
Aspartic acid	18	18,09	17	18,03	18	18,04	18	17.73	18	18.33	18	18.11
Threonine[b]	16	13,79	16	15,39	16	13,75	14	12,94	16	13,47	16	13,85
Serine[b]	15	12,46	16	13,13	16	13,83	16	13,62	17	12,39	16	14,57
Glutamic acid	16	17,05	16	16,84	16	16,96	16	16,63	16	17,11	16	17,04
Glycine	7	6,82	6	5,81	6	5,81	6	5,81	6	5,86	6	5,81
Alanine	14	15,14	14	13,70	14	14,16	14	14,09	14	14,05	14	14,16
Valine	14	12,70	14	13,41	14	13,43	14	13,66	14	14,25	14	13,60
Isoleucine[b]	9	7,81	9	7,99	9	7,65	10	7,60	9	7,08	9	7,93
Leucine	12	12,16	12	12,18	13	13,12	12	12,07	13	13,13	13	13,27
Tyrosine	4	3,84	4	3,92	4	3,89	4	3,86	4	3,90	4	3,82
Phenylalanine	8	7,89	8	7,94	8	7,93	8	7,93	8	7,94	8	8,01
Proline	8	8,19	8	8,40	7	7,47	8	8,54	6	5,77	7	7,46
Methionine	0	0	0	0	0	0	1	0,89	0	0	0	0

[a] Peptides were hydrolyzed in 6 M HCl for 20 hrs at 105°C. The tryptophan residues were partially protected from degradation by the addition of some thioglycollic acid.

[b] No corrections were made for hydrolytic losses or incomplete cleavage. The pair of isleucine residues in positions 4 and 5 of NI 568 protein, and positions 93 and 94 of all the proteins are not cleaved during hydrolysis.

Table 2.1 : Amino acid composition in molar ratios of the proteins of six mutants of TMV that have known amino acid exchanges (Henning and Wittmann, 1972).

Amino acid composition[a] of tryptic peptides[b] and a derivative[c] of peptide 12 of TMV protein

Amino Acid	Residues 1-41 Peptide 1		Residues 62-68 Peptide 4		Residues 93-112 Peptide 8		Residues 135-141 Peptide 11		Residues 142-158 Peptide 12		Residues 142-157 C.T.[c] peptide 12	
	Expected	found	Expected	found	Expected	found	Expected	found	Expected	found	Expected	found
Tryptophan[a]	1	0,51	0	0	0	0	0	0	1	0,04	1	0,35
Lysine	0	0,09	1	0,94	0	0,06	0	0	0	0,12	0	0,42
Arginine	1	0,93	0	0	1	0,97	1	1,52	0	0,02	0	0,37
Aspartic acid	4	4,92	2	2,26	3	2,95	1	0,74	0	0,93	0	0,76
Threonine[a]	4	2,67	0	0,42	4	3,65	1	1,04	2	1,72	1	0,62
Serine[a]	5	3,05	1	1,06	0	0,21	1	0,69	6	2,78	6	3,22
Glutamic acid	6	5,69	0	0,27	4	4,29	0	0,49	1	1,40	1	1,04
Glycine	1	1,33	0	0,13	0	0,30	2	2,05	2	1,53	2	1,95
Alanine	4	4,44	0	0,41	3	2,95	0	0	1	1,30	1	0,90
Valine	1	1,20	0	0,33	1	1,06	0	0	1	1,31	1	1,15
Isoleucine[a]	3	2,57	0	0,18	2	0,89	0	0	0	0,35	0	0,63
Leucine	4	4,77	0	0,25	1	0,95	0	0	1	1,76	1	2,71
Tyrosine	1	0,44	0	0,06	0	0	1	0,98	0	0,31	0	0,30
Phenylalanine	3	2,35	2	1,87	0	0	0	0	1	1,07	1	1,02
Proline	2	2,27	1	0,94	1	1,07	0	0	1	1,06	1	0.98

[a]Peptides were hydrolyzed in 6 M HCl for 20 hrs at 105°C. The tryptophan residues were partially protected from degradation by the addition of some thioglycollic acid. No corrections were made for hydrolytic losses or incomplete cleavage. The pair of isleucine residues in positions 93 and 94 of peptide 8 are not cleaved during hydrolysis.

[b]Peptide 1 was prepared by isoelectric precipitation at pH 4,5 and extensive dialysis against water, and peptides 4,8,11 and 12 by high voltage paper electrophoresis.

[c]Peptide 12 was treated with carbosypeptidase A to remove the C-terminal threonine.

Table 2.2 : Amino acid composition in molar ratios of five of the tryptic peptides of TMV protein that are used for immunochemical studies.

Amino acid composition[a] of tryptic peptides[b] of TMV mutants.

Amino Acids	Ni 118-Peptide 1 Residues 1-41 (Residue 20:Pro→Leu)		Ni 568-Peptide 1 Residues 1-41 (Residue 5:Thr→Ile)		414-Peptide 4 Residues 62-68 (Residue 65:Ser→Gly)		Ni 1688-Peptide 4 Residues 62-68 (Residue 63:Pro→Ser)		Ni 568-Peptide 8 Residues 93-112 (Residue 107:Thr→Met)		Ni 1688-Peptide 12 Residues 142-158 (Residue 156:Pro→Leu)		Ni 1927-Peptide 12 Residues 142-158 (Residue 156:Pro→Leu)	
	Expected	Found	Expected	Found	Expected	Found	Expected	Found	Expected	Found	Expected	Found	Expected	Found
Tryptophan[a]	1	0,64	1	0,49	0	0	0	0	0	0	1	0,01	1	0,04
Lysine	0	0,06	0	0,11	1	0,68	1	0,94	0	0,18	0	0,07	0	0,07
Arginine	1	0,56	1	0,29	0	0,22	0	0,19	1	0,89	0	0,18	0	0,19
Aspartic acid	4	4,36	4	4,90	2	1,79	2	2,63	3	3,36	0	0,99	0	1,08
Threonine[a]	4	3,09	3	2,37	0	0,57	0	0,40	3	2,81	2	1,32	2	1,40
Serine[a]	4	2,72	4	3,65	0	0,39	2	1,92	0	0,17	6	2,40	6	2,40
Glutamic acid	6	5,09	6	4,75	0	0,32	0	0,34	4	3,95	1	0,88	1	0,93
Glycine	1	1,15	1	1,32	1	1,08	0	0,25	0	0,30	2	1,01	2	1,02
Alananine	4	3,67	4	3,78	0	0,78	0	0,53	3	2,67	1	1,15	1	1,16
Valine	1	1,14	1	1,19	0	0,68	0	0,42	1	1,04	1	1,14	1	1,20
Isoleucine[a]	3	2,72	4	3,39	0	0,22	0	0,15	2	0,98	0	0.22	0	0,29
Leucine	5	5,37	4	4,84	0	0,48	0	0,46	1	0,96	2	2,01	2	2,02
Tyrosine	1	0,45	1	0,77	0	0,12	0	0,01	0	0	0	0,38	0	0,50
Phenylalanine	3	2,61	3	2,75	2	1,87	2	2,20	0	0	1	0.83	1	1,00
Proline	1	1,00	2	2,15	1	1,09	0	0,19	1	1,12	0	0,47	0	0,44
Methionine	0	0	0	0	0	0	0	0	1	1,05	0	0	0	0

[a]Peptides were hydrolyzed in 6M HCl for 20 hrs at 105°C. The tryptophan residues were partially protected from degradation by the addition of some thioglycollic acid. No corrections were made for hydrolytic losses or incomplete cleavage. The pair of isoleucine residues in positions 4 and 5 of Ni 568-peptide 1 and positions 93 and 94 of Ni 568-peptide 8 are not cleaved during hydrolysis.

[b]Both Ni 118 and Ni 568-peptide 1 were prepared by isoelectric precipitation at pH 4,5 and extensive dialysis against water, and all the other peptides by high voltage paper electrophoresis.

Table 2.3 : Amino acid composition in molar ratios of tryptic peptides, from the proteins of TMV mutants, which correspond to those peptides used for immunochemical studies.

The three tryptic I-peptides from TMV, Ni 118 and Ni 568, were submitted to SDS-polyacrylamide gel electrophoresis on 18% (w/v) slab gels (4.2.3) to test for virus protein contamination (fig. 2.7). All three I-peptides migrated as single bands with the same electrophoretic mobility, and, even when the gels were overloaded with the peptides, no sign of a virus protein band was detectable.

2.3 THE EFFECT OF CHANGES IN THE C-TERMINAL CONFIGURATION OF TMV PROTEIN ON THE CROSS-REACTION BETWEEN TMV AND TMV A-PROTEIN

2.3.1 CHANGES IN THE C-TERMINAL CONFIGURATION OF TMV A-PROTEIN BETWEEN pH 7,0 AND pH 8,0

Near-ultraviolet circular-dichroism absorption spectra of TMV A-protein at 20°C, pH 7,0 and pH 8,0, show a change in their 280-295 nm wavelength range (Vogel, 1973; Vogel and Jaenicke, 1974). This is likely to be associated with a localized structural change, as only tyrosine and tryptophan overlap in this spectral range, and tryptophan, which is the only species absorbing above 290 nm, shows only a slight change in the intensity of the 295 nm-band. This indicates that it is not likely to be involved in the structural change (Vogel, 1973). The involvement of the tyrosine in position 139, near the C-terminus, in the formation of the 20S disk (Ohno *et al.*, 1972), and the observation that the exchange of proline in position 156 for leucine (in the mutant Ni 1927) causes a comparable change in dichroic absorption (Vogel and Jaenicke, 1974), points to the C-terminal region of the TMV protein being involved in the localized structural change which occurs between pH 7,0 and pH 8,0 (Vogel and Jaenicke, 1974).

As the C-terminal residues of the TMV protein subunit in the virus are known to be involved in an antigenic determinant of the virus (Harris and

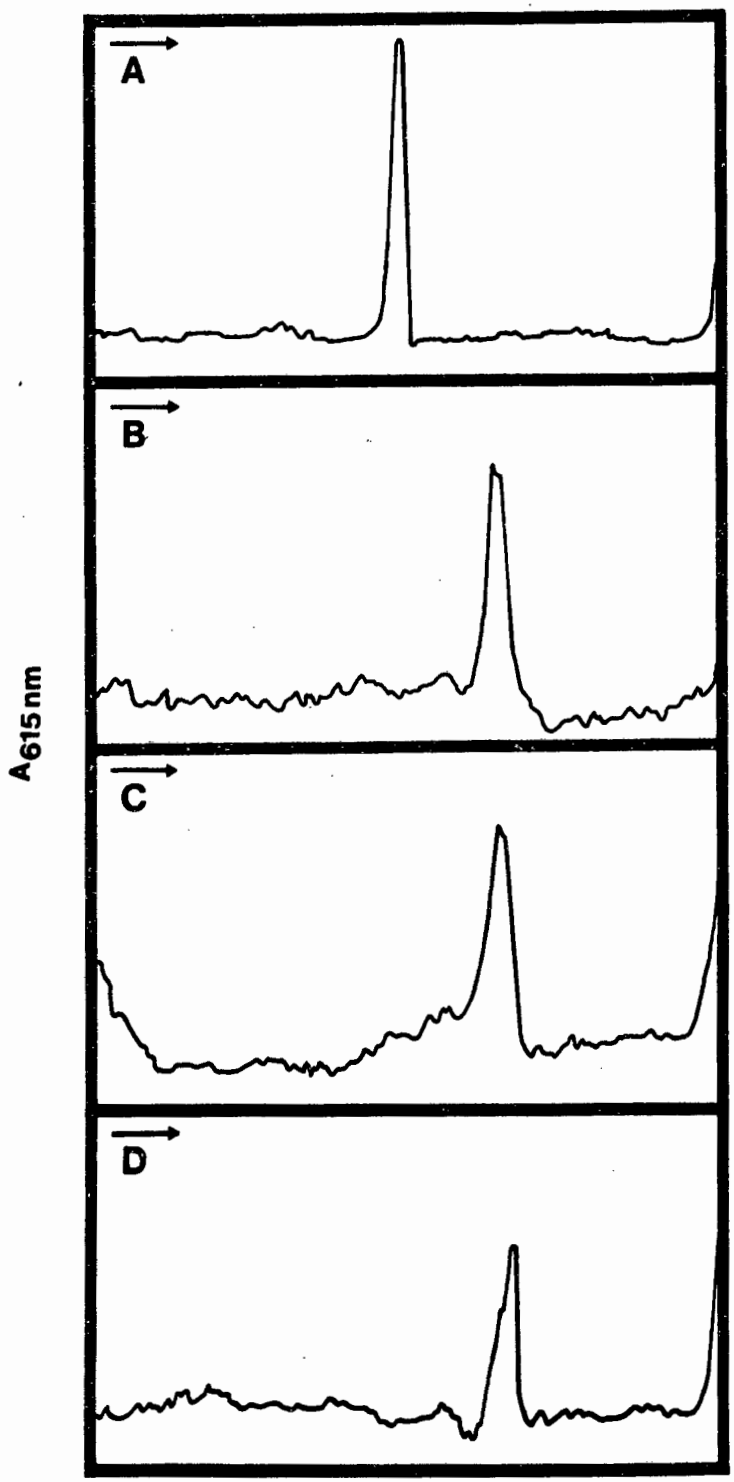

Fig. 2.7 : Densitometer traces of TMV protein and the three tryptic l-peptides electrophoresed in one slab of 18% (w/v) polyacrylamide 1% (w/v) SDS gel and the bands stained using Coomassie brilliant blue. The arrow indicates the direction of electrophoresis from the top of the gel on the left hand side to the bottom on the right. (A) 5 µl of a 1,0 mg/ml solution of TMV protein. (B) 20 µl of a stock solution of TMV l-peptide. (C) 20 µl of a stock solution of Ni 118 l-peptide. (D) 15 µl of a stock solution of Ni 568 l-peptide.

Knight, 1955; von Sengbusch and Wittmann,1965; von Sengbusch, 1965; and van Regenmortel, 1967), it was decided to investigate the effect of the conformational change in the C-terminal region of the TMV A-protein on the cross-reaction between the intact virus and the A-protein. To ensure the validity of the results, the problem of virus disaggregation and specificity of the reaction between the anti-TMV antibodies and the A-protein had to be solved.

2.3.2 A MICRO-PRECIPITIN ASSAY WITH RADIOLABELLED TMV TO ENSURE THAT ONLY THE SPECIFIC REACTION BETWEEN TMV AND ANTI-TMV ANTIBODIES IS MEASURED.

[4,6-^{3}H] uridine was incorporated into TMV RNA in infected plants by the same basic method that was used by Singer (1971) to incorporate radioactive amino acids into TMV protein (4.3.4.1.1). Once the radiolabelled virus had been extracted from the plants it was found that 10% of the (^{3}H-)uridine had been incorporated into the virus, which had a specific activity of 3,67 x 10^{6} dpm/mg. The ultraviolet spectrum of the radioactive virus was identical to that of unlabelled TMV.

Fig. 2.8 shows the precipitin reaction between 0,5 µg of (^{3}H)uridine-TMV and increasing dilutions of a TMV antiserum. After the antibody-TMV precipitate had formed, it was removed by low speed centrifugation and the amount of radioactivity in 1,0 ml of the supernatant, which is proportional to the amount of unbound (^{3}H)uridine-TMV, was plotted against the reciprocal of the antiserum dilution. The first part of the curve corresponds to the zone of antibody excess where all the (^{3}H)-uridine-TMV is bound by antibody and removed from the solution by precipitation. The inflection point of the curve indicates equivalence proportions, and from here on there is insufficient antibody to precipitate

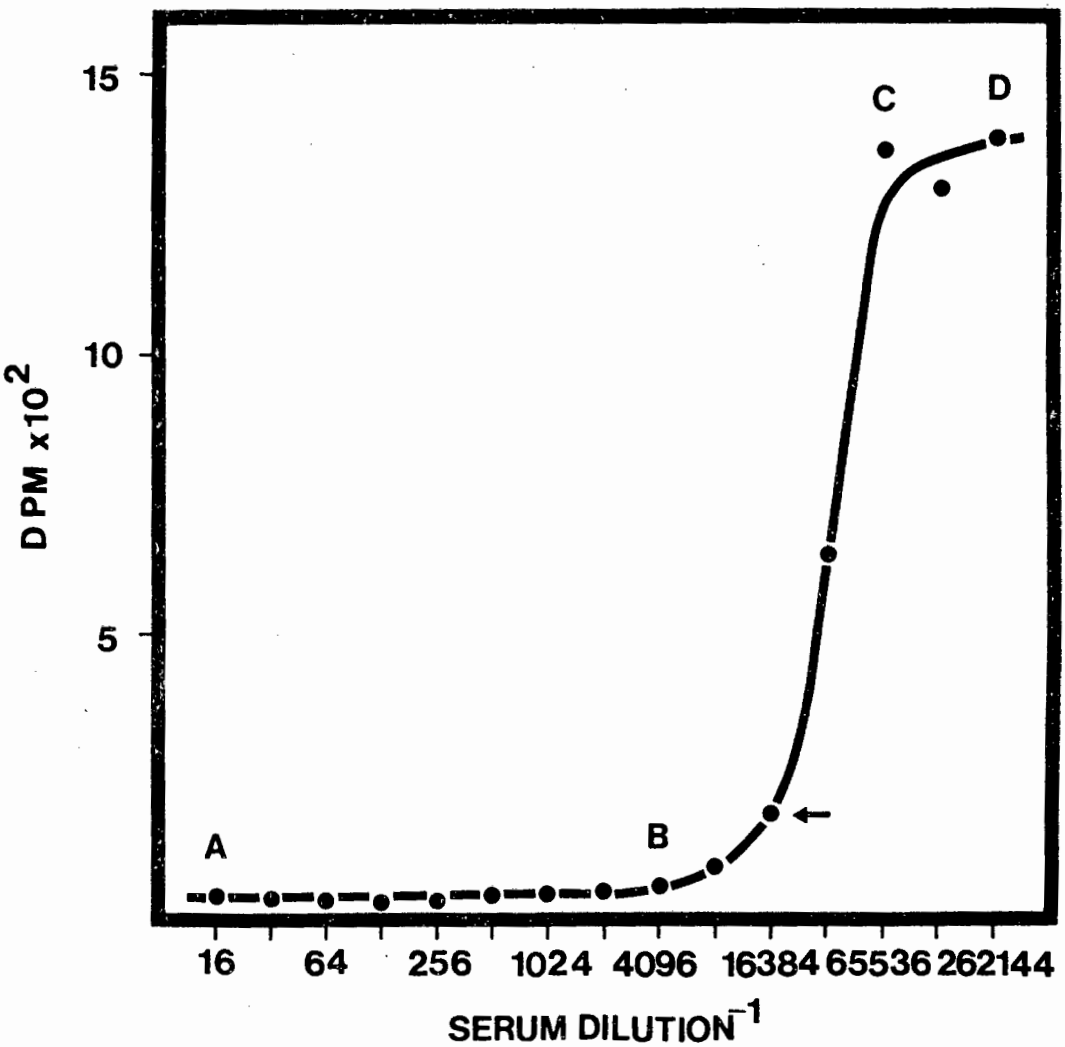

Fig. 2.8 : A precipitin curve of the reaction between 0,5 µg of (^{3}H) uridine-TMV and increasing dilutions of a TMV antiserum. The amount of radioactivity in 1,0 ml of the supernatant, after the removal of the precipitate by centrifugation, was plotted against the reciprocal of the antiserum dilution. The region of the curve between A and B corresponds to the zone of antibody excess, B indicates the zone of equivalence and the region between B and C corresponds to the zone of antigen excess. The region from C to D corresponds to the zone in which soluble antibody-antigen complexes occur. The antibody:antigen proportions chosen for the precipitation-inhibition tests are indicated by the arrow. This point is in the zone of antigen excess, near the equivalence region.

all the (^{3}H)uridine-TMV which starts appearing in the supernatant in increasing amounts. This is the zone of antigen excess. The proportions of (^{3}H)uridine-TMV and antiserum, used for inhibition tests, were chosen to be in the zone of antigen excess, near the equivalence point, so that antibodies would be divalently bound and thus more easily prevented from precipitating the radiolabelled antigen in the presence of inhibitor.

The advantage of this system, apart from the obvious ease of working with the supernatant rather than the precipitate, is that, due to the location of the radioactive label in the RNA, only the reaction between TMV and its specific antibodies is measured. Hence the reaction between anti-TMV protein antibodies and TMV protein subunits, dissociated from the virus, is not detectable and cannot influence the measurement of the TMV-anti TMV reaction. Thus the inhibition which is measured, is specific for the TMV-anti TMV system. Another advantage is that the inhibition of the specific precipitin reaction by non-haptenic molecules is possible with this system. Unlabelled antigens such as TMV protein and even intact TMV particles were found to be successful inhibitors of the reaction between (^{3}H)uridine-TMV and specific anti-TMV antibodies.

In experiments where the TMV-anti TMV reaction was inhibited, it was decided to express the concentration of the inhibitor in terms of "TMV equivalents", where one TMV equivalent is equal to the amount of TMV, used per test in n-moles, multiplied by 2130 (the number of subunits in a TMV capsid). The rationale behind this choice is that, although for reasons of steric hindrance the valence of the virus is approximately 800 (Hardie and van Regenmortel, 1975; van Regenmortel and Hardie, 1976),

each subunit of the virus has on its surface identical antigenic determinants which are equally attractive to the antibodies specific for them irrespective of the particular subunit. The antigenic regions of the inhibitor compete with every available antigenic determinant on the virus and the number of available virus antigenic determinants increases as inhibition tends towards a maximum.

2.3.3 THE EFFECT OF pH ON THE ABILITY OF TMV PROTEIN AND Ni 1927 PROTEIN TO INHIBIT THE REACTION BETWEEN RADIOLABELLED TMV AND SPECIFIC ANTI-TMV ANTIBODIES

The proportions of (^{3}H)uridine-TMV and TMV specific antibodies most suitable for inhibition experiments were determined as described in sections 2.3.2 and 4.3.4.2.5 (Fig. 2.8). The precipitin reaction between 0,5 µg of (^{3}H)uridine-TMV and 0,5 ml of a $^{1}/_{16384}$ dilution of a TMV antiserum was inhibited by increasing concentrations of TMV protein and Ni 1927 protein, which were preincubated with the antiserum for 16-18 hrs at +4°C. This was done under two different pH conditions for each of the inhibitors, viz. pH 6,8 and pH 8,0. A complete set of maximum and minimum (^{3}H)uridine-TMV binding controls was included at each pH and the percentage inhibition for each test was calculated from the controls carried out at the same pH as the test (4.3.4.3.1).

Fig. 2.9 illustrates the effect of pH, and hence the C-terminal configuration of the virus proteins (2.3.1), on the cross-reaction of TMV protein and Ni 1927 protein with (^{3}H)uridine-TMV. Complete inhibition of the reaction between (^{3}H)uridine-TMV and TMV specific antibodies was obtained with TMV protein at pH 6,8 and pH 8,0 as well as Ni 1927 protein at pH 8,0, but not with Ni 1927 protein at pH 6,8.

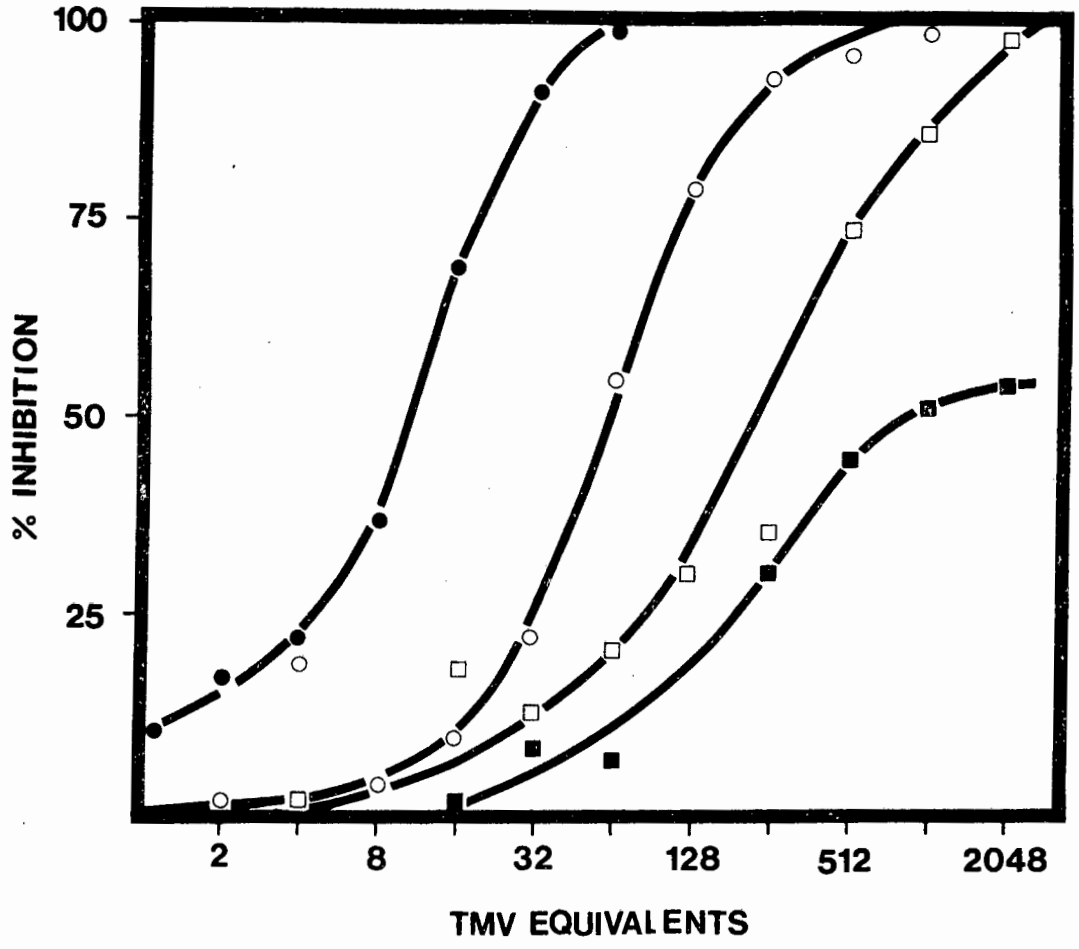

Fig. 2.9 : The effect of pH on the cross-reaction between TMV and TMV protein, and TMV and Ni 1927 protein. The reaction between radiolabelled TMV and specific anti-TMV antibodies was inhibited by increasing concentrations of TMV protein and Ni 1927 protein in different pH conditions. The amount of inhibitor is expressed in TMV equivalents, where one equivalent is equal to the amount of radiolabelled TMV used per test multiplied by 2130 - the number of subunits in a virus particle. The amount of inhibition per test was calculated from controls carried out at the same pH as the test. (●) TMV protein at pH 6,8, (○) TMV protein at pH 8,0, (■) Ni 1927 protein at pH 6,8, and (□) Ni 1927 protein at pH 8,0.

TMV protein at pH 6,8, which is in the conformational form closest to the subunits of the 20S disk (Vogel, 1973; Vogel and Jaenicke, 1974) and hence closest to the subunits of the intact virus (Graham and Butler, 1978), is the best inhibitor with 11,3 TMV equivalents (corresponding to a 24080 molar excess of TMV protein over virus) causing 50% inhibition. TMV protein at pH 8,0, which has a C-terminal configuration different from that of the subunits of the 20S disk (Vogel, 1973; Vogel and Jaenicke, 1974) and the subunits of the intact virus (Graham and Butler, 1978) is less efficient as an inhibitor of the TMV-anti-TMV reaction, for 64,0 TMV equivalents of protein (corresponding to a 136320 molar excess of TMV protein over virus) are required to achieve 50% inhibition.

In the case of Ni 1927 protein, which has the proline residue 156 replaced by leucine, the position is reversed. 215,3 TMV equivalents of Ni 1927 protein at pH 8,0 (i.e. a molar excess of 458589 protein subunits for every TMV particle) in contrast to 861,1 TMV equivalents of Ni 1927 protein at pH 6,8 (i.e. a molar excess of 1834143 protein subunits for every TMV particle) are required for 50% inhibition of the reaction between (^{3}H)uridine-TMV and TMV specific antibodies.

In an effort to determine whether the specific inhibition described above could possibly be due to the aggregation of the TMV and Ni 1927 proteins into 20S disks or RNA free helices (which would be antigenically identical to the virus) 0,5 mg/ml and 1,0 mg/ml solutions of the proteins at pH 6,8 and pH 8,0 respectively, were subjected to conditions identical to those of the inhibition assays and submitted to an ultracentrifugal analysis at 40000 rpm and 56000 rpm using an AnD rotor with a standard 12 mm single sector centrepiece in a Beckman model E analytical ultra-

centrifuge (4.2.4). The sedimentation coefficients of the TMV and Ni 1927 protein preparations were calculated by the method of Chervenka (1969) from photographs taken with the schlieren optical system at various intervals. No protein aggregates with sedimentation coefficients significantly greater than 4S could be detected in any of the protein preparations. The highest concentration of protein inhibitor used in the inhibition assay was 0,64 mg/ml, and it is therefore unlikely that any large aggregates were present during the tests.

When a different TMV antiserum with a lower precipitin titre was used in a similar inhibition assay, the results obtained were not substantially different from those illustrated in fig. 2.9, except that higher concentrations of the inhibitor were required to obtain equivalent degrees of inhibition of the radiolabelled TMV-anti-TMV system.

2.4 IMMUNOCHEMICAL STUDIES WITH THE PROTEINS OF TMV MUTANTS

2.4.1 A MEANS OF DETERMINING THE ROLE OF SPECIFIC AMINO ACIDS IN THE ANTIGENICITY OF TMV PROTEIN

Von Sengbusch (1965) concluded from serological studies with mutants of TMV that not all of the amino acid exchanges, which were serologically distinguishable from TMV, could be in antigenic determinants, as the surface of each protein subunit in the viral helix was not large enough. Some of the amino acid exchanges would affect the immunological reactions of the mutant virus indirectly by causing a subtle modulation of the conformation of the protein subunit. This was borne out by X-ray crystallographic studies on the virus (Holmes *et al.*, 1975) and the 20S disk (Champness *et al.*, 1976; Bloomer *et al.*, 1978) which showed that residue 107, which is serologically distinguishable when threonine is replaced by methionine (von Sengbusch, 1965), is located near the core of the viral

helix, far from the surface of the subunit (1.3.4).

It was thought that a similar situation may exist in the TMV protein system. In order to determine which amino acid exchanges affected the immunological reactivity of the TMV protein by altering its conformation, and which by directly changing antigenic determinants, six mutants of TMV, which had amino acid exchanges in one or more of the five tryptic peptides utilized in this work, were selected for study. An immunochemical comparison of the reactivity of these mutant proteins with TMV protein would determine which amino acid exchanges were serologically distinguishable in the TMV protein-anti-TMV protein system (2.4.3). Meanwhile, the immunochemical reactivity of mutant protein peptides, which contained an amino acid exchange, would be compared with the equivalent peptides from TMV protein using the same antisera (2.6.1). Serologically distinguishable amino acid exchanges which affect the reactivity of the mutant protein, but not the corresponding random coil peptide, would be likely to exert their influence by modulating the protein conformation, while those amino acid exchanges which affected the reactivity of both the protein and the peptide would be likely to form a part of an antigenic determinant.

In this way information about the role of specific amino acids in the conformation and the antigenicity of TMV protein would be obtained. This section deals with the results of the immunochemical comparison of the mutant proteins with the TMV protein.

2.4.2 A MICRO-PRECIPITIN ASSAY USING RADIOLABELLED TMV PROTEIN

The following tritiated amino acids were incorporated into TMV protein in infected plants by the method of Singer (1971): L-[2,3-^{3}H] alanine,

L-[4,5(n)-^{3}H] isoleucine, L-[4,5-^{3}H] leucine, L-[3-^{3}H] serine and L-[3,4(n)-^{3}H] valine (4.3.4.1.2). Once the virus had been extracted from the plants and TMV protein prepared, it was found that 4.1% of the radiolabelled amino acids had been incorporated into the virus protein which had a specific activity of $5,67 \times 10^{6}$ dpm/mg. The ultraviolet spectrum of the radiolabelled protein was identical to that of unlabelled TMV protein.

The micro-precipitin assay with the radiolabelled TMV protein was identical to the system described in section 2.3.2, except for the different antigen and pH at which the reaction took place. pH 7,4 was maintained in the radiolabelled TMV protein assays compared to pH 6,8 which was used in the (^{3}H)uridine-TMV assays (4.3.4.2.5). Fig. 2.10 shows the precipitin reaction between 0,5 µg of radiolabelled TMV protein and increasing dilutions of a TMV protein antiserum. The precipitate, which formed, was removed by low speed centrifugation and the amount of radioactivity in 1,0 ml of the supernatant was monitored and plotted against the reciprocal of the antiserum dilution. The first part of the curve, where there is relatively little radiolabelled antigen in the supernatant, corresponds to the zone of antibody excess. The region of the curve immediately before the inflection point corresponds to the equivalence zone and the next part of the curve, where the amount of radiolabelled antigen in the supernatant increases, corresponds to the zone of antigen excess. The final plateau of the curve where the amount of radiolabelled antigen in the supernatant is maximal, corresponds to the zone in which soluble antibody-antigen complexes are formed. Inhibition studies were performed using the proportions of antibody and radiolabelled antigen that corresponded to a point in the zone of antigen excess, near the equivalence zone, so that the competition for

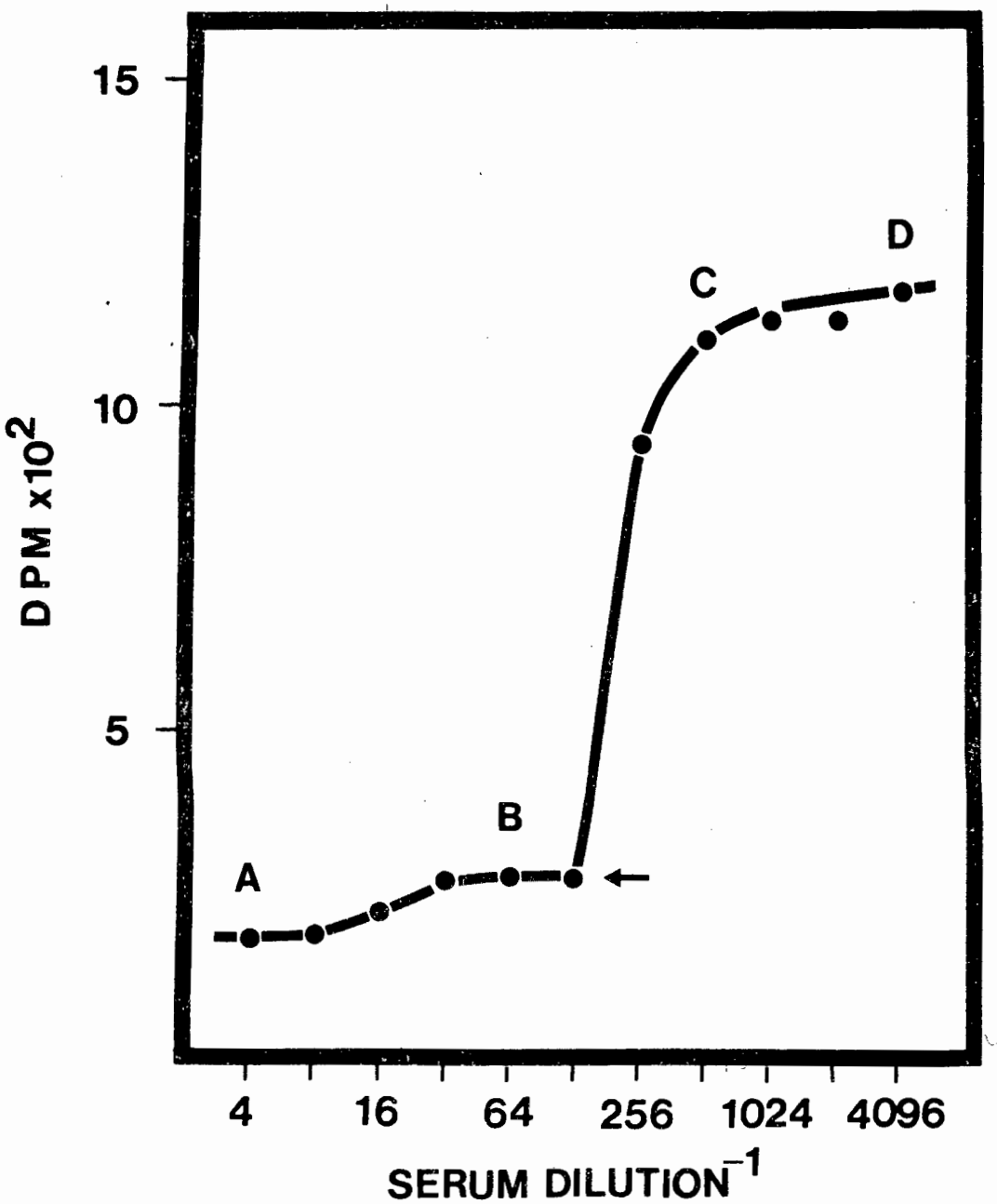

Fig. 2.10 : A precipitin curve of the reaction between 0,5 µg of radiolabelled TMV protein, into which tritiated amino acids were incorporated, and increasing dilutions of a TMV protein antiserum. The amount of radioactivity in 1,0 ml of the supernatant, after removal of the precipitate by centrifugation, was plotted against the reciprocal of the antiserum dilution. The region of the curve between A and B corresponds to the zone of the antibody excess, B indicates the zone of equivalence and the region between B and C corresponds to the zone of antigen excess. The region from C to D corresponds to the zone in which soluble antibody-antigen complexes occur. The antibody:antigen proportions chosen for the precipitation-inhibition tests are indicated by the arrow. This point is near the equivalence point, in the zone of antigen excess.

antibody between the inhibitor and the radiolabelled antigen would increase the sensitivity of the assay.

Similar to the (^{3}H)uridine-TMV micro-precipitin assay, the chief advantage of this system is that only the specific binding of antibodies to the radiolabelled TMV protein can be measured, and therefore inhibited. Also the system is not limited to hapten inhibition, so any unlabelled protein or peptide inhibitor can be tested for its cross-reaction with specific anti-TMV protein antibodies.

The potential aggregation of the radiolabelled TMV protein and other free virus proteins to the 20S disk or protein helices can be a problem if the concentration of the proteins is allowed to become too high (Klug and Durham, 1971). Ultracentrifugal studies on TMV protein (4.2.4) have shown that even a 1,0 mg/ml solution of the protein at pH 6,8 and physiological ionic strength had a sedimentation coefficient not greater than 4S (2.3.3). The total virus protein concentration in inhibition assays was kept as low as 1,0 µg/ml at the same ionic strength and a higher pH, so aggregation should be negligible in this system.

2.4.3 A COMPARISON OF THE ABILITIES OF TMV PROTEIN AND SIX TMV MUTANT PROTEINS TO INHIBIT THE PRECIPITIN REACTION OF RADIOLABELLED TMV PROTEIN WITH SPECIFIC ANTIBODIES

The proportions of radiolabelled TMV protein and anti-TMV protein antibodies most suitable for inhibition experiments were determined as described in sections 2.4.2 and 4.3.4.2.5 (fig. 2.10). The precipitin reaction 0,5 µg of radiolabelled TMV protein and 0,5 ml of a 1/128 dilution of a TMV protein antiserum was inhibited by increasing concentrations of TMV protein, or one of six TMV mutant proteins, which were

preincubated with the antiserum for 16-18 hrs at +4°C. The percentage inhibition was calculated from maximum and minimum radiolabelled TMV protein binding controls carried out under the same conditions as the inhibition assays (4.3.4.2.6).

The results of the inhibition assays are shown in fig. 2.11 A and B. The precipitin reaction between radiolabelled TMV protein and anti-TMV protein antibodies was efficiently inhibited by unlabelled TMV protein, with 75% inhibition being caused by 0,024 µg (1,4 p-moles) of the unlabelled protein. This is equivalent to a 0,047 molar excess of unlabelled over radiolabelled TMV protein. Complete inhibition was obtained with 1,0 µg of unlabelled TMV protein which is equivalent to a 2 molar excess of unlabelled TMV protein. The inhibition obtained with the mutant proteins, 414 (serine replacing glycine in position 65) and CP 415 (lysine replacing asparagine in position 140), was not significantly different from that obtained with unlabelled TMV protein. In both cases 75% inhibition was obtained with a 0,047 molar excess of mutant protein over radiolabelled TMV protein. The results with CP 415 protein concur with the finding of du Plessis and van Regenmortel (1977) that the exchange of lysine for asparagine in position 140 is not serologically distinguishable in TMV protein. In the case of 414 protein, if a serological difference between it and TMV protein exists, this assay is not sufficiently sensitive to clearly detect it.

The remainder of the mutant proteins are serologically distinguishable from TMV protein by this method, although the difference in their inhibitory behaviour is not as great as expected. This is understandable in view of the valence of 5-6 found by van Regenmortel and Lelarge (1973).

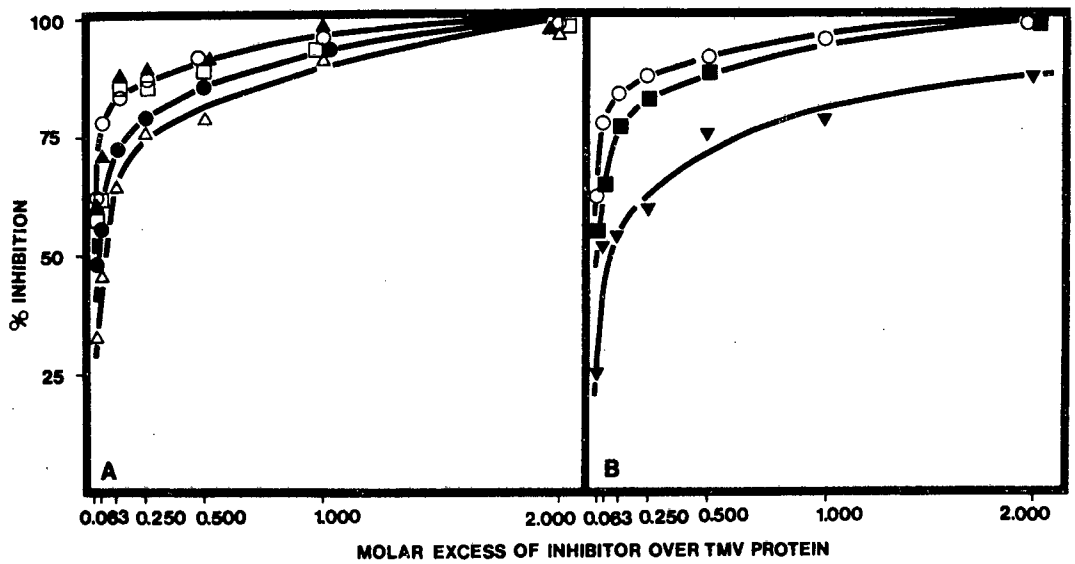

Fig. 2.11 : Quantitative inhibition of the precipitin reaction of radiolabelled TMV protein with a TMV protein antiserum by increasing concentrations of unlabelled TMV protein and mutant proteins. The concentration of the inhibitor is expressed as the molar ratio of inhibitor over the amount of radiolabelled TMV protein present in each test. (A). TMV protein (○); 414 protein (□); CP 415 protein (▲); Ni 118 protein (●); and Ni 568 protein (△). B. TMV protein (○); Ni 1688 protein (▼); and Ni 1927 protein (■).

Of the six mutant proteins, four have only one amino acid exchange (414, CP 415, Ni 118 and Ni 1927) and the rest have two amino acid exchanges (Ni 568 and Ni 1688). A maximum of two out of five antigenic determinants in the mutant proteins could therefore be affected, while specific antibodies react unhindered with the remaining antigenic determinants. Shepard et al., (1974) have also suggested that sequence homologies are more likely to be expressed in cross-reactions of the depolymerized coat proteins of plant viruses than in the undissociated virus.

In constrast to the 0,047 molar excess of unlabelled TMV protein required for 75% inhibition of the reaction of radiolabelled TMV protein and anti-TMV protein antibodies, a 0,063 molar excess (equivalent to 0,032 µg protein) of Ni 1927 protein (leucine replacing proline in position 156) was required for the same inhibition. A 0,119 molar excess (equivalent to 0,060 µg protein) of Ni 118 protein (leucine replacing proline in position 20) was required for 75% inhibition, while for Ni 568 protein (isoleucine replacing threonine in position 5 and methionine replacing threonine in position 107) and Ni 1688 protein (serine replacing proline in position 156) molar excesses of 0,250 (equivalent to 0,125 µg protein) and 0,663 (equivalent to 0,332 µg protein) respectively, were required for the same inhibition.

These results, with those of du Plessis and van Regenmortel (1977), are summarized in fig. 2.12 to show the relationship between serological distinguishability of the mutant and the position of the amino acid exchange in the sequence of the TMV protein.

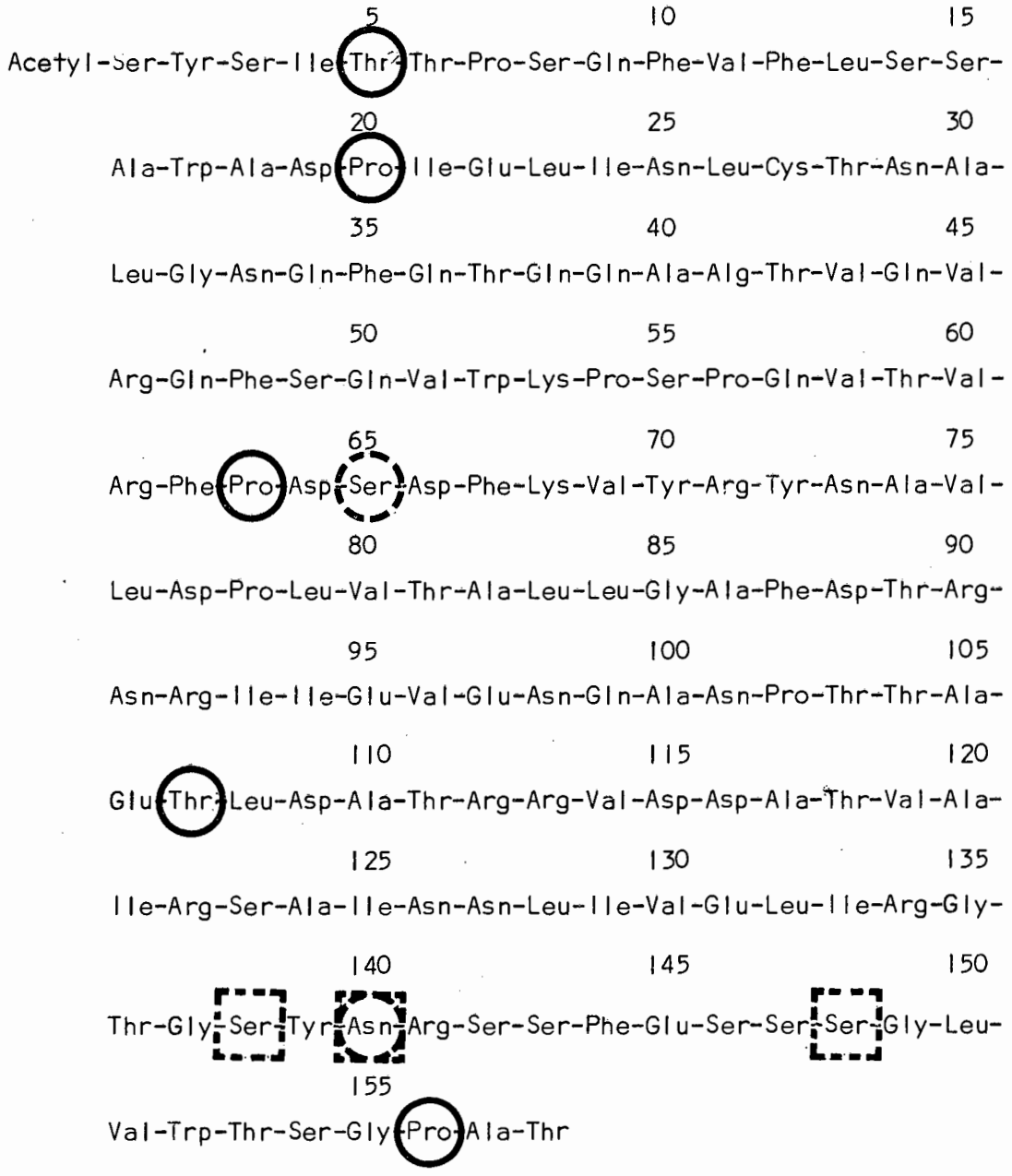

Fig. 2.12 : The primary sequence of TMV protein showing the amino acid exchanges studied in this work and by du Plessis and van Regenmortel (1977). The residues marked by circles were studied in this work, and those marked by squares, by du Plessis and van Regenmortel. Solid line symbols indicate serologically distinguisable residues while dashed symbols indicate those residues which were not serologically distinguishable from TMV protein.

2.5 IMMUNOCHEMICAL STUDIES WITH FIVE OF THE TRYPTIC PEPTIDES OF TMV PROTEIN

2.5.1 STUDIES WITH PEPTIDES IN THE TMV-ANTI TMV AND THE TMV PROTEIN-ANTI TMV PROTEIN COMPLEMENT FIXATION SYSTEMS

2.5.1.1 COMPLEMENT FIXATION AS A MEANS OF TESTING THE IMMUNOCHEMICAL ACTIVITY OF PEPTIDES

Antigen binding by certain classes and subclasses of antibody exposes a site on the Fc region of the antibody molecule which is able to bind the C'1 protein complex and activate the complement cascade. When the antibody is specific for the cell surface antigens of sheep erythrocytes this process results in cell lysis. Complement fixation by soluble antibody-antigen complexes, in the absence of sheep erythrocytes, leads to the inactivation of the initial products in the complement cascade as they are labile unless membrane bound. The principle underlying the complement fixation assay is the competition for complement between the primary reaction of antibody with soluble antigen (which is being studied) and the later reaction of specific antibodies with the membranes of the sheep erythrocytes (the indicator system).

Both IgM and IgG antibodies are able to fix complement, but unlike IgM, two adjacent molecules of IgG are required to initiate this process. As virtually all the antibodies in late-course antisera are of the IgG type, hapten binding will not result in complement fixation, but will cause a decrease in the amount of complement fixed in the presence of soluble antigen. The inhibition of complement fixation by the antibody and the intact antigen can be used as a measure of the haptenic activity of fragments of the antigen.

A particular peptide can behave in one of three possible ways with respect to complement fixation: If it has a single antigenic region it will inhibit the complement fixation caused by its antigen and specific antibody. If it contains two or more antigenic regions it will fix complement in its own right with the antibodies specific for the determinants of the whole antigen. Lastly, if the peptide is immunochemically inactive it will not affect complement fixation at all.

In the studies described below, it was found that a peptide with more than one antigenic region was able to react with antibodies specific for it and to fix complement, in the presence of the whole antigen, so to cause an enhancement of the complement fixation resulting from the reaction of the antigen with its antibodies.

2.5.1.2 COMPLEMENT FIXATION BY TMV AND TMV PROTEIN AND THEIR ANTISERA

Both TMV- and TMV protein-antibody complexes were found to fix complement in microcomplement fixation assays (4.3.2), based on the method of Levine (Wasserman and Levine, 1961; Levine and van Vunakis, 1968), irrespective of whether whole sera or globulin preparations from sera (4.3.1.3) were used. Serum titres, defined as that dilution of the antiserum which gave 75% fixation at the peak of the complement fixation curve (Champion et al., 1974), differed for the two antigens.

The titres of TMV antisera in complement fixation assays with TMV were invariably high. They ranged from $^{1}/1600$ - $^{1}/5000$ except for a globulin preparation which had been absorbed with TMV protein (4.3.1.2.2), to remove all the anti-protein activity, where the titre was reduced to $^{1}/500$. The range of antigen concentrations associated with maximal complement fixation was 0,125 - 0,500 µg TMV/ml.

The titres of TMV protein antisera were usually much lower than their anti-TMV counterparts, averaging $^{1}/300$. The lowest titre encountered was $^{1}/250$ for an anti-TMV protein globulin preparation which was absorbed with TMV (4.3.1.2.1) to remove all the anti-virus activity. Only one anti-TMV protein globulin preparation was outstanding in that it had a titre of $^{1}/1250$. The range of antigen concentrations associated with maximal complement fixation in the TMV-protein-anti TMV protein system was 0,063 - 0,500 µg/ml when whole sera or unabsorbed globulin preparations were used. The anti-TMV protein globulin preparation which was absorbed with TMV fixed the most in the 2,0 - 8,0 µg/ml antigen range.

Fig.s 2.13 and 2.14 illustrate the effect of antiserum dilution on complement fixation by the TMV-anti TMV and TMV protein-anti TMV protein immune systems respectively. In both cases increasing dilution of the antiserum caused a related reduction in the peak height of the complement fixation curves. 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). The slope (m) is defined by the equation

$$y = m \log x + b$$

where y = % complement fixed at the peak of the curve, x = antiserum concentration expressed as the reciprocal of its dilution and b is the y-intercept. The slope for the TMV-anti TMV system illustrated in fig. 2.13 is m = 231, while the slope for the TMV protein-anti TMV protein system in fig. 2.14 is m = 111.

The consistent difference of the slopes obtained with the TMV-anti TMV and TMV protein-anti TMV protein immune systems show their unrelatedness.

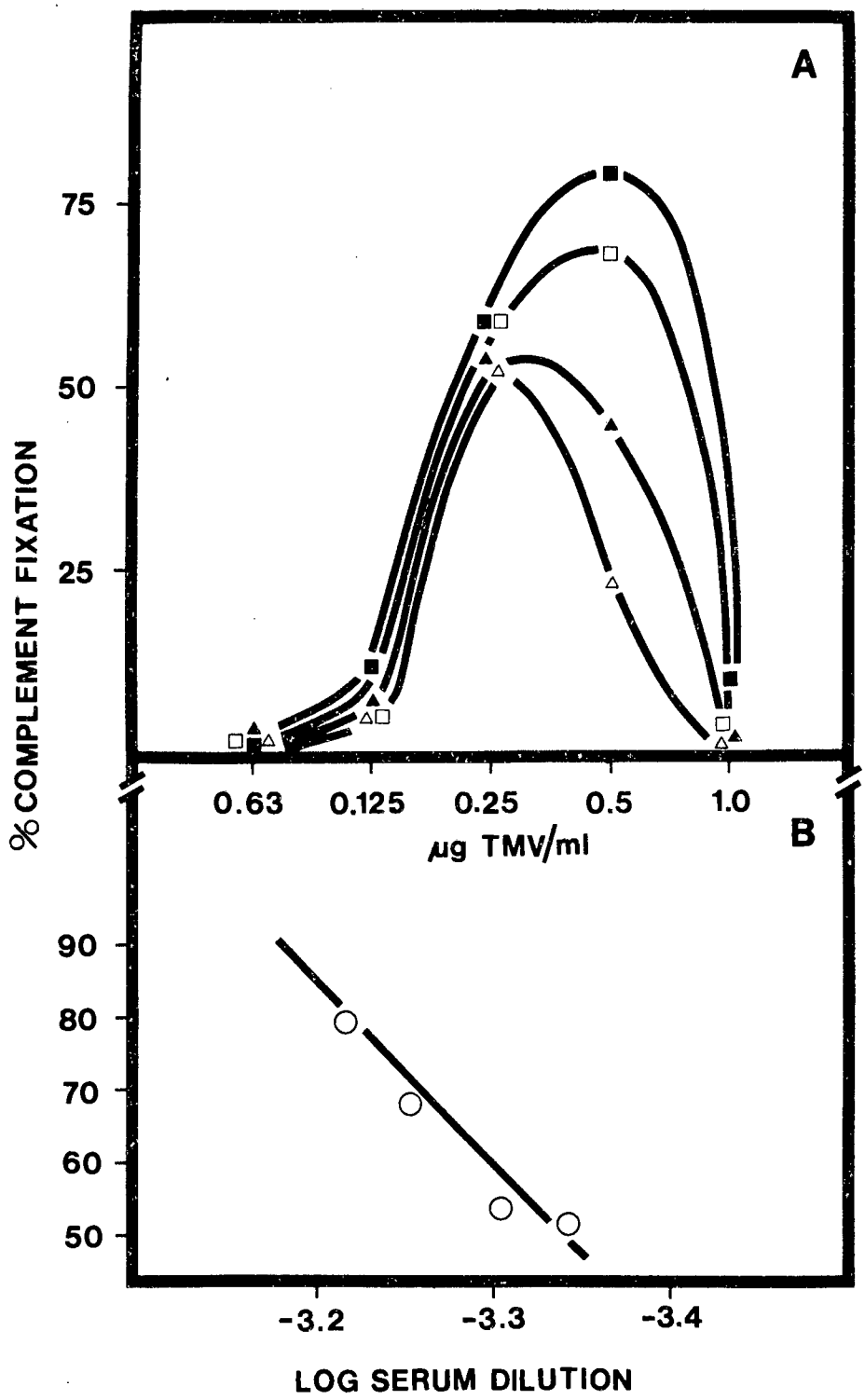

Fig. 2.13 : (A). Complement fixation curves with different dilutions of TMV antiserum. (■)1/1620, (□)1/1780, (▲)1/2000 and (△)1/2200. The test antigen was TMV. (B). Dependance of the peak height of the curves obtained in (A) on antiserum concentration. A slope, m=231, was calculated from the equation y=m log x + b where y = % complement fixed at the peak, x=antiserum concentration and b is the y-intercept (Champion et al., 1974).

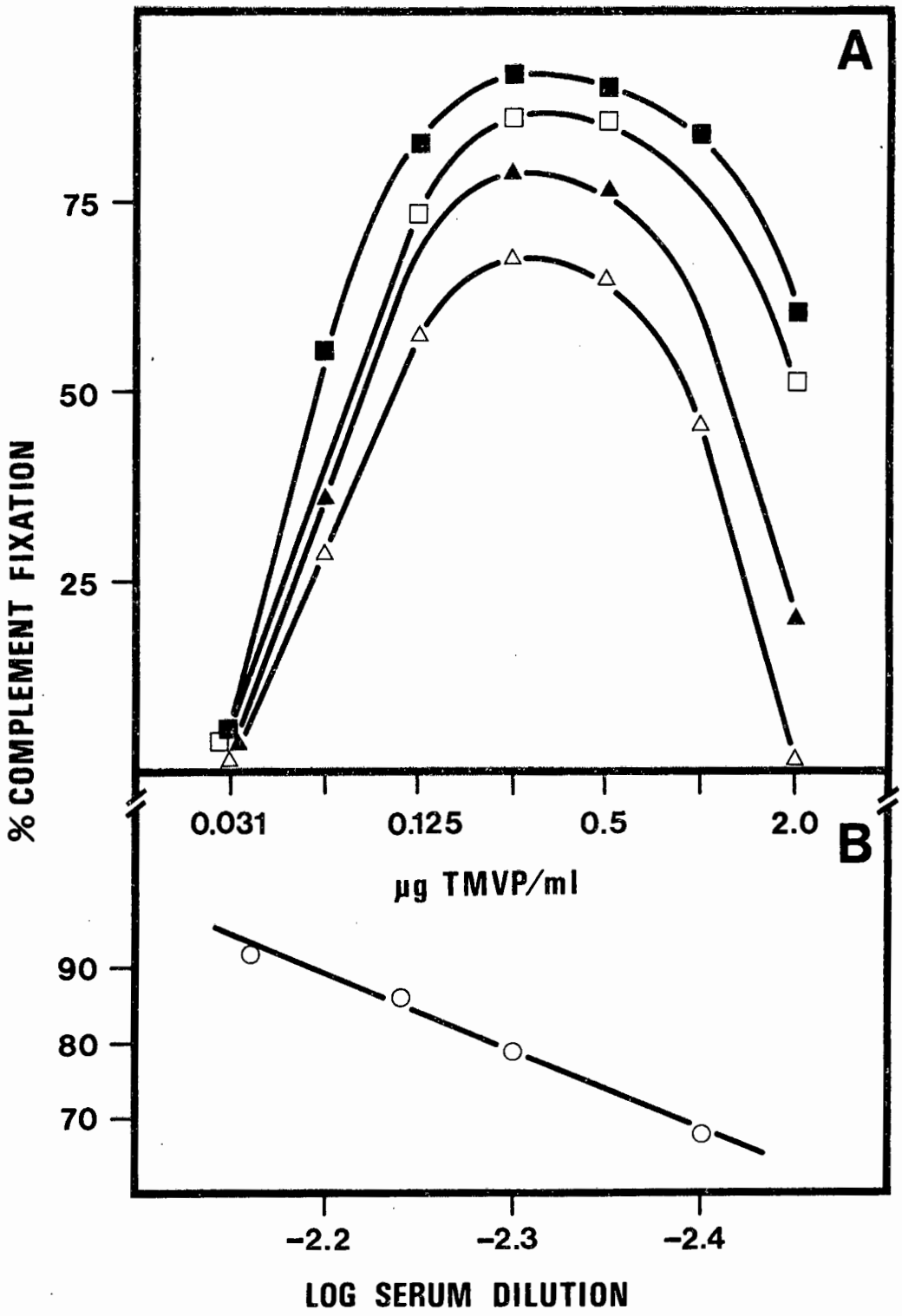

Fig. 2.14 : (A). Complement fixation curves with different dilutions of TMV protein antiserum. (■) 1/150, (□) 1/175, (▲) 1/200 and (△) 1/250. The test antigen was TMV protein. (B). Dependance of the peak height of the curves obtained in (A) on antiserum concentration. A slope, m=111, was calculated from the equation y=m log x + b where y= % complement fixed at the peak, x= antiserum concentration and b is the y-intercept (Champion et al., 1974). (From Milton and van Regenmortel, 1979).

2.5.1.3 THE EFFECT OF PEPTIDES ON COMPLEMENT FIXATION BY TMV AND ITS ANTISERA

Increasing concentrations of tryptic peptide 1 (residues 1-41), 4 (residues 62-68), 8 (residues 93-112), 11 (residues 135-141) and 12 (residues 142-158) were incubated with three late-course antisera from different rabbits, prior to the addition of TMV antigen, in complement fixation assays (4.3.2.1.9). None of these peptides, even when present in amounts corresponding to 800 TMV equivalents (2.3.2), i.e. a molar excess of 1704000:1 of peptide over virus, was able to cause any significant inhibition or enhancement of the complement fixation by TMV and its specific antibodies.

The possibility existed that the lack of activity by any of the peptides was due to their combination with anti-TMV protein antibodies present in the TMV antisera because of virus breakup prior to, or after, injection of the immunogen. To rule out this factor, the same peptides were preincubated with an anti-TMV globulin preparation (4.3.1.3), which had been absorbed with TMV protein to remove all the anti-protein activity (4.3.1.2.2), before the addition of TMV to the complement fixation assay. Again the peptides failed to affect the fixation of complement by TMV and anti-TMV antibodies even when 800 TMV equivalents of peptide were used in the assay.

2.5.1.4 THE EFFECT OF PEPTIDES ON COMPLEMENT FIXATION BY TMV PROTEIN AND ITS ANTISERA

An antiserum dilution, giving approximately 70% maximum complement fixation with TMV protein, was incubated at 37°C for 15 min with increasing concentrations of the four pH 4,5 soluble tryptic peptides, prior to the addition of antigen, in compelement fixation assays (4.3.2.1.9).

Fig.s 2.15, 2.16 and 2.17 show the ability of peptides 4 (residues 62-68), 8 (residues 93-112) and 12 (residues 142-158) to inhibit complement fixation in this system. The specificity of the inhibition was established by showing that similar molar excesses of the various peptides caused no inhibition of complement fixation in a control system consisting of H3 histone and anti-H3 serum (Sapieka et al., 1976).

Fig. 2.18 shows the comparative inhibitory power of the four peptides. Peptide 12 demonstrated the most activity with 5,72 n-moles of peptide causing 80% inhibition of complement fixation. An equal amount of peptide 4 caused 72% inhibition while peptide 8 caused only 21% inhibition. Peptide 11 (residues 135-141) did not inhibit complement fixation significantly.

When the peptides were used to inhibit complement fixation in a system comprising TMV protein and anti-TMV protein globulins (4.3.1.3), absorbed beforehand with TMV to remove all the anti-virus activity (4.3.1.2.1), the results were qualitatively the same as for the whole serum. In fig. 2.19 D it can be seen that 11,43 n-moles of peptide 12 caused 90% inhibition of complement fixation with a cross-absorbed anti-TMV protein globulin preparation. Similarly, 22,87 n-moles of peptides 4 and 8 caused 75% and 47% inhibition of complement fixation, respectively, with the same absorbed globulin preparation (fig. 2.19 C and D).

The hapten inhibition caused by peptides 4,8 and 12 with both absorbed and whole anti-TMV protein sera points to the existence of a single antigenic region corresponding to an antigenic determinant or part thereof, in each of these peptides. Peptide 11, which showed no immunological activity, probably corresponds to a region of the TMV protein sequence which is not antigenic in the free protein subunit. It is

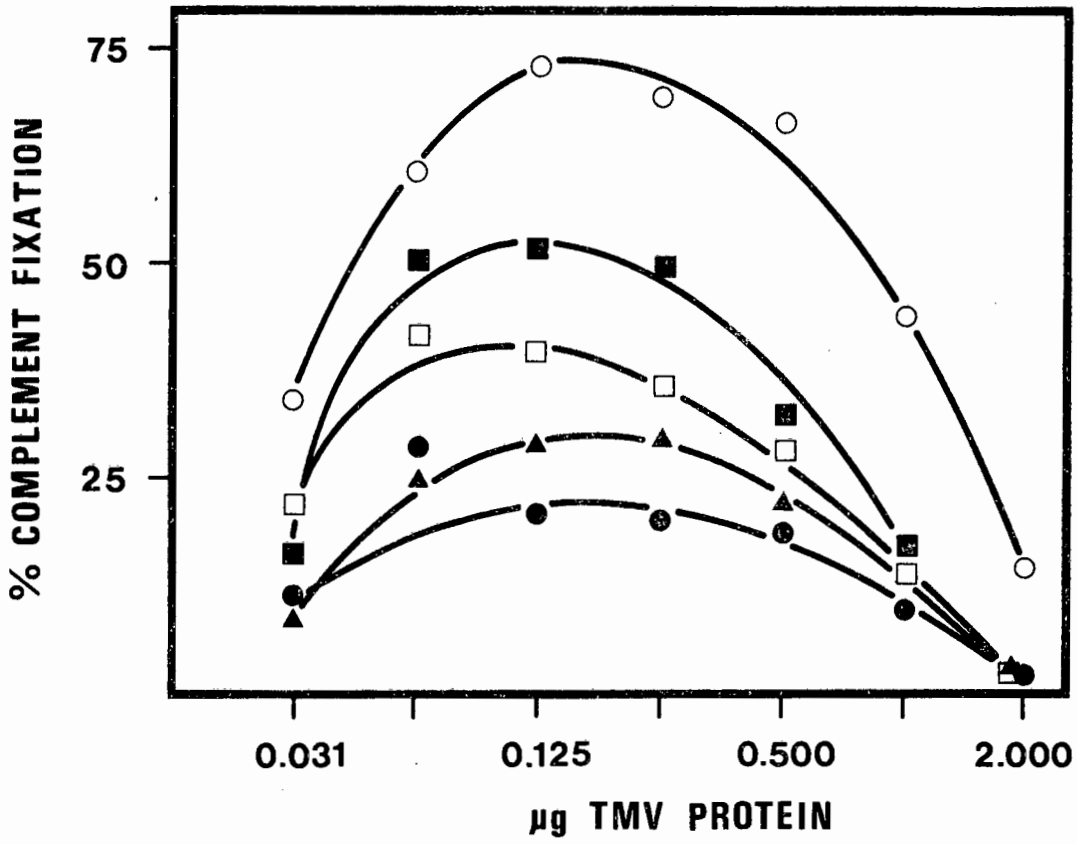

Fig. 2.15 : Inhibition of complement fixation in the TMV protein-anti TMV protein system by tryptic peptide 4 (residues 62-68). A TMV protein antiserum diluted 1/200 was used. Prior to the addition of TMV protein, the antiserum was incubated for 15 min at 37°C with the inhibitor. (○) no inhibitor, (■)0,29 n-moles of peptide equivalent to a 40 molar excess of peptide over TMV protein, (□)0,57 n-moles peptide equivalent to an 80 molar excess of peptide over TMV protein, (▲)2,86 n-moles of peptide equivalent to a 400 molar excess over TMV protein, and (●) 5,72 n-moles of peptide equivalent to an 800 molar excess of peptide over TMV protein.

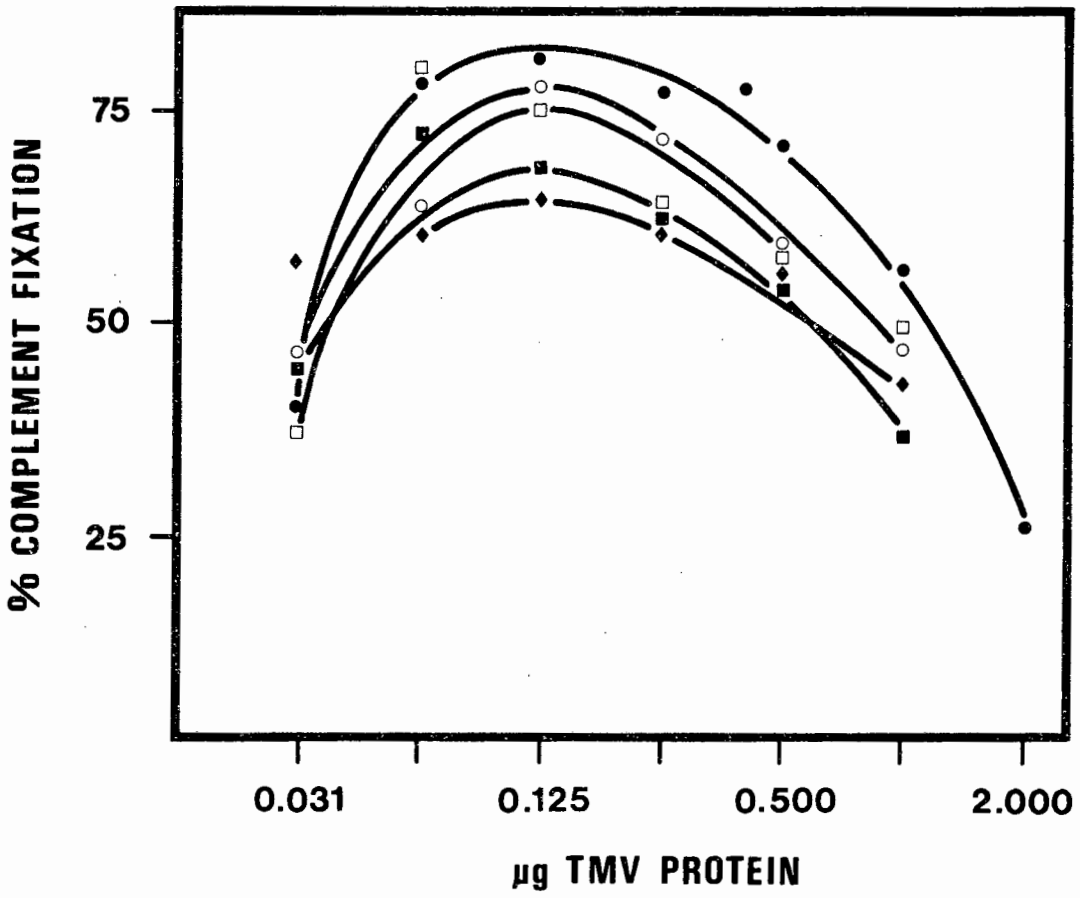

Fig. 2.16 : Inhibition of complement fixation in the TMV protein-anti TMV protein system by tryptic peptide 8 (residue 93-112). A TMV protein antiserum diluted 1/200 was used. Prior to addition of the TMV protein, the antiserum was incubated for 15 min at 37°C with the inhibitor. (●)no inhibitor, (○)0,30 n-moles of peptide equivalent to a 40 molar excess of peptide over TMV protein, (□)0,60 n-moles of peptide equivalent to an 80 molar excess of peptide over TMV protein (■)3,00 n-moles of peptide equivalent to a 400 molar excess of peptide over TMV protein, and (◆)6,74 n-moles of peptide equivalent to an 800 molar excess of peptide over TMV protein.

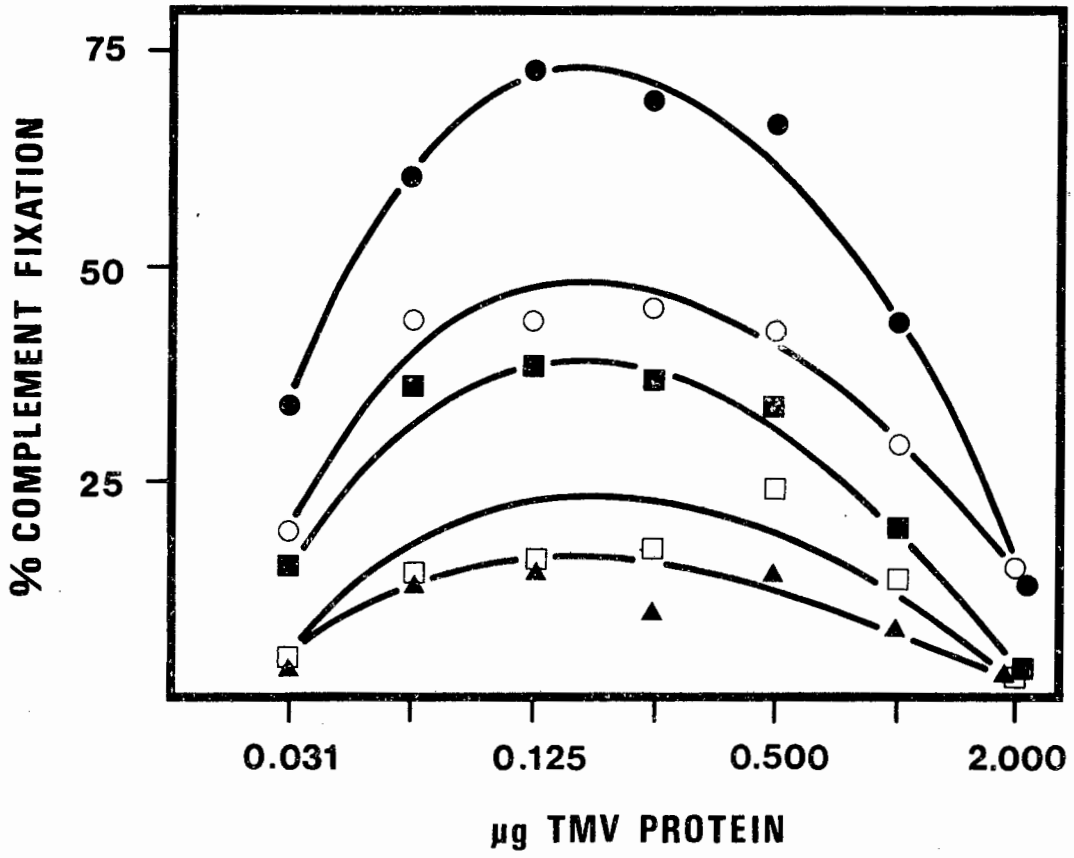

Fig. 2.17 : Inhibition of complement fixation in the TMV protein-anti TMV protein system by tryptic peptide 12 (residues 142-148). A TMV protein antiserum diluted 1/200 was used. Prior to the addition of TMV protein, the antiserum was incubated for 15 min at 37°C with the inhibitor. (●)no inhibitor, (○)0,29 n-moles of peptide equivalent to a 40 molar excess of peptide over TMV protein, (■)0,57 n-moles of peptide equivalent to an 80 molar excess of peptide over TMV protein, (□)2,86 n-moles of peptide equivalent to a 400 molar excess of peptide over TMV protein, and (▲)5,72 n-moles of peptide equivalent to an 800 molar excess of peptide over TMV protein.

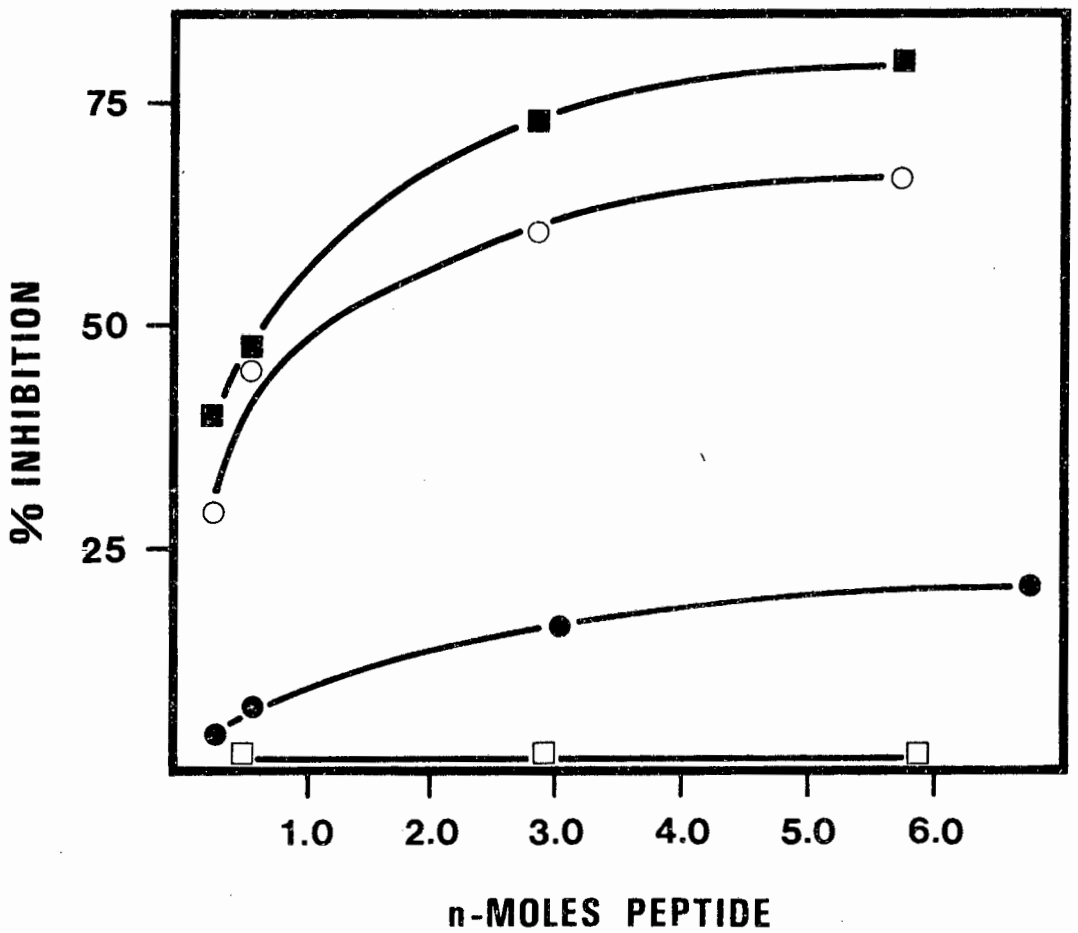

Fig. 2.18 : Inhibition of complement fixation in the TMV protein-anti TMV protein system by increasing quantities of TMV tryptic peptides. A TMV protein antiserum diluted 1/200 was used in all tests. (■)tryptic peptide 12, (○)tryptic peptide 4, (●)tryptic peptide 8, and (□)tryptic peptide 11. (Milton and van Regenmortel, 1979.)

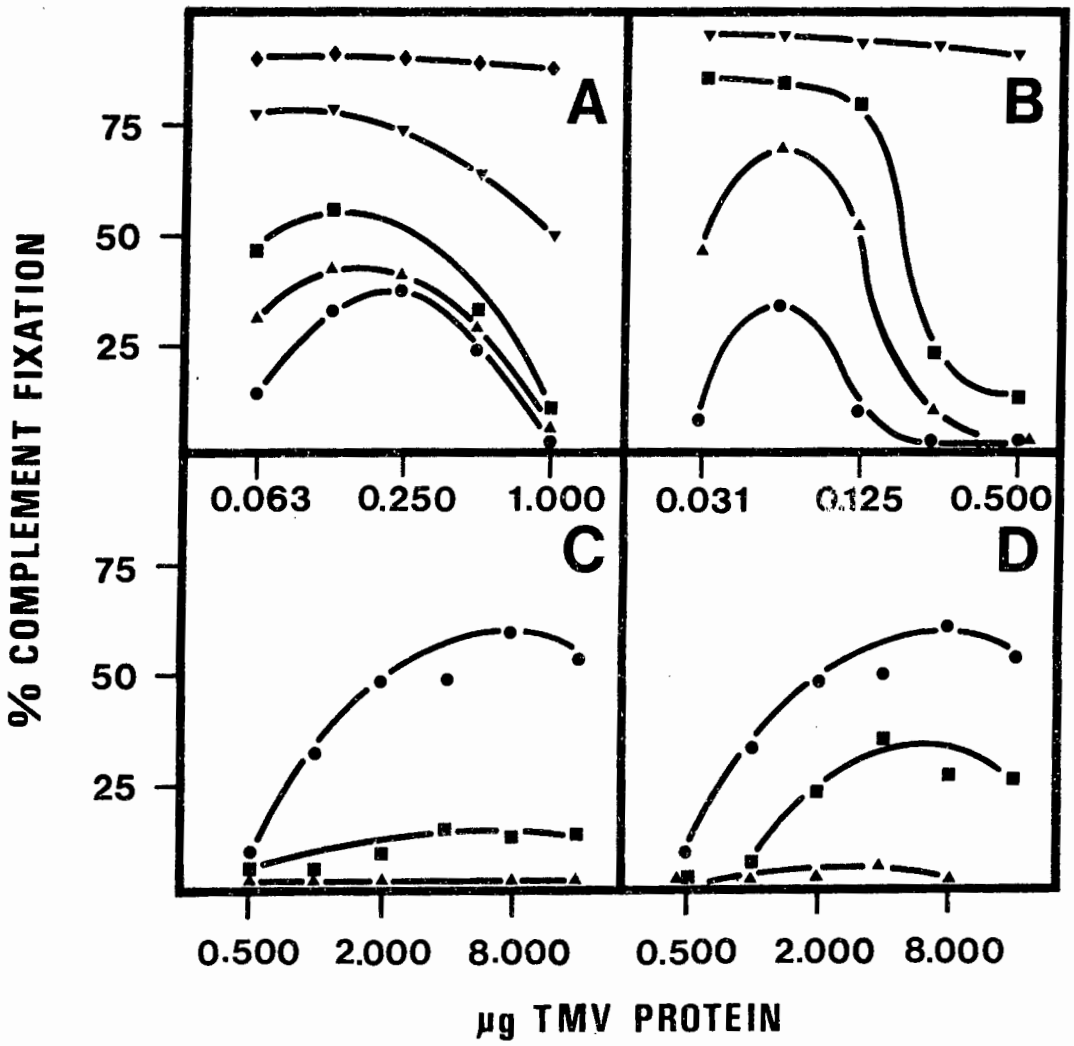

Fig. 2.19 : (A). Enhancement of complement fixation in the TMV protein-anti TMV protein system by peptide I (residues 1-41). (●)no enhancement, (▲)0,29 n-moles of peptide per test, equivalent to a 40 molar excess of peptide over TMV protein, (■)0,57 n-moles of peptide per test, equivalent to an 80 molar excess of peptide over TMV protein, (▼)2,86 n-moles of peptide per test, equivalent to a 400 molar excess of peptide over TMV protein, and (◆)5,72 n-moles of peptide per test equivalent to an 800 molar excess of peptide over TMV protein. (B). Enhancement of complement fixation in the TMV protein-anti TMV protein system by peptide I using a different antiserum from that in (A) (●)no enhancement, (▲)0,72 n-moles of peptide per test, equivalent to a 200 molar excess of peptide over TMV protein, (■)1,43 n-moles of peptide per test, equivalent to a 400 molar excess of peptide over TMV protein, and (▼)2,86 n-moles of peptide per test, equivalent to an 800 molar excess of peptide over TMV protein. (C). Inhibition of complement fixation in the TMV protein-anti TMV protein system by peptides I (residues 1-41) and 4 (residues 62-68) using a TMV protein antiserum absorbed with TMV. (●)no inhibitor, (▲)11,43 n-moles of peptide I per test, equivalent to a 100 molar excess of peptide over TMV protein, and (■)22,87 n-moles of peptide 4

per test, equivalent to a 200 molar excess of peptide over TMV protein. (D). Inhibition of complement fixation in the TMV protein-anti TMV protein system by peptides 8 (residues 93-112) and 12 (residues 142-158) using a TMV protein antiserum absorbed with TMV. (●)no inhibitor, (■)22,87 n-moles of peptide 8 per test, equivalent to a 200 molar excess of peptide over TMV protein, and (▲)11,43 n-moles of peptide 12 per test, equivalent to a 100 molar excess of peptide over TMV protein. (Milton and van Regenmortel, 1979.)

possible, however, that peptide 11 contains too small a part of an antigenic determinant to react with antibodies.

Peptide 1 (residues 1-41) did not inhibit complement fixation in the TMV protein-anti TMV protein system, but, on the contrary, enhanced it. In fig. 2.19 A and B, the results obtained with two antisera show that increasing amounts of peptide 1 progressively raised the peak height of the complement fixation curve. However, this phenomenon was observed only with non-absorbed TMV protein antisera. When the same antiserum had been absorbed with TMV, 11,43 n-moles of peptide 1 caused 96% inhibition of complement fixation (fig. 2.19 C). Thus it is expected that peptide 1 contains at least two antigenic regions of which only one is antigenic in the free TMV protein subunit. Antibodies specific for the other antigenic region were removed by the absorption of the serum with TMV so that peptide 1 could behave as a hapten with the absorbed serum.

Two other TMV protein antisera have been examined in inhibition of complement fixation experiments and the results were not substantially different from those illustrated in figs. 2.15 to 2.18. Although the amount of inhibition or enhancement caused by an equal molar excess of a particular peptide varied with each antiserum, the order, relative to each other, in which the peptides caused maximal inhibition remained the same.

2.5.2 INHIBITION STUDIES WITH PEPTIDES IN THE TMV-ANTI TMV AND THE TMV PROTEIN-ANTI TMV PROTEIN PRECIPITIN SYSTEMS

2.5.2.1 THE MICRO-PRECIPITIN ASSAY WITH RADIOLABELLED ANTIGENS AS A MEANS OF TESTING THE IMMUNOCHEMICAL ACTIVITY OF PEPTIDES

The advantages of inhibition studies with the precipitin systems consisting of radiolabelled TMV and TMV protein and their antisera have been pointed out in sections 2.3.2 and 2.4.2 respectively. These advantages are equally applicable to inhibition studies with peptides. In both systems only the specific reaction of the radiolabelled antigen and its antibodies is measured without having to absorb the antisera. Any inhibition by a peptide would therefore be specific inhibition of the binding of a particular antigenic determinant of the system, be it TMV-anti TMV or TMV protein-anti TMV protein. Cross-reactions between the peptides and contaminating antibodies (due to the depolymerization of the virus or aggregation of the protein in the animal after immunization) cannot be measured. This means that more peptide would be required for the same amount of inhibition than would be the case with an absorbed serum, but, on the other hand, antibodies which react with the same determinant in both the virus and the protein subunit are not lost.

The method of antiserum titration is given in section 4.3.4.2.5 and typical curves of the amount of radiolabelled antigen in the supernatant of the micro-precipitin system are shown in Figs. 2.8 and 2.10. An explanation of the shape and nature of the curves is given in sections 2.3.2 and 2.4.2 respectively.

2.5.2.2 THE EFFECT OF PEPTIDES ON THE PRECIPITIN REACTION OF RADIO-LABELLED TMV AND ITS SPECIFIC ANTIBODIES

Increasing concentrations of tryptic peptide I (residues 1-41), 4 (residues 62-68), 8 (residues 93-112), 11 (residues 135-141) and 12 (residues 142-158) as well as mixtures of these peptides and even a whole tryptic digest of the TMV protein were incubated with 0,5 ml of five late-course antisera, from different rabbits, prior to the addition of 0,5 µg of radiolabelled TMV in micro-precipitin assays (4.3.4.2.6). The antisera had previously been titrated with radiolabelled TMV (4.3.4.2.5) and the antiserum dilutions giving antibody: antigen proportions in the zone of antigen excess, near the equivalence point, had been chosen so that the system would be most sensitive to inhibition (2.3.2). In no case, either with the individual, or a combination of peptides, could any significant inhibition of the specific radiolabelled TMV-anti TMV reaction be detected, even when the peptides were present in amounts corresponding to 8000 TMV equivalents (2.3.2), i.e. a molar ratio of $1,7 \times 10^7$:1 of peptide over virus. All of these sera had been used for micro-precipitin assays with radiolabelled TMV which had been successfully inhibited by unlabelled TMV protein at pH 6,8 (2.3.3), the same pH at which the peptide inhibition studies were carried out.

A similar situation was encountered when attempts were made to inhibit the TMV-anti TMV reaction with peptides in the complement fixation system (2.5.1.3).

2.5.2.3 THE EFFECT OF PEPTIDES ON THE PRECIPITIN REACTION OF RADIO-LABELLED TMV PROTEIN AND ITS SPECIFIC ANTIBODIES

A TMV protein antiserum was titrated with radiolabelled TMV protein (4.3.4.2.5) and a dilution was chosen to give antibody:antigen

proportions in the zone of antigen excess, near the equivalence point, so that the system would be most sensitive to inhibition (2.4.2). Increasing amounts of the five tryptic peptides were incubated with 0,5 ml of the antiserum dilution prior to the addition of 0,5 µg of radiolabelled TMV protein in micro-precipitin assays (4.3.4.2.6). Fig. 2.20 shows the ability of tryptic peptides I (residues 1-41), 4 (residues 62-68), 8 (residues 93-112) and 12 (residues 142-158) to inhibit the specific reaction between radiolabelled TMV protein and anti-TMV protein antibodies.

Peptide I demonstrated the most activity with 57,6 n-moles of peptide (equivalent to a molar excess of 1600:1 over TMV protein) causing 60% inhibition of the precipitin reaction. An equal amount of peptide 12 caused 33% inhibition, while peptides 4 and 8 caused 20% and 18% respectively. At the lower peptide concentrations the difference in the inhibition caused by peptides 4 and 8 was more evident. Peptide 11 (residues 135-141) did not inhibit the reaction of anti-TMV protein antibodies and the radiolabelled TMV protein significantly. The comparative inhibitory power of the four pH 4,5 soluble tryptic peptides was the same as was found when these peptides were used to inhibit complement fixation by TMV protein and its antisera (2.5.1.4).

The specificity inherent in the micro-precipitin assay system with radiolabelled TMV protein (2.4.2) points to each of the active peptides containing an antigenic region corresponding to an antigenic determinant of the TMV protein subunit, and these results confirm the conclusions reached from the inhibition of complement fixation experiments with a TMV protein antiserum absorbed with TMV (2.5.1.4). In addition, the comparatively large inhibition caused by peptide I in this

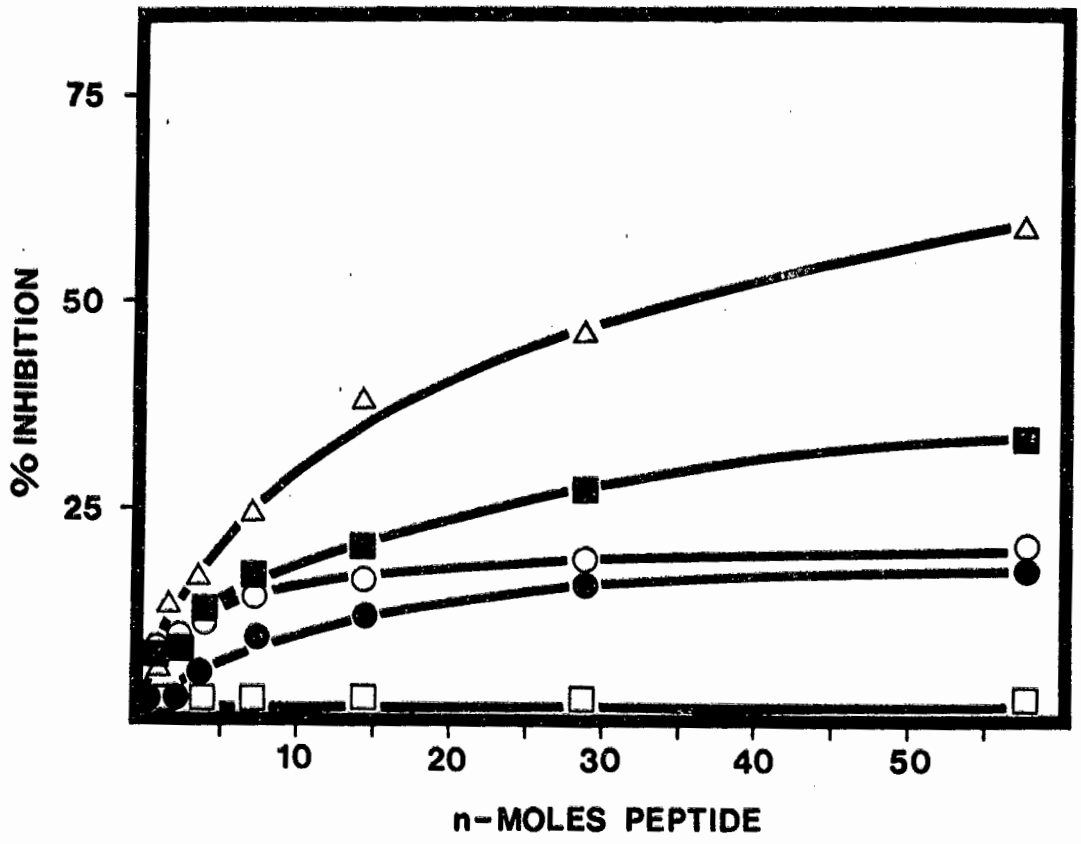

Fig. 2.20 : Inhibition of the precipitin reaction of 0,5 µg radiolabelled TMV protein and its specific antibodies by increasing amounts of TMV tryptic peptides. A TMV protein antiserum diluted 1/128 was used in all tests. Prior to the addition of the radiolabelled antigen the antiserum was incubated for 15 min at 37°C with the inhibitors and then left at +4°C for 17 hours. (△) tryptic peptide 1 (residues 1-41), (■) tryptic peptide 12 (residues 142-158), (○) tryptic peptide 4 (residues 62-68), (●) tryptic peptide 8 (residues 93-112), and (□) tryptic peptide 11 (residues 135-141).

system points to the conclusion that its second antigenic region is recognized by antibodies which can bind this region in both the intact virus and the protein subunit, as these antibodies are removed by absorption with TMV (2.5.1.4 and Fig. 2.19 B and C).

Two other TMV protein antisera have been examined in micro-precipitin assays with peptide inhibitors. The results obtained were not substantially different from those illustrated in Fig. 2.20 although the amount of inhibition caused by an equal molar excess of a particular peptide varied with each antiserum.

2.5.3 BINDING STUDIES WITH N-(^{3}H)ACETYL PEPTIDES

2.5.3.1 THE VALUE OF THE DIRECT BINDING ASSAY

A peptide that contains an antigenic region will be bound by antibodies specific for the corresponding antigenic determinant on the intact protein to form soluble antibody-hapten complexes, so long as the peptide conformation can be induced to fit the antibody binding site (Atassi, 1977 b). In this system there is no competition between the peptide and the conformationally intact antigenic determinant, as there is in inhibition experiments, so, low affinity peptide-antibody binding should be more apparent if the amount of bound peptide can be quantitated. This can be accomplished by attaching a radioactive label to the peptide and precipitating the soluble immune complexes.

The double-antibody method for separating the hapten-antibody complexes from the free hapten is sensitive, gentle and does not disturb the equilibrium to any significant extent (Ransom, 1976). It is based on the finding that the antigenic sites of an antibody are separate from its binding site and, therefore, an antibody molecule can form a complex

with its antigen and then itself be complexed to a second antibody specific for it (Hunter, 1978).

A particular disadvantage of this method with respect to the tryptic peptides of TMV protein, studied in this work, is that peptide I (residues 1-41) already has an acetyl group blocking its N-terminus. It therefore cannot be acetylated with tritiated acetic anhydride as the four pH 4,5 soluble tryptic peptides of TMV protein can be.

2.5.3.2 THE LABELLING OF FOUR pH 4,5 SOLUBLE TRYPTIC PEPTIDES WITH ^{3}H-ACETIC ANHYDRIDE

Tryptic peptides 4 (residues 62-68), 8 (residues 93-112), 11 (residues 135-141) and 12 (residues 142-158), as well as a synthetic peptide corresponding to residues 134-141 of the TMV strain U2 (a region equivalent to peptide 11 in the common strain), were acetylated with tritiated acetic anhydride (4.3.3.2) by the method of Benjamini et al., (1965).

The molar concentrations of the acetylated derivatives of the peptides were determined by amino acid analysis (4.2.2) and their specific activities as described in section 4.3.3.1. Typical activities of the peptides were: peptide 4 = 1718007 cpm/n-mole; peptide 8 = 149523 cpm/n-mole; peptide 11 = 2365957 cpm/n-mole; peptide 12 = 403653 cpm/n-mole; and the synthetic peptide octomer = 1060195 cpm/n-mole (4.3.3.2).

2.5.3.3 THE BINDING OF N-(^{3}H)ACETYL PEPTIDES TO ANTIBODIES

Typical results of the direct binding assays (4.3.3.3) using the globulin fraction (4.3.1.3) of TMV and TMV protein antisera are summarized in Tables 2.4 and 2.5. In an attempt to determine the contribution of

GLOBULINS	RABBIT	TOTAL cpm/TEST	SPECIFIC cpm/TEST[a]	% PEPTIDE BOUND
anti-TMV protein	1	140[b]	31	0,48
		144[b]	34	0,55
"	2	155	71	1,14
		164	80	1,29
"	3	139	22	0,33
		137	20	0,31
"	4	123	22	0,36
		129	29	0,45
"	5	237	61	0,4
anti-TMV protein absorbed with TMV	5	250	76	0,5
anti-TMV	6	196	0	<0,05
"	7	111	7	<0,05
"	8	212	36	0,2
anti-TMV absorbed with TMV protein	8	176	0	<0,05

[a]Rabbit globulins obtained from unimmunized animals and animals immunized with human serum albumin served to establish the degree of non-specific binding which was subtracted from the total cpm.

[b]Results obtained in duplicate experiments with globulins from rabbits 1-4 are presented in order to illustrate the reproducibility of measurements.

Table 2.4 : Binding of N-(^{3}H)acetyl tryptic peptide 8 (residues 93-112) to specific TMV and TMV protein antibodies.

virus-specific and protein-specific antibodies to the overall reactivity of the antisera, the sera were also tested after absorption with depolymerized protein or intact formalinized virus (van Regenmortel and Lelarge, 1973) as described in section 4.3.1.2.

The results obtained with tryptic peptide 8 (residues 93-112) are shown in Table 2.4. The amount of labelled peptide specifically bound by different TMV protein antisera varied between 0,31 and 1,29% of the amount used in the test. Absorbing a particular serum with virus did not affect its ability to bind the peptide. On the other hand, the prior absorption of a TMV antiserum with TMV protein subunits reduced the amount of binding to an insignificant level. Most of the anti-virus sera examined showed no significant binding of labelled peptide 8. These results suggest that the peptide 8 determinant is functional only in the TMV protein-anti TMV protein system, i.e. that it is a cryptotope.

The specificity of the antibody binding of labelled peptide 8 was demonstrated by inhibition with unlabelled peptide 8 (Fig. 2.21). Even the small amount of binding (0,6%) obtained with the antiserum from rabbit no. 1 could be inhibited by increasing concentrations of unlabelled peptide 8. This illustrates that the low percentage binding obtained with certain antisera is nevertheless significant.

Table 2.5 shows the results of binding assays with the remaining N-(^{3}H) acetyl peptides. The amount of labelled peptide bound varied with each particular antiserum. By comparing the ability of a single antiserum to bind various peptides, it can be seen that peptide 12 (residues 142-158) showed the most activity followed by peptide 4 (residues 62-68) and

PEPTIDE	GLOBULINS	RABBIT	TOTAL cpm/TEST	SPECIFIC cpm/TEST	% PEPTIDE BOUND
4	anti-TMV protein	2	2058	1332	1,8
	"	3	1270	544	0,8
	"	9	2045	1319	1,8
	"	10	1027	301	0,4
	"	5	1825	1099	1,5
	anti-TMV protein absorbed with TMV	5	1773	1047	1,5
	anti-TMV	6	831	105	0,1
	"	8	1274	548	0,8
	anti-TMV absorbed with TMV protein	8	747	22	<0,05
11	anti-TMV	6	802	167	0,1
	"	8	697	62	>0,05
	anti-TMV absorbed with TMV protein	8	679	43	<0,05
synthetic U2 octomer	anti-U2 protein	13	546	326	0,1
	"	14	470	0	<0,05
	anti-U2	11	548	45	<0,05
	"	12	503	3	<0,05
	anti-TMV	8	516	17	<0,05
	anti-TMV absorbed with TMV protein	8	515	15	<0,05
12	anti-TMV protein	10	1015	814	4,8
	"	5	2720	2496	14,7
	anti-TMV protein absorbed with TMV	5	1940	1716	10,1
	anti-TMV	6	281	81	0,5
	"	8	1185	962	5,7
	anti-TMV absorbed with TMV protein	8	532	309	1,8

Table 2.5 : Binding of N-(^{3}H)acetyl peptides 4 (residues 62-68), 11 (residues 135-141), 12 (residues 142-158) and the synthetic U2 octomer (residues 134-141 of U2) to antibodies.

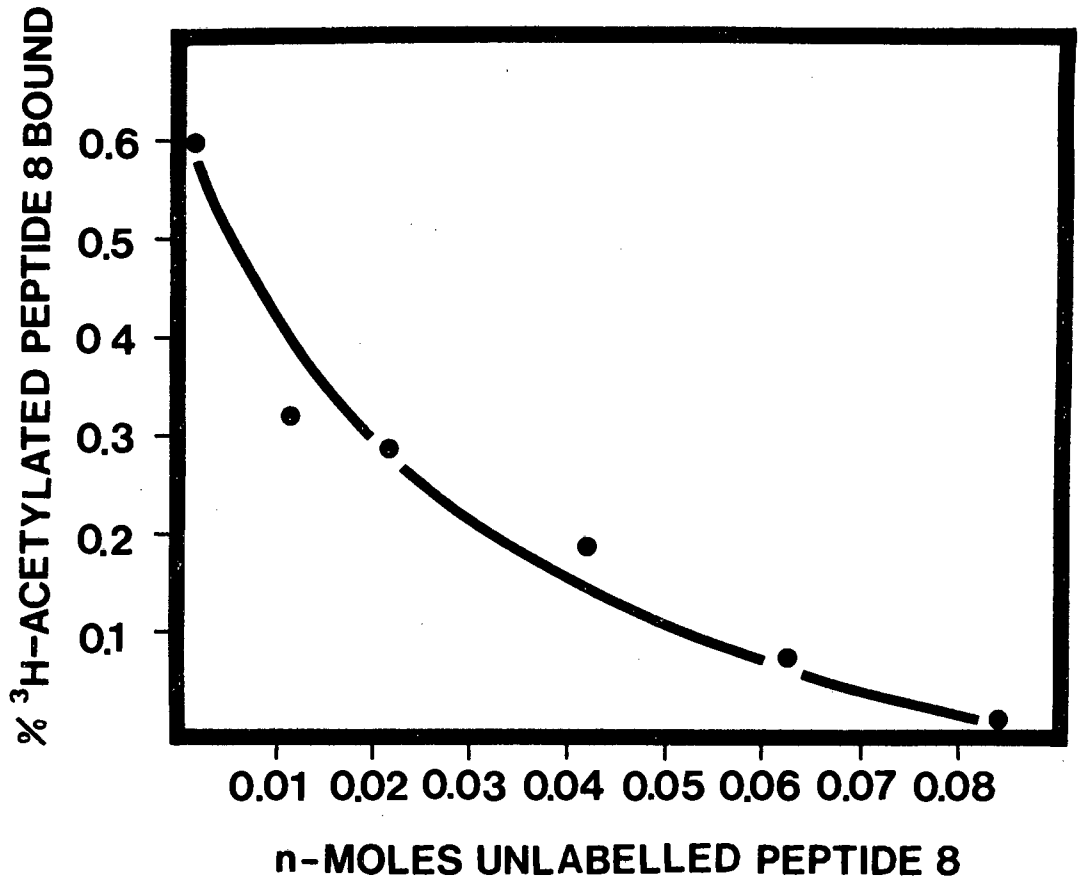

Fig. 2.21 : Specific inhibition of the antibody binding of N-(^{3}H) acetyl peptide 8 (residues 93-112) by unlabelled peptide 8. The amount of N-(^{3}H)acetyl peptide 8 bound by specific antibodies, expressed as the percentage of 0,042 n-mole, decreases in the presence of increasing concentrations of unlabelled peptide 8.

peptide 8 (Tables 2.5 and 2.4). Peptide 11 (residues 135-141) and the synthetic octomer (residues 134-141) of the U2 strain of TMV showed some weak activity only with certain anti-TMV sera and it is difficult to ascertain the significance of these low figures. Peptide 11 was found unable to inhibit the TMV protein-anti TMV protein reaction by complement fixation (2.5.1.4) and micro-precipitin assays (2.5.2.3).

N-(^{3}H)acetyl peptide 4, like peptide 8, showed more binding with anti-TMV protein sera than with anti-TMV sera. Absorption of an anti-TMV protein serum with virus did not affect the ability of the antibodies to bind peptide 4, whereas the absorption of an anti-TMV serum with protein subunits reduced the amount of binding to an insignificant level. This indicates that the determinant in peptide 4 probably also functions as a cryptotope.

With peptide 12 it was found that both the absorption of a TMV anti-serum with protein subunits, and the absorption of a TMV protein anti-serum with virus, caused a decrease in the amount of binding observed. However, as the amount of residual binding after cross-absorption was still considerable, it appears that peptide 12 is recognized by both anti-TMV and anti-TMV protein antibodies. It should be emphasized that the binding observed with peptide 12, which ranged from 4,18-14,7% with different whole antisera, was at least twice, and at most seven times, as great as that of any of the other active peptides.

2.6 IMMUNOCHEMICAL STUDIES WITH MUTANT HOMOLOGUES AND A DERIVATIVE OF THE ACTIVE TRYPTIC PEPTIDES OF TMV PROTEIN

2.6.1 IMMUNOCHEMICAL STUDIES WITH TRYPTIC PEPTIDES FROM TMV MUTANTS

2.6.1.1 TRYPTIC PEPTIDES FROM TMV MUTANTS AS A MEANS OF LOCATING AMINO ACID RESIDUES THAT FORM PART OF ANTIGENIC DETERMINANTS

In section 2.4.1 a strategy was outlined whereby the role of specific amino acid residues in the antigenicity of TMV protein could be determined. The ability of the proteins of six TMV mutants to inhibit the precipitin reaction of radiolabelled TMV protein and its specific antibodies was compared with that of unlabelled TMV protein (2.4.3). In the second part of the strategy, tryptic peptides containing the amino acid exchanges which caused the antigenic behaviour of the mutant proteins to be altered, were compared with the active peptides from wild type TMV using the same system, viz. inhibition of precipitation between radiolabelled TMV protein and the same TMV protein antisera. Mutant peptides which display inhibitory behaviour not significantly different from the wild type peptide, even though the whole protein was serologically distinguishable from TMV protein, would suggest a particular amino acid exchange exerts its influence through conformational modulation of the whole protein, although it is not proof that this is the case (3.2). On the other hand, a decrease in inhibition relative to the active wild type peptide indicates that the exchange of a particular residue makes the recognition of the antigenic region in that peptide more difficult for the antibodies specific for the corresponding antigenic determinant on the TMV protein. Because it is improbable that the conformational state of the peptide will approach the native mode of folding of that region of the protein sequence (Atassi and Singhal, 1970; Atassi, 1977 b), especially in the case of the shorter peptides which have less than twenty amino acids, it can be assumed that a change in

the immunochemical behaviour of a mutant peptide implicates that particular residue in the antigenic region of the peptide. The validity of this assumption can be tested by immunochemical studies with overlapping synthetic peptides corresponding to the proposed antigenic region (Atassi, 1975, 1977 b).

2.6.1.2 A COMPARISON OF THE IMMUNOCHEMICAL BEHAVIOUR OF WILD TYPE AND TMV MUTANT TRYPTIC PEPTIDES BY PRECIPITIN INHIBITION ASSAYS WITH RADIOLABELLED TMV PROTEIN

The inhibition assays were performed exactly as described in sections 2.4.2 and 2.4.3 using the same antisera. Figs. 2.11 and 2.22 - 2.25 illustrate the inhibition of the precipitin reaction of radiolabelled TMV protein caused by the unlabelled TMV protein and the mutant proteins as well as the wild type and mutant peptides with the same pooled bleedings of antiserum from a single rabbit. The results shown are therefore comparable.

Fig. 2.22 shows the inhibition obtained with the I-peptide (residues 1-41), peptide I of Ni 118 (proline in position 20 replaced by leucine) and peptide I of Ni 568 (threonine in position 5 replaced by isoleucine). No significant difference between the inhibition caused by these three peptides is evident, even though Ni 118 protein and Ni 568 protein are serologically distinguishable from TMV protein (2.4.3). A similar situation was encountered with TMV peptide 8 (residues 93-112) and Ni 568 peptide 8 (threonine in position 107 replaced by methionine), which also gave inhibition curves that were not significantly different (fig. 2.24). This is in accord with the findings of Benjamini and co-workers (Young et al., 1968; Benjamini et al., 1969) who defined the antigenic region in peptide 8 from residues 108-112 by the use of synthetic peptides (1.6.2).

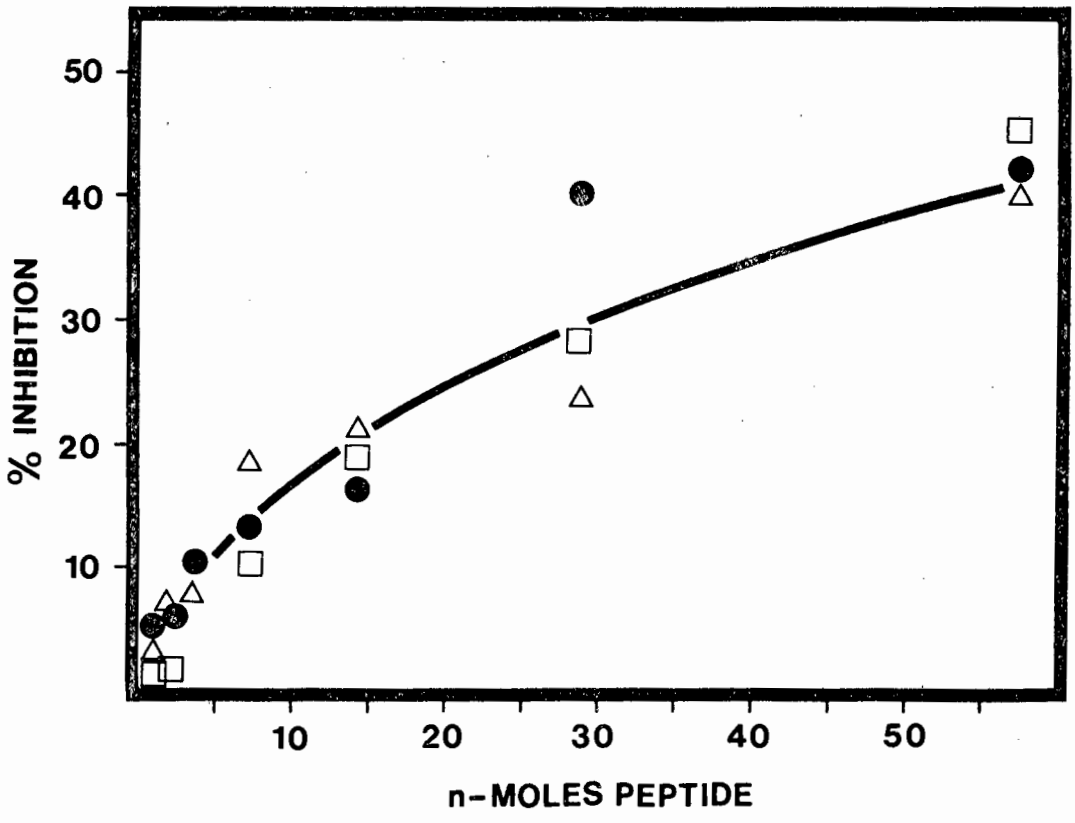

Fig. 2.22 : Inhibition of the precipitin reaction of 0,5 µg radiolabelled TMV protein and its specific antibodies by increasing amounts of tryptic peptide I (residues 1-41) from TMV (●), Ni 118 (□) and Ni 568 (△). No significant difference in the amount of inhibition caused by these peptides was discernable. A 1/128 dilution of a TMV protein antiserum was used in all tests.

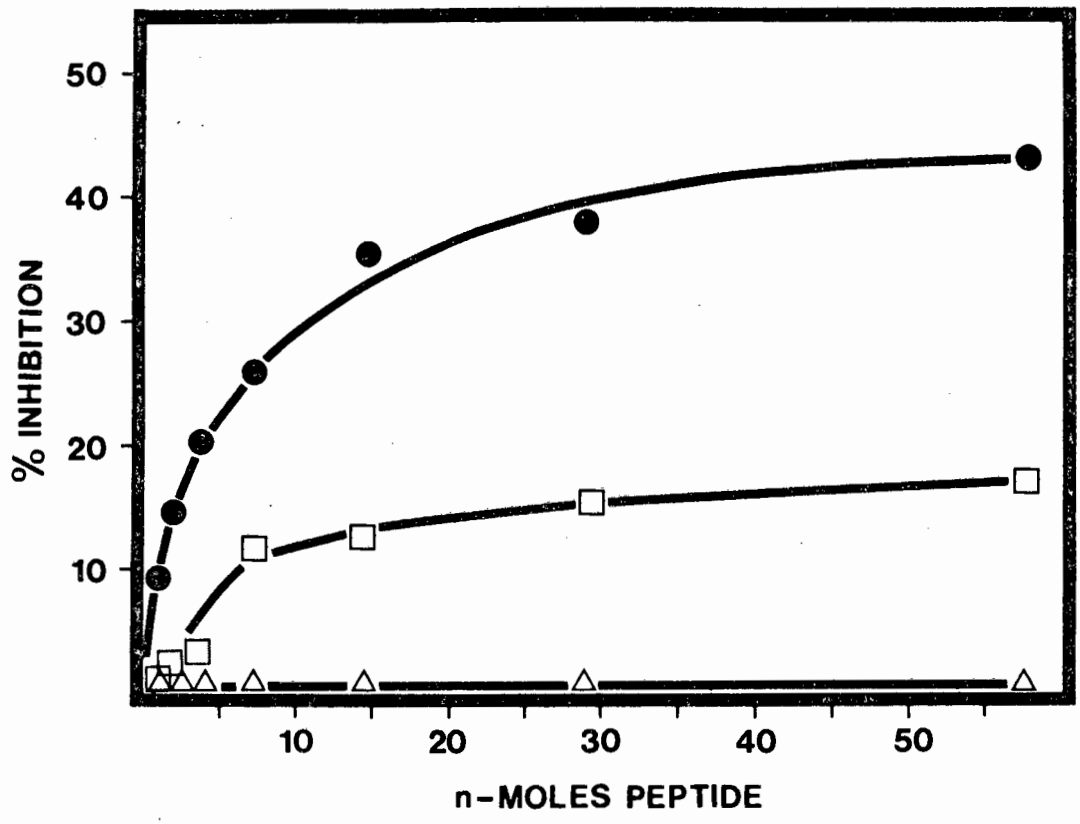

Fig. 2.23 : Inhibition of the precipitin reaction of 0,5 µg of radio-labelled TMV protein and its specific antibodies by increasing amounts of tryptic peptide 4 (residues 62-68) from TMV (●), 414 (□) and Ni 1688 (△). The peptides from the two mutant viruses are less successful inhibitors of the TMV protein-anti TMV protein reaction than the corresponding peptide from the wild type virus. A $^{1}/_{128}$ dilution of a TMV protein antiserum was used in all tests.

From these results it must be concluded that the amino acid exchanges in the mutant proteins of Ni 118 and Ni 568 are likely to exert their influence on immunochemical behaviour by altering the protein conformation sufficiently to affect the shape of one or more of the protein's antigenic determinants, but not sufficiently to affect the other biological properties of the proteins (3.2).

The situation with the mutant peptides homologous with peptides 4 and 12 was found to be completely different. Fig. 2.23 shows that peptide 4 (residues 62-68) of the mutants 414 and Ni 1688 have a reduced ability to inhibit the TMV protein-anti TMV protein reaction compared with wild type peptide 4. 57,6 n-moles of peptide 4 from the mutant 414 (serine in position 65 replaced by glycine) caused only 16% inhibition, compared to 38% inhibition by wild type peptide 4, while no significant inhibition was obtained with peptide 4 of the mutant Ni 1688 (proline in position 63 replaced by serine). Similarly, 28,8 n-moles of peptide 12 from the mutants Ni 1688 and Ni 1927 (proline in position 156 replaced by leucine in both cases) caused 20% inhibition compared to 39% inhibition by wild type peptide 12 (residues 142-158) (Fig. 2.25). The inhibition curves of the two identical mutant peptide homologues of peptide 12 are not significantly different and the "tailoff" seen at the highest peptide concentration is probably due to the reduced solubility of the mutant peptides causing aggregate formation.

The result with peptide 4 of the mutant 414 was remarkable in that the whole protein was not serologically distinguishable from TMV protein (2.4.3). This could be due to a "masking effect" caused by the other antigenic determinants on the rest of the TMV protein subunit reacting with their specific antibodies in the normal way. Thus, the small

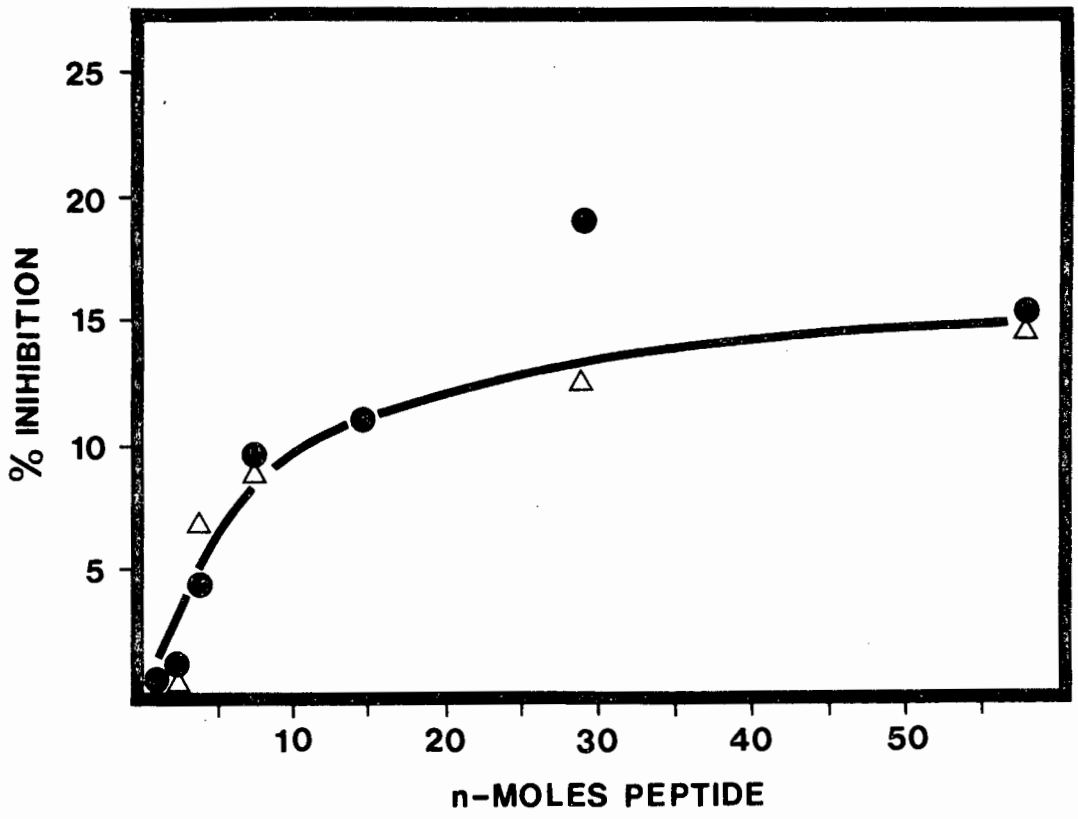

Fig. 2.24 : Inhibition of the precipitin reaction of 0,5 µg of radiolabelled TMV protein and its specific antibodies by increasing amounts of peptide 8 (residues 93-112) from TMV (●) and Ni 568 (△). No significant difference in the amount of inhibition caused by these two peptides was discernable. A $^{1}/_{128}$ dilution of a TMV protein antiserum was used in the tests.

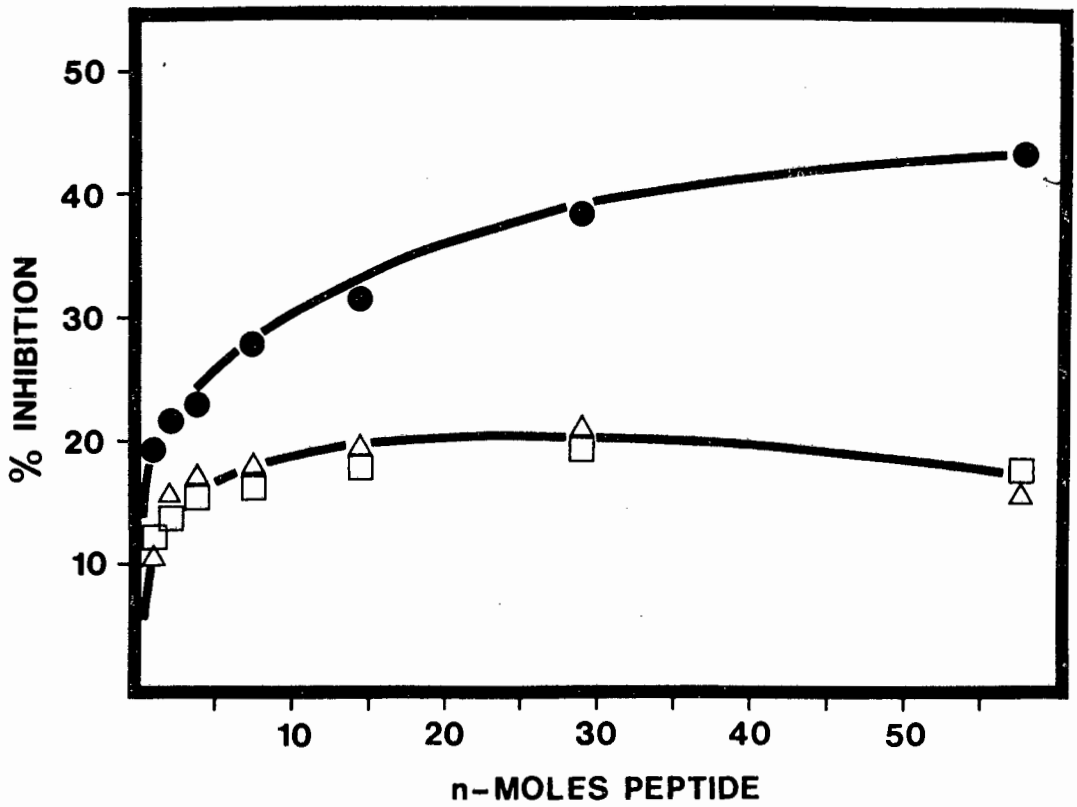

Fig. 2.25 : Inhibition of the precipitin reaction of 0,5 µg of radiolabelled TMV protein and its specific antibodies by increasing amounts of tryptic peptide 12 (residues 142-158) from TMV (●), Ni 1927 (□) and Ni 1688 (△). The two identical peptides from the mutant viruses cause the same amount of inhibition which is nevertheless smaller than the inhibition caused by the corresponding peptide from the wild type virus. A 1/128 dilution of a TMV protein antiserum was used in all tests.

difference in inhibition (about half the inhibition of the wild type peptide) caused by the replacement of serine (residue 65) by glycine in a single determinant out of five (van Regenmortel and Lelarge, 1973) may be beyond the sensitivity of the micro-precipitin inhibition assay (3.2).

The difference in the abilities of Ni 1688 and Ni 1927 protein to inhibit the reaction of radiolabelled TMV protein and its antibodies (4.2.3) is easily understood in terms of the results with the mutant peptide homologues of peptide 4 and 12. Although these two mutants share the amino acid exchange of proline to leucine in position 156, it is the replacement of proline by serine in position 63 that causes the decrease in inhibition by Ni 1688 protein relative to Ni 1927 protein (Fig. 2.11 B).

2.6.2 A COMPARISON OF THE IMMUNOCHEMICAL BEHAVIOUR OF CARBOXYPEPTIDASE A TREATED PEPTIDE 12 (RESIDUES 142-157) WITH WILD TYPE PEPTIDE 12 (RESIDUES 142-158) BY PRECIPITIN INHIBITION ASSAY WITH RADIOLABELLED TMV PROTEIN

Peptide 12 (residues 142-158) was treated with carboxypeptidase A (4.1.5 and 2.1.4) in order to remove the C-terminal threonine residue (table 2.2). The immunochemical behaviour of this derivative (residues 142-157) was compared with that of wild type peptide 12 using a micro-precipitin inhibition assay with radiolabelled TMV protein and a TMV protein antiserum. The antiserum was titrated and the inhibition assay was performed as described in section 4.3.4.2.

Fig. 2.26 shows that the inhibition of the radiolabelled TMV protein-anti TMV protein reaction caused by the wild type peptide 12 and the derivative, from which the C-terminal threonine had been removed, was not distinguishable over a range of peptide concentrations corresponding

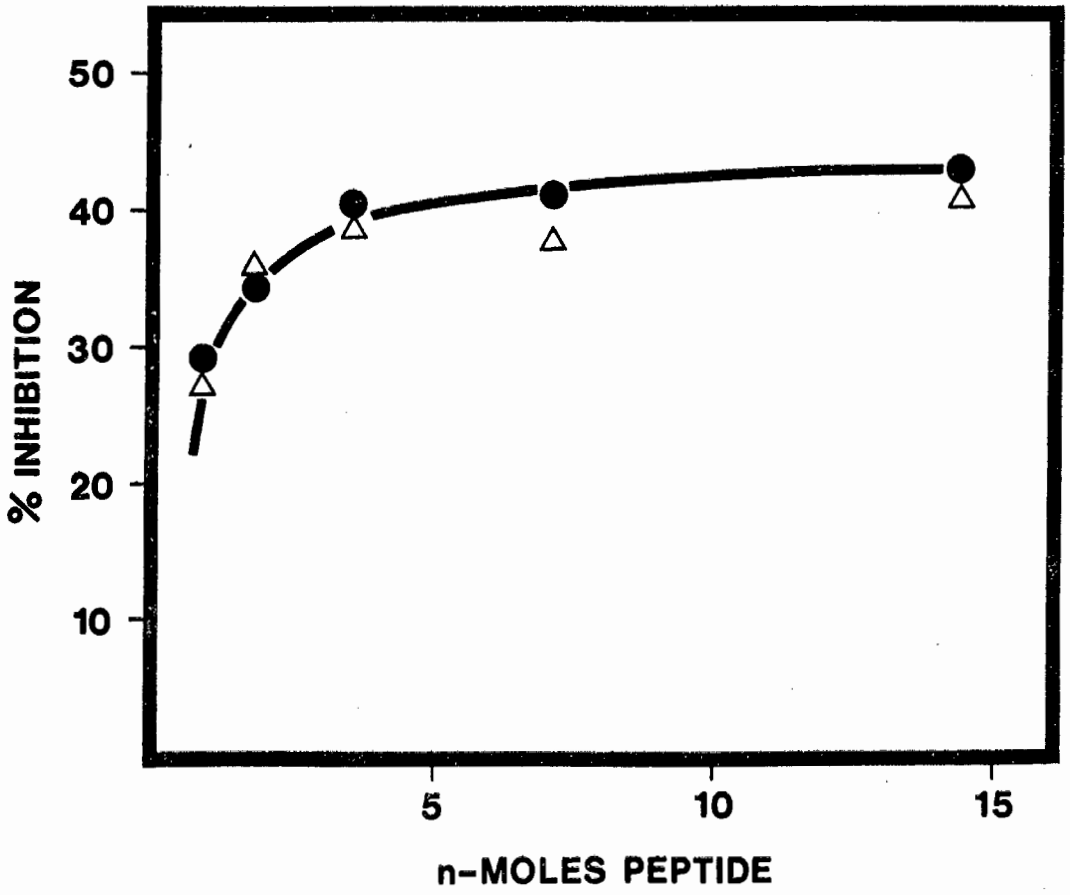

Fig. 2.26 : Inhibition of the precipitin reaction of 0,5 µg radiolabelled TMV protein and its specific antibodies by increasing amounts of peptide 12 (residues 142-158) from TMV (●) and a derivative of tryptic peptide 12 from which the C-terminal threonine had been removed (residues 142-157) (△). No significant difference in the amount of inhibition caused by these two peptides was discernable. A 1/128 dilution of a TMV protein antiserum was used in the tests.

to a molar excess of 25-400:1 of peptide over TMV protein. It is, therefore, unlikely that the threonine residue in position 158 at the C-terminus of the protein is a part of the cryptotope that is located in peptide 12.

PART 3. DISCUSSION

3.1 EVALUATION OF THE IMMUNOCHEMICAL TECHNIQUES USED TO DETERMINE THE ACTIVITIES OF PEPTIDES

Of the three immunochemical techniques used for demonstrating the antigenic activity of TMV peptides, inhibition of the micro-complement fixation system (Wasserman and Levine, 1961) (2.5.1.4) proved to be the most sensitive. The molar concentrations of the reactants in the complement fixation system were found to average 2×10^{-9} moles/liter, a value which compares well with the results from other laboratories using this technique (Reichlin and Noble, 1977). This sensitivity, which means that immunochemical activity can be detected even with very small amounts of peptide, results from the amplification effect which is implicit in the complement fixation system. It is thought to result from a small number of immune complexes causing the inactivation (fixation) of large numbers of early complement components, so preventing their participation in the lysis of sheep erythrocytes. When the formation of some of these immune complexes is inhibited by a peptide, the early complement proteins, which would normally be unavailable, can be activated by the haemolysin antibodies bound to the erythrocytes to cause extensive lysis. The high inhibitions obtained with individual peptides (2.5.1.4 - fig. 2.18) must be seen in the context of the serum dilution used in the complement fixation assay. A quantitative measure of the proportion of the complement fixing antibody population, that reacts with a particular peptide, is the difference between the two serum dilutions that cause an equal amount of complement fixation in the presence of a fixed quantity of peptide. It was probably this amplification effect which caused Benjamini and co-workers to conclude (Benjamini et al, 1964; Benjamini, 1977) that peptide 8 contained the immunodominant antigenic determinant of the TMV protein.

antigen. Antibodies specific for an antigenic determinant bind the corresponding random coil peptide with less affinity than they would the same region of the polypeptide chain in the protein and thus the resultant antibody-peptide complexes would dissociate more readily. As a result of this, the quantity of peptide bound by antibody, at a specific time, may be small, even though a considerable amount of binding has taken place during the experiment.

Another explanation for the low percentage binding with the N-(^{3}H)acetyl peptides could be framed in terms of the concept of a conformational equilibrium of protein fragments in solution (Sachs et al., 1972; Habeeb, 1977). However, this hypothesis may not be applicable to all peptide solutions (3.2).

The value of the direct binding technique lies in the fact that it provided evidence of activity in peptides that appeared to be inactive in inhibition experiments: Antibodies reactive with N-(^{3}H)acetyl peptide 12 could only partly be removed from the TMV antisera by absorption with TMV protein using this technique (2.5.3.3 - table 2.5). No inhibitory activity could be detected with this peptide, though it has a known viral determinant (Harris and Knight, 1955; von Sengbusch and Wittmann, 1965; von Sengbusch, 1965; and van Regenmortel, 1967), in the TMV-anti TMV system with either complement fixation, using TMV protein absorbed sera, or the micro-precipitin technique. Direct binding tests may thus be of considerable importance for elucidating the nature of antigenic determinants in viruses and perhaps other antigens with quaternary structure.

The sensitivity of the direct binding technique is comparable with that

of microcomplement fixation and the micro-precipitin assay in that the molar concentrations of the reactants was of the order of 10^{-8} moles/liter. It must be borne in mind, however, that the percentage bindings of the individual N-(^{3}H)acetyl peptides were small.

3.2 SUMMARY AND INTERPRETATION OF THE IMMUNOCHEMICAL RESULTS IN TERMS OF THE KNOWN STRUCTURE OF THE TMV PROTEIN SUBUNIT

A diagrammatic representation of the course of the polypeptide chain of the TMV protein subunit (Bloomer et al., 1978), showing the locations of five of the tryptic peptides used in this work, is given in fig. 3.1 and will be referred to throughout the ensuing discussion. The approximate locations of the residues where amino acid exchanges have occurred in the TMV mutants that were used, as well as the threonine residue in position 158, that was removed from peptide 12 (residues 142-158), are also indicated.

Peptide 1 (residues 1-41) was remarkable in that it could react with antibodies of a TMV protein antiserum to fix complement (2.5.1.4 - fig. 2.19). This effect was not due to aggregation of the peptide, as the same preparation of peptide 1 was able to inhibit complement fixation in the normal manner, when the TMV protein antiserum had first been absorbed with TMV to remove antibodies capable of reacting with the intact virus. This suggests that peptide 1 contains two seperate antigenic regions: one being a cryptotope (1.2.1), while the other is recognized by antibodies capable of binding to the viral capsid.

In an antiserum raised against TMV protein, antibodies specific for cryptotopes, neotopes and determinants common to both virus and depolymerized protein subunits can exist (1.2.3). If two IgG antibodies,

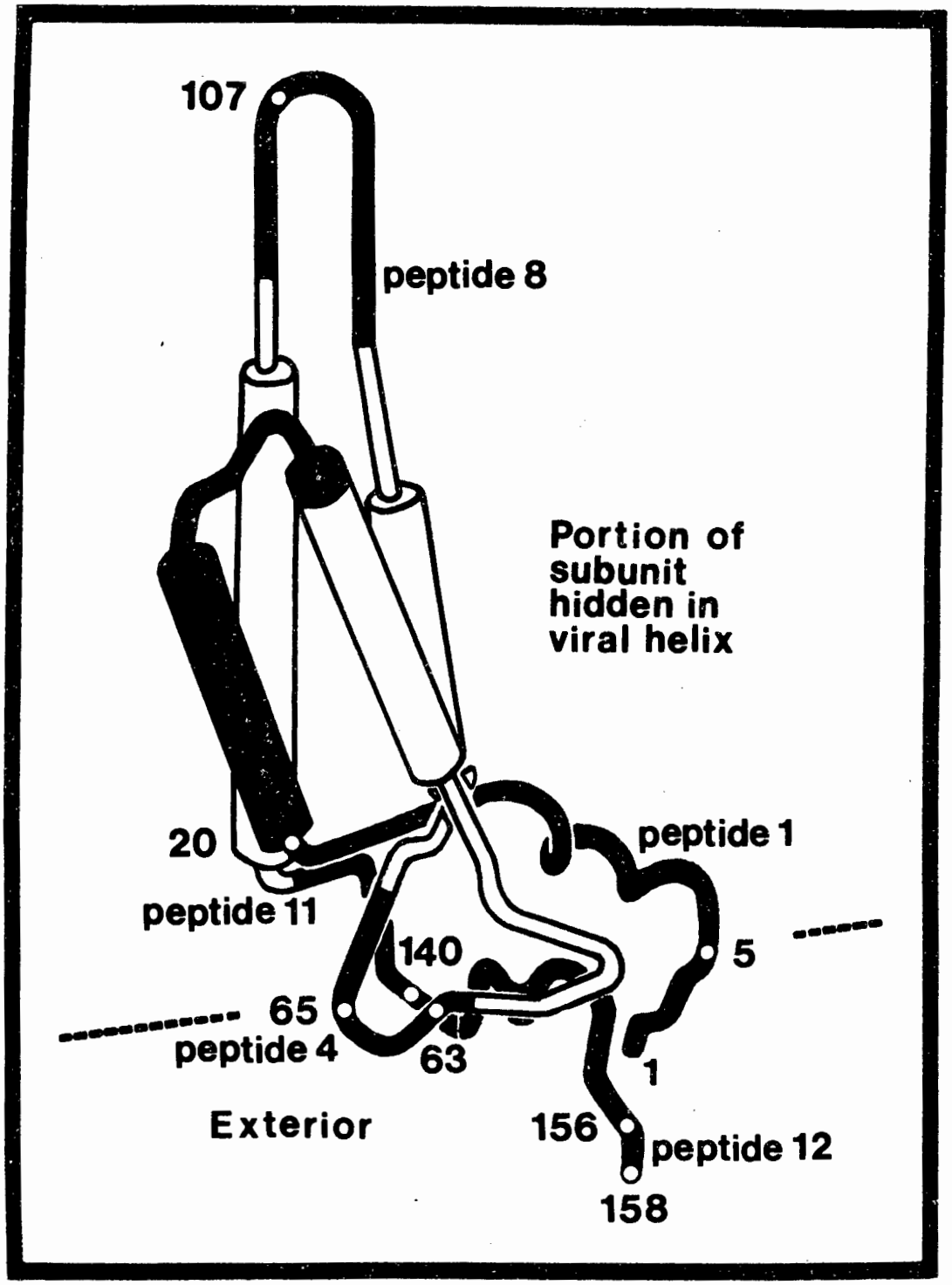

Fig. 3.1 : A diagramatic representation of the secondary and tertiary structure of the TMV protein subunit in the 20S disk. The four approximately parallel helices are represented as cylinders (based on Bloomer et al., 1978; Argos et al., 1977). The regions of the polypeptide chain shown in black represent the five tryptic peptides used in this study. The approximate locations of the amino acid exchanges of the mutants are shown as numbered open circles. The portion of the protein subunit that is exposed in the viral capsid is beneath the dotted lines.

with anti-cryptotope and anti-viral specificities, respectively, were to bind to a single peptide, the resultant immune complex would be capable of fixing complement. Absorption of the antiserum with TMV would remove the antibodies capable of reacting with both virus and peptide and only the antibodies specific for cryptotopes would remain. With respect to the absorbed TMV protein antiserum, peptide I would then behave as a monovalent hapten and cause inhibition of complement fixation. (Milton and van Regenmortel, 1979.)

The first 5-6 residues of peptide I have been shown to be located on the surface of the protein subunit in the 20S disk (Bloomer et al., 1978) and this is likely to be the case in the viral helix as well (Graham and Butler, 1978). This region, therefore, is most likely to contain the antigenic site that is recognized by anti-viral antibodies as no other part of peptide I is close enough to the viral surface. The cryptotope of peptide I is likely to be found in the C-terminal half of the peptide which is hidden by neighbouring subunits in the viral helix (Milton and van Regenmortel, 1979).

Whether the N-terminal antigenic region of peptide I is a neotope or a determinant common to both virus and depolymerized protein subunit, is still in doubt. The amount of enhancement of complement fixation caused by peptide I with TMV protein antisera (2.5.1.4 - fig. 2.19), as well as the results obtained with the precipitin inhibition experiments with radiolabelled TMV protein, where peptide I invariably caused inhibition almost twice as great as that of any other single peptide (2.5.2.3 - fig. 2.20), suggests that this determinant is common to both the intact virus and the depolymerized protein subunit. The results obtained in the inhibition of the precipitin reaction with radiolabelled

TMV protein (2.5.2) seem to support this conclusion, as antibodies to neotopes are not measured by this technique. The high level of inhibition caused by peptide I was taken to represent the involvement of antibodies specific for both antigenic regions of the peptide.

On the basis of this argument it was expected that a homologue of peptide I from the TMV mutant Ni 568, where the threonine in position 5 is replaced by isoleucine, would not react as well with antibodies specific for the depolymerized protein subunit as the wild type peptide. However, the mutant peptide was found to inhibit the precipitin reaction of radiolabelled TMV protein and its specific antibodies to the same extent as the wild type peptide I (2.6.1.2 - fig. 2.22). Unless the N-terminal antigenic determinant of peptide I is less than five amino acids in size, or the replacement of threonine by isoleucine does not affect antibody recognition of this site — and both these explanations seem unlikely in terms of current knowledge of antigenic determinants (Atassi, 1977 b; 1978) — the possibility of the N-terminal region (residues 1-5) of the TMV protein subunit comprising a neotope (1.2.1) of the viral capsid, must be considered.

On the other hand, it is possible that in TMV protein antisera the proportion of antibodies specific for the peptide I cryptotope is very large compared to the antibodies specific for the antigenic site in the region of residues 1-5. The effect of the replacement of serine by isoleucine, in position 5, would then be minimized and may not even be distinguishable with cetain antisera using the micro-precipitin technique. Further immunochemical studies with the isolated N-terminal and C-terminal regions of peptide I are needed to resolve this question.

In the preliminary experiments, an attempt was made to obtain two peptides (corresponding to the sequences 1-19 and 20-41 respectively) which would contain the cryptotope and the viral antigenic determinant of peptide I on seperate polypeptide chains. TMV protein and peptide I were hydrolysed in 66% (v/v) formic acid solution to cleave the aspartyl-proline bond between residues 19 and 20 (Baltz and van Regenmortel, 1974). Unfortunately, neither of these two peptides was soluble under the conditions of the assay (2.1.4).

The bend in the polypeptide chain at the beginning of the LS α-helix may be regarded as a likely location for the cryptotope in peptide I, especially in view of the reported strong inhibition of the TMV-anti TMV precipitin reaction by a peptide corresponding to residues 18-23 (Anderer, 1963 b). It is not clear how this result can be reconciled with the known location of residues 18-23 away from the surface of the virus rod (Stubbs et al., 1977; Bloomer et al., 1978). It is possible that contamination of the antivirus precipitin system with depolymerized viral subunits is responsible for this effect. The protein of the TMV mutant Ni 118, which has a single amino acid exchange, proline to leucine in position 20, was found to be distinguishable from the wild type TMV protein by its reduced ability to inhibit the precipitation of radiolabelled TMV protein by anti-TMV protein antibodies (2.4.3 - fig. 2.11). The Ni 118 homologue of peptide I, however, caused inhibition that was not significantly different from wild type peptide I in the same system (2.6.1.2 - fig. 2.22). This binding can be explained if it is assumed that although the amino acid exchange in position 20 causes a conformational change in the protein, the same exchange does not affect the conformation of the peptide to the same extent owing to its random coil structure. Thus the replacement of

proline by leucine in the protein would exert its influence on the antigenicity by modulating the conformation of neighbouring residues that are part of an antigenic determinant, in much the same way as suggested by von Sengbusch (1965) for the virus-anti virus system. The exact location of the cryptotope of peptide 1 is, however, still to be established.

Tryptic peptide 4 (residues 62-68) was found to inhibit the TMV-anti TMV precipitin reaction (Anderer, 1963 b). In addition, amino acid exchanges in position 65 and 66 resulted in TMV mutants that are serologically distinguishable from the wild type virus (von Sengbusch, 1965; van Regenmortel, 1967). The replacement of proline in position 63 by serine, however, did not affect serological behaviour (von Sengbusch, 1965). When these results are interpreted in terms of the location of this peptide on the surface of the protein subunit in the viral helix, it seems likely that the C-terminal part of peptide 4 contains an antigenic determinant of the intact virus (1.4.2).

In the present work, however, peptide 4 behaved as a cryptotope in all the relevant experiments. Antibodies specific for the antigenic region of peptide 4 could not be removed from TMV protein antisera by absorption with virus, as shown by both inhibition of complement fixation (2.5.1.4 - fig. 2.19) and direct binding assays (2.5.3.3 - table 2.5). In fact, N-(^{3}H)acetyl peptide 4 was bound equally well by a particular TMV protein antiserum before and after it was absorbed with TMV (table 2.5). Absorption of a TMV antiserum with TMV protein, on the other hand, completely eliminated the antibodies capable of binding to N-(^{3}H)acetyl peptide 4 (table 2.5). Peptide 4 also strongly inhibited the specific precipitin reaction of radiolabelled TMV protein

and anti-TMV protein antibodies (2.5.2.3 - fig. 2.20).

Two possible explanations for the discrepancy between the results with peptide 4 reported in this work and the earlier findings (von Sengbusch, 1965; van Regenmortel, 1967) can be suggested: it is possible that the amino acid exchanges in positions 65 and 66 exert their influence through some modulation effect on neighbouring residues, or alternatively, that the short peptide 4 contains, in addition to the cryptotope, a part of a viral antigenic determinant too small to be recognized by the corresponding antibodies (Milton and van Regenmortel, 1979).

An attempt was made to obtain a rough delineation of the peptide 4 cryptotope by comparing the activity of the wild type peptide 4 with that of the tryptic peptide homologues of two mutants (2.6.1.2 - fig. 2.23). The mutants were 414 (serine in position 65 replaced by glycine) and Ni 1688 (proline in position 63 and 156 replaced by serine and leucine respectively). The peptide 4 homologue with serine in position 63 did not inhibit the precipitation of radiolabelled TMV protein by its specific antibodies, while the peptide homologue with glycine in position 65 showed a greatly reduced inhibition compared to the wild type peptide 4. This suggests that the peptide 4 cryptotope is located in the N-terminal half of the peptide and extends to at least residue 65. Part of the cryptotope may be found in the C-terminal region of tryptic peptide 3 (residues 47-61), but an accurate delineation would require a study with overlapping synthetic peptides (1.6.1) corresponding to this sequence: (Val-Arg)-Phe-Pro-Asp-Ser-(Asp-Phe). In terms of the structure of the TMV protein subunit (Bloomer et al., 1978), this sequence is located in a projecting loop of the polypeptide chain at the 80 Å radius (best illustrated by comparing fig. 3.1 with the side elevation in fig. 1.4 B). Such surface configurations were also found to be associated with anti-

genic sites in the case of myoglobin (Atassi, 1975; 1977 b).

The mutant 414 protein is indistinguishable from wild type TMV protein in its ability to inhibit the precipitation of radiolabelled TMV protein by its specific antibodies (2.4.3 - fig. 2.11). On the other hand, the same amino acid exchange at position 65, in the case of the peptide, leads to a mutant peptide 4 with decreased inhibitory capacity compared to wild type peptide 4. This implies that residue 65 is located within an antigenic determinant. However, if this determinant is a cryptotope, as was concluded from the inhibition of complement fixation and direct binding studies, the serological distinguishability of amino acid exchanges at positions 65 (von Sengbusch, 1965; van Regenmortel, 1967) and 66 (van Regenmortel, 1967) would mean that they modulate the conformation at the surface of the viral helix. This conformational modulation effect, which would occur in the depolymerized protein subunit as well, may lead to a small change of overall activity in the monomers, because of the large proportion of the free protein surface that contains other protein determinants which are not affected.

In the case of the amino acid exchange in position 63, the complete loss of the inhibitory capacity of the corresponding mutant peptide also demonstrates that it is a part of the peptide 4 cryptotope, but because of the nature of the exchange, from proline to serine, it is also expected to lead to a major conformational change in the protein subunit. This is illustrated by the differing inhibitory power of wild type TMV protein and Ni 1688 protein in the radiolabelled TMV protein-anti TMV protein precipitin system (2.4.3 - fig. 2.11). The difference in the behaviour of Ni 1927 protein (proline in position 156 replaced by leucine) and Ni 1688 protein (proline in position 63 replaced by serine as

well as proline in position 156 replaced be leucine) shows that the amino acid exchange in position 63 is responsible for the greater part of the reduction in the inhibitory capacity of Ni 1688 protein.

It is not clear why von Sengbusch (1965) failed to find the amino acid exchange of proline for serine in position 63 serologically distinguishable in the TMV-anti TMV system, especially since a less extreme exchange of serine for glycine, only two residues away at position 65, was distinguishable. It would be expected that the nature of the amino acid exchange in position 63 would affect an adjacent viral antigenic determinant, even if residue 63 were not a part of it, especially in the quaternary structure of the viral capsid. Also, if the amino acid exchange in position 65 caused a conformational modulation of the viral subunit surface, the replacement of proline in position 63 should do the same.

The antigenic site in tryptic peptide 8 (residues 93-112) has been fully characterized by Benjamini and co-workers (1.4.3; 1.5.2; and 1.6.2) and found to be a pentapeptide (residues 108-112: Leu-Asp-Ala-Thr-Arg) (Young et al., 1967). The location of this antigenic determinant in the region of the 20 Å radius (Bloomer et al., 1978), where it would border on the central hole in the viral helix (Stubbs et al., 1977), prevents it from being considered as anything but a cryptotope. It is obvious that it could not be antigenically expressed in the intact virus except at the extremities of the viral rod (Milton and van Regenmortel, 1979). The results obtained in the present work support this conclusion. Antibodies specific for peptide 8 could not be removed from antisera raised to depolymerized TMV protein by absorption with virus as determined by both inhibition of complement fixation (2.5.1.4 - fig. 2.19) and

direct dinding studies (2.5.3.3 - table 2.4). On the other hand, the absorption of TMV antisera with protein sununits removed all the anti-peptide 8 activity that the antisera possessed (table 2.4). In this respect the results are similar to those obtained with peptide 4.

In view of these findings, it is somewhat surprising that the replacement of threonine by methionine in position 107 was found to be serologically distinguishable in the TMV-anti TMV system (von Sengbusch, 1965). Similarly, the protein of the TMV mutant Ni 568, which has the same amino acid exchange, but in addition has the threonine in position 5 replaced by isoleucine, was found to inhibit the precipitation of radio-labelled TMV protein far less efficiently than unlabelled wild type protein (2.4.3 - fig. 2.11 A). In this case also, neither of the mutant peptide homologues containing the amino acid exchanges in positions 5 and 107 were distinguishable from the wild type peptides in this system, utilizing the same antiserum (2.6.1.2 - fig.s 2.22 and 2.24). In view of von Sengbusch's (1965) findings with two TMV mutants which have the same amino acid replacement at position 107, it is thought likely that the exchange of threonine for methionine is responsible for the differing serological behaviour of the protein of the mutant Ni 568 (rather than the amino acid replacement in position 5). The incorporation of methionine in this region of the protein sequence causes a far-reaching conformational modulation of the whole protein subunit, affecting antigenic determinants on the viral surface.

The results obtained with the proteins of the mutants Ni 118 and Ni 568, showing them to be serologically distinguishable from the wild type TMV protein (2.4.3 - fig. 2.11), have been interpreted in terms of the amino acid exchanges in these proteins having a likely conformational effect,

rather than a direct involvement in antigenic determinants. In the case of Ni 118 protein, this interpretation is supported by circular dichroism data (Budzynski, 1970) which shows conformational differences in comparison with wild type protein. Peptide homologues from these mutant proteins were not distinguishable from the corresponding wild type peptides (2.6.1.2 - figs. 2.22 and 2.24) while comparable amino acid exchanges in other peptides were detectable (figs. 2.23 and 2.25). As these mutants retain their biological properties, the envisaged conformational changes must be relatively small. It has been suggested (Atassi et al., 1970) that drastic conformational changes are not permissible in an evolutionary sense, as they would disrupt the biological functions of proteins. However, small conformational changes, which may not be detected by the rather coarse physical methods now available for examining protein conformation, could be tolerated and exert a measurable effect on the antigenic reactivity of the protein. On the other hand, when certain amino acid exchanges are not distinguishable in the protein or a peptide, there is always the possibility that the change in a particular amino acid side chain is insufficient to affect the immunochemical role of that residue in an antigenic site (1.3.1).

An alternative multideterminant hypothesis must be considered as well (White et al., 1978). This suggests that there may be many other antigenic determinants, beside those identified so far in proteins such as myoglobin and lysozyme, that embrace collectively almost the entire surfaces of these proteins and elicit the production of small amounts of antibodies which are only detectable with very sensitive techniques like the micro-complement fixation assay (White et al., 1978). The fact that mutant peptide homologues cannot be distinguished from a bivalent wild type peptide such as peptide 1, whereas the corresponding mutant

The protein of the mutant CP 415, which has the asparagine residue in position 140 replaced by lysine, was not distinguishable from wild type TMV protein when tested for its ability to inhibit the precipitation of the radiolabelled TMV protein by its specific antibodies (2.4.3 - fig. 2.11 A). This is in accordance with the results of du Plessis and van Regenmortel (1977), who showed that this mutant protein could not be distinguished from wild type TMV protein in a modified phage assay (1.3.3).

The location of peptide 12 (residues 142-158) at the surface of the protein subunit which is also exposed in the intact virus, would lead one to expect this region to be antigenically very active (Milton and van Regenmortel, 1979). The C-terminus of the TMV polypeptide chain has been implicated in an antigenic determinant of the virus ever since it was found that the serological specificity changes when the threonine in position 158 is removed from the subunits of the intact virus by carboxypeptidase A. Although antisera raised to the carboxypeptidase A treated virus and untreated TMV showed strong mutual cross-reactions, cross-absorption of the antisera completely prevented the heterologous reaction (Harris and Knight, 1955). Similar studies with the carboxypeptidase A treated derivative of the TMV mutant Ni 1927 (proline in position 156 replaced by leucine), which has lost the three terminal amino acids (Leu-Ala-Thr), showed that this derivative was still able to react with both TMV and Ni 1927 antisera after they had been absorbed with Ni 1927 virus (von Sengbusch and Wittmann, 1965). The existence of heterospecific antibodies in TMV antisera (Loor, 1971; van Regenmortel, 1978) was confirmed by the observations that absorption of TMV antisera with TMV did not remove the capacity for reaction with the mutants Ni 1927, Ni 1688 (von Sengbusch, 1965) and 171 (van Regenmortel,

1967), which all have the proline in position 156 replaced by leucine. (1.3.2.)

Another explanation for the presence of heterospecific antibodies in TMV antisera, based on the existence of two conformational forms of the protein subunit (Vogel, 1973; Vogel and Jaenicke, 1974), was proposed in section 1.3.2. It was suggested that the C-terminal region of TMV protein contains two antigenic sites, a neotope and a cryptotope. The neotope is associated with the pH 7,0 conformational form of the protein subunit, which has the same C-terminal configuration as the 20S disk (Vogel, 1973; Vogel and Jaenicke, 1974), and hence the intact virus (Graham and Butler, 1978). This antigenic site, which is immunogenic only in the virus, is most likely located at the C-terminus of the polypeptide chain and involves the threonine residue in position 158 (Harris and Knight, 1955; von Sengbusch and Wittmann, 1965). The cryptotope, on the other hand, exists only by virtue of the configuration of the region comprising residues 151-156 in the pH 8,0 form. The present interpretation is based on the assumption that residues 157 and 158 are not part of the cryptotope. Thus the neotope and the cryptotope sites are expected to overlap.

When intact TMV is injected into an animal, partial dissociation of the viral rods may release free protein subunits in the pH 8,0 conformational form. This would lead to the formation of antibodies specific for the peptide 12 cryptotope. These anti-cryptotope antibodies would not be able to bind to the C-terminal configuration of the intact virus because of the rigidity resulting from the proline residue in position 156, and hence would not be removed from a TMV antiserum by absorption with TMV. The replacement of the proline in position 156 by leucine, as in the

TMV mutants Ni 1688, Ni 1927 and 171, would lend flexibility to the C-terminal configuration of the virus (von Sengbusch and Wittmann, 1965) and allow some of the anti-cryptotope antibodies to bind to their antigenic site. Although the three C-terminal amino acids may still cause steric hindrance, it could be less than when the rigid proline is present (von Sengbusch and Wittmann, 1965), as the presence of proline allows no flexibility in the configuration of the two terminal residues. It is possible that the steric hindrance effect is completely removed by splitting off the three terminal amino acids of the mutants (von Sengbusch and Wittmann, 1965) to expose the major part of the cryptotope which is expected to extend from residues 151-156.

The existence of a cryptotope in peptide 12 was confirmed by the results of inhibition of complement fixation experiments with TMV protein antisera absorbed with TMV (2.5.1.4 - fig. 2.19). The removal of virus specific antibodies from TMV protein antisera did not lessen the ability of peptide 12 to inhibit the remaining specific TMV protein-anti TMV protein reaction. Similarly, absorption with the intact virus could not remove about 70% of the antibodies in a TMV protein antiserum which reacted with N-(^{3}H)acetyl peptide 12 in direct binding assays (2.5.3.3 - table 2.5). In another experiment, when a TMV antiserum was absorbed with TMV protein subunits to remove all the anti-protein activity, about 30% of the antibodies reactive with the N-(^{3}H)acetyl peptide 12 remained (2.5.3.3 - table 2.5). These latter antibodies, which could not be removed by absorption with TMV protein, are specific for the viral capsid and are directed against a neotope. Thus peptide 12 contains a neotope in addition to the cryptotope described above. The threonine in position 158 is likely to be a part of this neotope.

Milton and van Regenmortel (1979) suggested that peptide 12 exists, in solution, in a mobile configuration and that it can assume the native conformation present in the viral capsid. If this is the case, it is this conformation which would allow anti-neotope antibodies to combine with peptide 12.

The location of the peptide 12 cryptotope was probed by the use of mutant peptide homologues from Ni 1688 and Ni 1927 (proline in position 156 replaced by leucine in both peptides), and a carboxypeptidase A treated derivative of peptide 12 (2.1.4) with the C-terminal threonine removed (residues 142-157). The two mutant peptides, which were identical except for their source, caused the same amount of inhibition of the precipitation of radiolabelled TMV protein by its specific antibodies. This inhibition was only half that caused by the wild type peptide 12 (2.6.1.2 - fig. 2.25). Ni 1688 protein and Ni 1927 protein were also found to be less efficient inhibitors of the precipitin reaction of radiolabelled TMV protein than the unlabelled protein of wild type TMV (2.4.3 - fig. 2.11). This decrease in the efficiency of inhibition by the mutant peptides and proteins, compared to their wild type counterparts, suggests that the proline residue in position 156 is a part of the cryptotope of peptide 12. On the other hand, the replacement of proline by leucine does not completely prevent specific antibodies from reacting with the cryptotope (in the same way that a similar replacement of proline by serine in position 63 of the mutant peptide 4 homologue of Ni 1688 prevents the reaction of specific anti-cryptotope antibodies —— fig. 2.23). This leads to the assumption that the proline residue in position 156 is not absolutely essential for antibody binding of the peptide 12 cryptotope. It may well be the "boundary" residue of the antigenic site. The preliminary result with the peptide 12 derivative from

which the C-terminal threonine had been removed (2.1.4), supports the idea that the cryptotope sequence ends at residue 156 and this derivative was found to cause the same amount of inhibition of the radiolabelled TMV protein precipitin reaction as the wild type peptide 12 (2.6.2 - fig. 2.26). This result has to be viewed with a certain amount of circumspection since the quality of the separation of the undigested peptide 12 from peptide 12 minus threonine on Sephadex G50 (2.1.4 - fig. 2.4) was much better than expected from the gel matrix characteristics. However, the amino acid analysis of the pooled fractions of the second peak was characteristic of a peptide 12 derivative which had lost the C-terminal threonine (table 2.2). The observation that the peptide 12 minus threonine reacted positively with ninhydrin in a spot test, while peptide 12 did not (2.1.4), also favours the view that both peaks do not represent peptide 12 in different states of aggregation. Immunochemical studies with overlapping synthetic peptides and cross-absorbed antisera will be necessary to obtain a more precise definition of the peptide 12 neotope and cryptotope.

A finding, which is not explicable in terms of the overlapping cryptotope-neotope model, is the fact that carboxypeptidase A treated Ni 1927 virus reacts better with TMV antiserum than both intact TMV and Ni 1927 (von Sengbusch, 1965).Because of the absence of the rigid proline residue at position 156, it can be argued that intact Ni 1927 virus expresses configurations in the C-terminal region that are necessary for the reaction of antibodies specific for both the neotope and the cryptotope. If threonine 158 is a part of the viral antigenic determinant as concluded by von Sengbusch and Wittmann (1965), carboxypeptidase A treated Ni 1927 virus, which has lost the three C-terminal residues, would only be able to react with the anti-cryptotope antibodies. It would, there-

fore, be expected to react less strongly than Ni 1927 with TMV antisera in terms of the model described in section 1.3.2. If, however, the carboxypeptidase derivative of Ni 1927, used by von Sengbusch (1965), had not lost these amino acids from all the subunits in each viral capsid, the situation would not be significantly different from that described for Ni 1927 virus: The anti-cryptotope antibodies would react with the exposed cryptotopes on the subunits lacking the C-terminal residues, while the anti-neotope antibodies would combine with the uncleaved subunits in the favourable conformation. Thus the reaction of Ni 1927 and a partially digested carboxypeptidase treated derivative of this mutant with TMV antisera would be expected to be approximately equal. In fact, the carboxypeptidase treated Ni 1927 derivative, used by von Sengbusch , was only slightly superior to the untreated Ni 1927 virus in its precipitin reaction with a TMV antiserum, and both were almost twice as active as wild type TMV (von Sengbusch, 1965). A test of this model, based on the existence of two conformational forms of the protein subunit, would consist in letting Ni 1927 virus and its carboxypeptidase A treated derivative react with TMV antiserum after it had been fully absorbed with TMV protein at pH 8,0.

In both the inhibition of complement fixation experiments with the TMV protein-anti TMV protein system and the inhibition of the precipitin reaction with radiolabelled TMV protein, peptide 12 was shown to be antigenically very active (2.5.1.4 - figs. 2.17 and 2.18, and 2.5.2.3 - fig. 2.20). It is not clear why Benjamini *et al.* (1964) failed to demonstrate activity in this peptide.

The results of cross-reaction studies with TMV and Ni 1927 protein at pH 6,8 and pH 8,0, in the precipitin system with radiolabelled TMV and

specific anti-TMV antibodies (2.3.3 - fig. 2.9), were especially surprising in view of the lack of activity displayed by all five tryptic peptides in both inhibition of complement fixation (2.5.1.3) and inhibition of micro-precipitin (2.5.2.2) assays performed with the same antibodies. On the other hand, both TMV protein at pH 6,8 and pH 8,0 as well as Ni 1927 protein at pH 8,0 were able to cause complete inhibition of the specific TMV-anti TMV reaction (fig. 2.9). The simplest explanation of this phenomenon is that small amounts of TMV and Ni 1927 protein aggregates, 20S and larger, are present in sufficient quantity to react with all the TMV specific antibodies. The pH dependance of the inhibition could be ascribed to the greater ease of aggregate formation at lower pH, except in the case of Ni 1927 protein at pH 6,8 which is far less efficient an inhibitor of the TMV-anti TMV reaction than the same protein at pH 8,0 (fig. 2.9). This would imply that the aggregation behaviour of Ni 1927 protein is the reverse of wild type TMV protein. Also, analytical ultracentrifugation of identically treated TMV protein solutions at the same pH (4.2.4), and with up to double the concentrations used in the assays, did not show any sign of the convective disturbances, seen on the baseline of the schlieren pattern (Schachman, 1959), associated with trace amounts of the higher aggregates (Dr. A. Polson - personal communication).

Assuming, for the moment, that subunit aggregation is not entirely responsible for the complete inhibition of the TMV-anti TMV reaction observed with the TMV and Ni 1927 protein solutions, a tentative hypothesis, based on the mobility of the polypeptide chain in cowpea chlorotic mottle virus, is suggested. It was found by fluorescence studies with pyridoxal-5'-phosphate labelled protein subunits and capsids of cowpea chlorotic mottle virus, that the mobility of the polypeptide chain in the free subunit was greater than in the intact capsid (Krüse et al., 1978).

If a similar situation exists with TMV and its subunit, it would be expected that the quaternary structure of the capsid imposes a far more rigid conformation on the constituent subunits than is found in the depolymerized TMV protein. Continuing this line of reasoning, peptides would have the least structure, approaching the random coil state, depending on their size. It has been shown that peptides resulting from the cleavage of myoglobin at proline and arginine residues have an improved immunochemical reactivity when their helical content increased (Atassi, 1977 b). If this is a general phenomenon, it is suggested that antibodies specific for a "continuous site" (Atassi, 1978) conformational determinant (Sela et al., 1967) of the viral capsid may be able to react with the same, less rigidly structured sequence in the depolymerized protein subunit (even if this region is no longer immunogenic in the absence of the quaternary structure), but with a low binding affinity. This means that a very large molar ratio of subunits to virus specific antibodies would be required for substantial binding. A proportionately larger molar excess of a random coil peptide, containing the same sequence, would be required to achieve an equivalent binding with this type of antibody. On the other hand, antibodies raised to the virus will bind a neotope having the same sequence with high affinity because of the conformation induced by the quaternary structure of the viral helix. Then, theoretically, the molar ratio of a single species of neotope to antibody will be 2:1, assuming monogamous bivalent binding of the virus and ignoring the steric hindrance between antibody molecules.

The results obtained by Sachs et al. (1972) imply that a conformation identical to the native conformation of staphylococcal nuclease is required before peptides could be bound by antibodies specific for a conformational determinant of the protein. However, the specificity of the antibodies used in these experiments is in doubt. Habeeb (1977)

has argued that the method of Sachs and co-workers for isolating conformation specific antibodies is likely to give rise to an antibody population capable of cross-reacting with both native nuclease and the disordered form of the determinant. Nevertheless, a conformational equilibrium may not be a feature of all peptides in solution, and conformation specific antibodies may still react in a reversible manner with random coil peptides. In the case of a conformational equilibrium, the difficulty would be to detect the low affinity reaction with the disordered peptide in the presence of peptide in the native conformational form, as the specific antibodies would bind in an irreversible manner to the latter form of the peptide.

In any case, the hypothesis, based on the reaction of virus conformation specific antibodies with equivalent less structured sequences in protein subunits and peptides, should not be seen as a contradiction of the concept of the conformational cryptotope or the neotope (van Regenmortel, 1966; van Regenmortel and Lelarge, 1973), as these would relate to the immunogenicity of a particular region of the polypeptide chain when it is associated with a defined conformational form. In a more relaxed configuration the same sequence would not necessarily elicit the formation of antibodies with the same specificity, or even elicit the formation of antibodies at all. In fact it may react poorly with antibodies which bind its quaternary conformational form with a high affinity. Regions of the protein sequence which cause the formation of antibodies with the same specificity in both the subunit and the quaternary structure, would be involved in those cross-reactions that can be detected at equivalence proportions.

This hypothesis, although it would explain the complete inhibition with TMV protein and the inactivity of the peptides in the TMV-anti TMV system

at the molar excesses that were used, is unsatisfactory in that it is difficult to devise experimental situations to test its postulates. Studies, similar to those done by Krüse et al. (1978) on cowpea chlorotic mottle virus, could be carried out to assess the freedom of mobility of the polypeptide chains of the subunits in the various aggregates of TMV. Another approach would be to release antibodies specific for neotopes from TMV rods and use these in direct binding studies with radiolabelled N-bromosuccinimide (NBS) treated TMV protein (Ohno et al., 1972; du Plessis and van Regenmortel, 1977) and peptides I and 12. NBS treatment, which only modifies the tyrosine residue at position 139 (Ohno et al., 1972) is not likely to affect the sequences of the TMV protein associated with the neotopes in peptides I and 12. The configuration of the peptides could be stabilized by attaching them to carrier molecules.

To return to the cross-reaction studies with radiolabelled TMV and unlabelled TMV protein (2.3.3 - Fig. 2.9), an alternative interpretation to that of aggregation of the protein subunits, can be formulated in terms of the two conformational forms of the A-protein (Vogel, 1973; Vogel and Jaenicke, 1974) to explain the difference in the inhibitory efficiency of TMV protein at pH 6,8 and pH 8,0. If the peptide 12 cryptotope is exposed by the C-terminal configuration at pH 8,0, but not by the virus-like configuration at pH 7,0, antibodies, specific for the cryptotope (present in the TMV antiserum as a result of partial TMV dissociation after injection into the animal) would bind to the pH 8,0 protein subunits and in so doing would sterically hinder any reaction of anti-viral antibodies with the C-terminus of the polypeptide chain. In this way less protein subunits would be available to inhibit the TMV-anti TMV reaction and greater molar excesses of TMV protein would be required for inhibition (the amount of anti-cryptotope

antibodies remaining the same). At pH 6,8, however, the virus-like C-terminal configuration of the subunits would cause the peptide 12 cryptotope to be sterically hindered from reacting with its specific antibodies, and moreover, the C-terminal region would be conformationally more suited to react with the anti-viral antibodies specific for this region. The net result would be that a smaller molar excess of the subunits at pH 6,8 would be required, than at pH 8,0, to achieve an equivalent amount of inhibition of the TMV-anti TMV reaction. This was in fact observed. The similarity of the slopes of the two TMV protein inhibition curves at pH 6,8 and 8,0, can be accounted for by assuming that the same anti-viral antibody population is reacting with the same sequences of the protein subunits in both conformational states to cause inhibition of the TMV-anti TMV reaction.

Ni 1927 protein is a less efficient inhibitor of the TMV-anti TMV reaction than TMV protein (2.3.3 - Fig. 2.9), as would be expected from the location of the amino acid exchange in the C-terminal region. What is not understood is the reversal of the inhibitory behaviour of this protein, at pH 6,8 and pH 8,0, compared to TMV protein. This would imply either that its aggregation behaviour (at least from pH 7,0 - 8,0) is the opposite of that of wild type TMV protein, or that the two conformational forms of the A-protein in Ni 1927 are the reverse of those of TMV protein, depending on the explanation that has been accepted for the behaviour of TMV protein in these experiments.

The results that Anderer (1963 b) obtained by inhibition studies with peptides in the TMV-anti TMV precipitin system have been discussed in section 1.4.2. Two of the most active peptides, corresponding to residues 18-23 and 123-134 respectively, are located too far from the surface of the protein subunit in the viral helix to be available to

antibodies (Stubbs et al., 1977; Bloomer et al., 1978). A third active peptide, tryptic peptide 4 (residues 62-68), was found to behave like a cryptotope in the present work (2.5.1.4 - Fig. 2.19 and 2.5.3.3 - table 2.5). Anderer (1963 b) also found tryptic peptide 12 (residues 142 - 158) to be active, and this peptide was shown in the present work to contain a neotope and a cryptotope (2.5.3.3 - table 2.5). In contrast to Anderer's results, none of the five tryptic peptides (including peptides 4 and 12), showed any ability to inhibit the TMV-anti TMV reaction with either the micro-precipitin or complement fixation techniques, even though molar ratios of peptide to virus, far in excess of those used by Anderer, were used (2.5.1.3 and 2.5.2.2). Since three of the so called "active" peptides, of Anderer, seem to owe their inhibitory properties to non-specific effects, it is possible that all the other inhibitions obtained with peptide 12 and C-terminal octa- and hexapeptides are also non-specific in nature. The experiments showing that antibodies specific for hexa-, penta-, tetra- and tripeptides, corresponding to the C-terminal sequence of the TMV polypeptide chain bind to TMV particles (Anderer, 1963 a; Anderer and Schlumberger, 1965), only demonstrate that this region is on the surface of the subunit in the viral helix. The low level of antibodies specific for the C-terminal hexapeptide, that is found in TMV antisera (Anderer, 1963 a; Anderer and Schlumberger, 1965; Anderer et al., 1971; and Anderer and Ströbel, 1972) allows one to predict that the use of this peptide to inhibit the reaction between the conformational C-terminal determinant of the virus and its specific antibodies is likely to prove difficult.

It is not suggested that antibodies specific for neotopes cannot bind to peptides that contain the sequence corresponding to the neotope. On the contrary, it has been shown by direct binding studies with a

protein-absorbed TMV antiserum that peptide 12 reacts with specific antiviral antibodies (2.5.3.3 - table 2.5). It is thought, however, that the binding of random coil peptides by antibodies which recognize the conformation rather than only the specific sequence of an antigenic determinant (Sela et al., 1967) is by nature weak and transitory. In a peptide inhibition assay with any type of indicator immune system involving multivalent viruses, it is likely that the practically irreversible (Hardie and van Regenmortel, 1975) reaction between high affinity antibodies and virus will be favoured compared to the dissociating antibody-peptide interaction. This could lead to a situation where all the antibodies that are capable of reacting with the virus in a monogamous bivalent fashion (van Regenmortel and Hardie, 1976) would appear, within a very short time, to be unable to react with the peptide. The result would be that active peptides would seem to have no inhibitory capacity. Of course the probability for the antibody-virus reaction to occur can be decreased by increasing the molar ratio of peptide to virus, but there is a limit to the concentration of peptide solutions that can be utilized.

If this argument holds, techniques that rely on competitive inhibition are unsuitable for peptide studies with quaternary proteins such as TMV. Assays which measure the actual binding reaction between the antibody and the peptide (such as direct binding of radiolabelled peptides, equilibrium dialysis and affinity chromatography for example) would be expected to be more reliable for determining the amino acid sequences associated with conformational antigenic determinants.

In conclusion, two antigenic determinants have been detected in peptide 1 (residues 1-41), a cryptotope and a viral antigenic determinant. Peptides 4 (residues 62-68) and 8 (residues 93-112) contain cryptotopes in the region of residues 63-65 and 108-112, respectively. Peptide 12

(residues 142-158) contains both a cryptotope and a neotope which are likely to be located in the C-terminal region, residue 156 being associated with the cryptotope of peptide 12, and residue 158 with the neotope. No antigenic activity could be demonstrated in peptide 11 (residues 135-141).

3.3 TMV AS A MODEL FOR THE STUDY OF ANTIGENICITY IN QUATERNARY PROTEIN STRUCTURES

It is commonly accepted that antibody molecules have discrete binding sites (Poljak, 1975) and it is likely that antigenic determinants should also be associated with discrete regions of a protein's primary structure (Crumpton, 1974) as was found by Atassi and co-workers (Atassi, 1975; 1977 b; 1978; Atassi and Habeeb, 1977). Such a discrete antigenic determinant has at least two attributes associated with it: an ordered arrangement of amino acid residues and a specific conformation. It is difficult to assess which of these attributes is more important to the immunogenicity and the antibody binding in the case of any particular antigenic determinant, but it is clear that both attributes are involved. The "continuous site" (Atassi, 1978) determinants of myoglobin illustrate the importance of the amino acid sequence to some antigenic sites (Atassi, 1975; 1977 b), while in lysozyme spatially adjacent surface residues, which are not necessarily in direct peptide bond linkage, form "discontinuous site" (Atassi, 1978) determinants which rely on the surface conformation of the protein for their integrity (Atassi and Habeeb, 1977; Atassi, 1978).

It is quite conceivable that the physical methods now available for examining protein conformation, especially in solution, are not capable of determining the subtle changes in conformation that may accompany a change in antigenicity (Atassi et al., 1970), but a strategy of approaches (1.1),

that can determine the amino acid composition and order in antigenic determinants, is available (Atassi, 1975; 1977 a; 1977 b). Once the antigenic determinants of various proteins have been defined and mapped, the results of conformational studies can be interpreted more easily.

In quaternary protein structures a new class of conformational determinants, comprising portions of separate polypeptide chains from different subunits that are adjacent in space ("discontinuous site" determinants— Atassi, 1978) become possible (Crumpton, 1974), as well as new determinants resulting from conformational change in the individual subunits that comprise the quaternary structure. These neotopes (van Regenmortel, 1966) may be recognized on the basis that homologous antisera contain specific antibodies against the quaternary structure which do not arise in antisera raised to the dissociated subunits.

Although no evidence has been found to show that TMV has conformational determinants arising from the juxtapositioning of protein subunits in the viral capsid, neotopes associated with the quaternary structure of the virus have been demonstrated. It has been shown that antibodies specific for these neotopes are able to react with peptides free in solution (fig. 2.19 and table 2.5). This implies that these conformational determinants of TMV may be amenable to Atassi's strategy (1.1) and that the amino acid sequences, of which these determinants are composed, can be determined. This information, along with the structural data arising from X-ray crystallography of the protein subunit and the virus, would shed light on the nature of neotopes in TMV and hence quaternary antigenicity in general.

Part 4. MATERIALS AND METHODS

4.1 THE PREPARATION OF ANTIGENS AND PEPTIDES

4.1.1 SOURCES AND PROPAGATION OF VIRUS

The type strain and the following mutants of TMV were utilized in this work: TMV vulgare and the n-bromosuccinimide mutant 414 were supplied by Prof. M.H.V. van Regenmortel of the Department of Microbiology, University of Cape Town (van Regenmortel, 1967); the spontaneous mutant CP 415 and the nitrous acid mutants Ni 118, Ni 568, Ni 1688 and Ni 1927 were obtained from Prof. H.G. Wittmann of the Max Planck Institute for Molecular Genetics, Berlin (Hennig and Wittmann, 1972).

The viruses were propagated in Nicotiana tabacum cultivar soulouk at 25°C in fully controlled growth chamber conditions. Infected leaves were harvested five days after innoculation.

4.1.2 EXTRACTION OF VIRUS FROM INFECTED LEAVES

The method of von Wechmar and van Regenmortel (1970) was used for the extraction and purification of TMV strains and mutants from the leaves of infected plants.

Leaf material, mixed in a weight to volume ratio of 2:1 with a buffer comprised of equal volumes of 0,05M sodium phosphate buffer and 0,01M E D T A (ethylene-diamine-tetra-acetic acid), adjusted to a final pH of 9,5 with 1,0M NaOH, was homogenized and strained through cheesecloth. The pH of this filtrate, which had dropped to pH 5-6, was immediately adjusted to pH 7,5 with 1,0M NaOH before centrifugation at 16000 g for 10 minutes. Powdered activated charcoal and Celite, each to a concentration of 5 g/100 ml of the filtrate, were stirred with the supernatant for 30 seconds and the slurry was filtered by suction

through Whatman No. 1 filter paper, covered with a 0,5 cm layer of damp Celite, to remove contaminating plant material.

The virus in this second filtrate was precipitated by the addition of MW 6000 polyethylene glycol and NaCl, each to a final concentration of 4,0 g/100 ml of the second filtrate, and pelleted by centrifugation at 16000 g for 10 minutes. The virus pellets were resuspended in 0,05M sodium phosphate buffer pH 7,5.

If necessary, further clarification of the virus solution could be achieved by a second precipitation with polyethylene glycol and NaCl followed by differential centrifugation.

4.1.3 PREPARATION OF VIRUS PROTEIN

4.1.3.1 THE COLD ACETIC ACID METHOD

The basis of the cold acetic acid method of Fraenkel-Conrat (1957) for the preparation of TMV protein is the dissociation of TMV in a 66% (v/v) solution of glacial acetic acid in water.

TMV, resuspended in cold distilled water to a concentration of approximately 10 mg/ml, was mixed with two volumes of glacial acetic acid at 17°C. The mixture was kept in chipped ice with occasional stirring for 30 minutes, or until the opalescence of the solution had cleared and strings of precipitated RNA were seen. After centrifugation at 12000 g for 10 min, to pellet the RNA, the supernatant was immediately decanted and dialysed against distilled water at +4°C, changing the water every day, until the protein had precipitated at its isoelectric point in the dialysis bags. The pH of the cold distilled water was approximately pH 5.

The precipitated protein was separated from the supernatant by centrifugation at 12000 g for 10 min and resuspended in distilled water. A drop or two of 1,0M ammonia solution was used to aid dissolution and to raise the pH to 8,0. The protein solution was centrifuged at 150000 g for one hour, to remove undegraded virus and denatured protein, and lyophilized.

4.1.3.2 THE ETHANOLAMINE - DEAE METHOD

This method, which was developed by Durham (1972), involves the dissociation of the virus at high pH followed by the removal of the RNA by filtration through an ion-exchange resin.

A solution of 10 mg TMV/ml was dialysed against 0,1M ethanolamine pH 11,0, at +4°C, until the opalescence had completely cleared. After a further 2-3 hours of dialysis against 0,012M TRIS (Tris(hydroxymethyl)-amino-methane) HCl buffer pH 8,3, the solution containing dissociated protein and RNA was centrifuged at 150000 g for one hour to remove any undegraded virus still present. The supernatant was then applied to a column of Whatman DE-52 diethylaminoethane cellulose consisting of 1 ml bed volume for every 50 mg of original virus, equilibrated with 0,12M TRIS-HCl buffer pH 8,3. The column was eluted with the same buffer and fractions, pooled on the basis of the elution profile (fig. 2.1 , page 62), were dialysed against distilled water for 3-4 days with frequent changes of water before they were lyophilized.

If a particular preparation of virus, or a particular mutant could not be disrupted by the ethanolamine buffer, 0,1M NaOH was substituted for it to good effect.

4.1.4 THE GENERATION AND PURIFICATION OF THE TRYPTIC PEPTIDES OF TMV PROTEIN

4.1.4.1 TRYPTIC DIGESTION OF TMV AND TMV MUTANT PROTEINS

Tryptic digestion was done by a modification of the method described by Benjamini et al. (1964).

The trypsin used was a twice-recrystalized preparation manufactured by Miles-Seravac(code 36-555 batch 1455). The TMV proteins, 100 mg in 20 ml water, were digested with 2 mg of trypsin at 37°C while the pH was maintained at 8,0 with 1,0M ammonia solution by means of a pH stat (Radiometer TTT 60 titrator). After an hour of digestion an additional 2 mg of trypsin was added (fig. 4.1) and, after a further hour, peptide 1 (residues 1 - 41) was separated from the other peptides in the digest by precipitation at pH 4,5 (Funatsu et al., 1964), using 2,0M acetic acid, and centrifugation at 48000 g for 15 minutes. The soluble peptides were lyophilized and stored at -20°C.

4.1.4.2 PURIFICATION OF THE 1-PEPTIDE (RESIDUES 1-41)

Following its separation from the tryptic digest, the 1-peptide was purified by three repeated isoelectric precipitations at pH 4,5 (using 2,0M acetic acid and 1,0M ammonia solution) and dialysis against frequent changes of distilled water (in cellophane tubing boiled for 10 minutes to reduce the pore size) for at least 7 days. After this period, the 1-peptide had reprecipitated due to the low pH of the water containing dissolved CO_2. This precipitate was pelleted by centrifugation at 48000 g for 15 minutes, redissolved with a few drops of 1,0M ammonia solution, and centrifuged again at the same speed to remove any insoluble material present. Thereafter the supernatant was lyophilized and stored at -20°C.

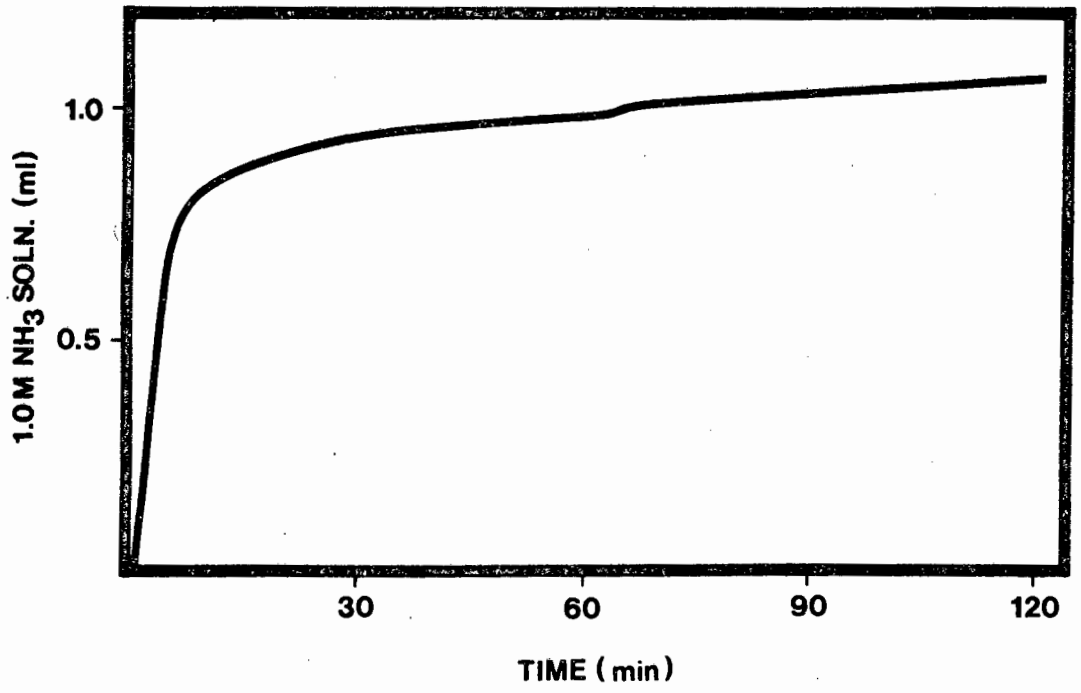

Fig. 4.1 : Tryptic digestion of 410,0 mg of TMV protein. Time of digestion is plotted against the volume of 1,0M ammonia solution required to maintain the pH at 8,0. 8,2 mg of twice recrystalized trypsin was added initially and after 60 minutes. The temperature was maintained at 37°C throughout the digestion.

4.1.4.3 FRACTIONATION OF THE pH 4,5 SOLUBLE TRYPTIC PEPTIDES

4.1.4.3.1 PREPARATIVE ION-EXCHANGE CHROMATOGRAPHY OF THE pH 4,5 SOLUBLE TRYPTIC PEPTIDES

AG1-X2 resin (Bio-Rad Laboratories, Richmond, California) was activated by sequential washing of every 200 ml of packed resin with 3,0 litre of distilled water, 0,5 litre of 1,0M HCl, 3,0 litre of distilled water, 1,0 litre of 0,5M NaOH and 2,0 litre of distilled water at 60°C. The activated resin was resuspended in a 1% (v/v) pyridine, 1% (v/v) collidine buffer, adjusted to pH 8,4 with glacial acetic acid, 1,0 litre for every 200 ml of packed resin. The "fines" were removed by suction.

A 150x0,9 cm jacketed column was packed with the activated resin and equilibrated with the pH 8,4 buffer. The column was maintained at a temperature of 37°C and run at a presure of 10-20 bar to assure a flow rate of 40 ml/hr.

Samples of 100-200 mg of the pH 4,5 soluble portion of the tryptic digest were dissolved in 5,0 ml of the pH 8,4 buffer by raising the pH of the solution to 10,0 with 0,5M NaOH. The samples were applied to the column and eluted with two different pH gradients.

The first, recommended by Funatsu (1964), was formed by a 9-chamber Autograde (Technicon Chromatography Corp.) and consisted of the following volumes of buffers (volume percentages) in sequence: (1) 250 ml 1% pyridine, 1% collidine pH 8,2; (2) 360 ml 1% pyridine, 1% collidine pH 7,3; (3) 240 ml 0,02M acetic acid; (4) 120 ml 0,2M acetic acid; (5) 120 ml 0,35M acetic acid; (6) 120 ml 0,5M acetic acid; (7) 240 ml 0,65M acetic acid; (8) 120 ml 1,7M acetic acid; and (9) 120 ml 5,0M acetic acid.

The pH of the pyridine-collidine buffers was adjusted with glacial acetic acid. 0,1 ml of each 3,3 ml fraction, that was collected, was quantitated by the Folin-Lowry reaction (Campbell et al., 1970) and the extinction at 700 nm was plotted against the fraction number (fig. 2.2). A characteristic elution profile (Funatsu, 1964) was obtained. The peaks corresponding to peptides 8 (residues 93-112) and 10 (residues 123-134) had a small amount of precipitate in the corresponding fractions.

The second pH gradient was identical to that described by Wittmann (1965). Formed with two mixing flasks, it consisted of the following volumes of buffers (volume percentages) in sequence: (1) ∿ 125 ml 1% pyridine, 1% collidine pH 8,4; (2) 125 ml 2% pyridine, 1% collidine pH 7,5; (3) 600 ml 1% pyridine, 1% picoline, 1% lutidine pH 7,2; (4) 600 ml 1% pyridine, 1% picoline, 1% lutidine pH 6,0; (5) 600 ml 2% pyridine, 2% picoline, 2% lutidine pH 5,0; (6) 600 ml 3% pyridine, 3% picoline, 3% lutidine pH 4,0; (7) 150 ml 2,0M acetic acid. The pH of the pyridine-collidine and pyridine-picoline-lutidine buffers was adjusted with glacial acetic acid. 0,2 ml of each 4,0 ml fraction, that was collected, was subjected to alkaline hydrolysis in 2,5M NaOH at 110°C and reacted with ninhydrin reagent (Benson and Patterson, 1971). The extinction at 570 nm was plotted against the fraction number (fig. 2.3), and the characteristic elution profile was obtained (Wittmann, 1965).

In both cases the fractions comprising each peak, corresponding to a particular peptide, were pooled, evaporated to dryness and redissolved in a small volume of distilled water preparatory to peptide mapping (4.2.1).

4.1.4.3.2 PREPARATIVE HIGH VOLTAGE ELECTROPHORESIS OF THE pH 4,5 SOLUBLE TRYPTIC PEPTIDES

Whatman 3 Chroma paper (35x40 cm) was washed, by descending chromatography, with 250 ml 50% (v/v) glacial acetic acid and water, 250 ml 2,0M ammonia solution and 1,0 l litre distilled water, and dried. A light pencil line was drawn at the centre across the breadth of each sheet. Preparatory to electrophoresis the paper was dampened with the electrophoretic buffer, which contained 200 ml acetone, 80 ml pyridine and 720 ml water. The pH of the buffer was adjusted to 6.4 by the addition of glacial acetic acid. 25 mg of the lyophilized pH 4,5 soluble tryptic digest of virus protein was dissolved in 600 µl of distilled water (a micro-drop of 25% (w/v) ammonia solution (Merck) was added if there was difficulty with solubilization) and applied along the pencil line in the centre of the damp paper. The digest was electrophoresed at approximately 1000 V and 45 mA in a Hormuth-Vetter pherograph (model 64) for 5 hours at +4^{o}C, using wicks. After this the paper was dried and two strips, with a breadth of 3 cm, were cut from the long edges of the paper and dried. The strips were stained with 0,2% (w/v) ninhydrin in ethanol containing 5% (v/v) collidine, and with Ehrlichs reagent — 10% (w/v) p-dimethylaminobenzaldehyde in 20% (v/v) concentrated HCl-acetone (Jepson, 1963) - to locate the positions of the peptides (fig. 4.2). Vertical bands corresponding to individual peptides were cut from the unstained portion of the dried paper.

The peptides were extracted from these bands of paper by subjecting 8 cm lengths of each band to ascending chromatography with the pH 6,4 buffer, followed by low speed centrifugation (2360 g for 15 min) of the wet paper wrapped in aluminium foil and suspended in a centrifuge

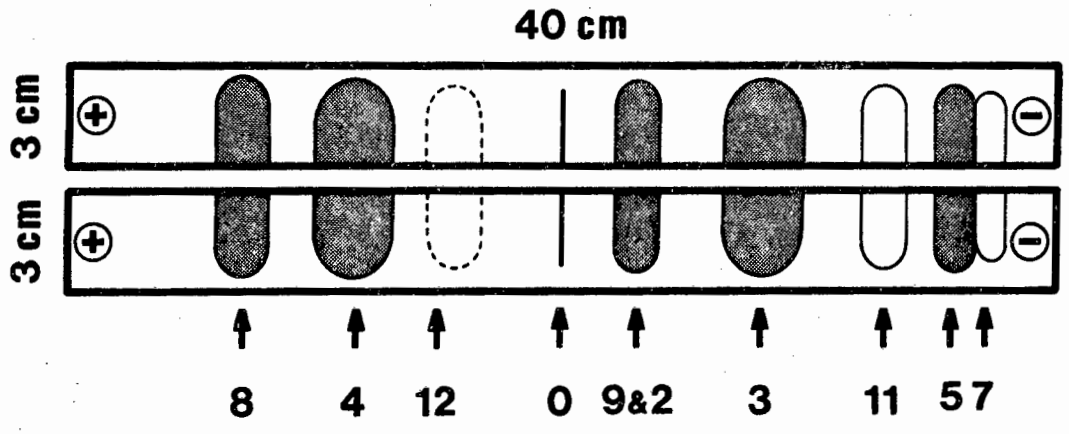

Fig. 4.2 : Separation of the pH 4,5 soluble tryptic peptides of TMV protein by high-voltage paper electrophoresis. A tryptic digest of TMV protein (25 mg) was applied across the middle of a 35x40 cm sheet of Whatman 3 Chroma paper, at the position marked 0. After 5 hours electrophoresis (1000 v;45 mA) at pH 6,4 the paper was dried and two 3 cm strips were cut off along the edges. The strips were stained with ninhydrin and Erlichs reagent. Numbers refer to the tryptic peptides. Staining colour of peptides 8, 4, 9 & 2, 3 and 5 was purple; peptide 11 : yellow; peptide 7: grey. Peptide 12 stained only with Erlichs reagent (10% (w/v) p-dimethylaminobenzaldehyde in 20% (v/v) concentrated HCl-acetone).

tube. The resultant peptide solutions were evaporated to dryness in a vacuum dessicator using P_2O_5 and NaOH pellets. In some instances, if the degree of purity of a peptide was insufficient, the peptide was submitted to electrophoresis a second time. The peptides were dissolved in distilled water, again evaporated to dryness and finally dissolved in a small volume of distilled water.

The purity and yield of each peptide was determined by amino acid analysis (4.2.2).

4.1.5 DIGESTION OF TRYPTIC PEPTIDE 12 WITH CARBOXYPEPTIDASE A

Carboxypeptidase digestion was done by a modification of a method described by Ambler (1972).

Carboxypeptidase A (Mannheim Boehringer) was prepared by washing 10 µl of a 25 mg/ml suspension of the crystals three times with distilled water at +4°C by centrifugation at 3896 g for 10 minutes, and then completely drying the crystals in a vacuum. The dried crystals were dissolved in 40 µl 1,0M $(NH_4)HCO_3$ and the solution was made up to 250 µl with N-ethylmorpholine buffer pH 8,1 (1,15 ml N-ethylmorpholine, 1,50 ml of a 10% (v/v) aqueous solution of glacial acetic acid and 48,0 ml water, adjusted to pH 8,1 with the 10% (v/v) glacial acetic acid solution).

Approximately 2,6 mg of purified peptide 12 (residues 142-158) (4.1.4.3.2) was dried in a vacuum and dissolved in 1,3 ml of N-ethylmorpholine buffer pH 8,1. 5 µl of the prepared solution of carboxypeptidase A was added to the peptide solution and the mixture was kept

at 37°C for two hours with continuous stirring. Thereafter it was stored overnight at +4°C.

The carboxypeptidase A digest (1,3 ml) was applied to a 65x1,6 cm column of Sephadex G50 fine equilibrated with distilled water. The column was run with distilled water and a flow rate of 30 ml/hr was maintained. 1,5 ml fractions were collected and monitored for their absorbance at 210 nm and 280 nm using a distilled water blank (fig. 2.4). 20 µl of every second fraction was spotted onto Whatman 3 Chroma paper, dried, and sprayed with 0,2% (w/v) ninhydrin in ethanol containing 5% (v/v) collidine to test for colour development.

The fractions under each peak of the elution profile were pooled, dried and redissolved in 2,5 ml of 0,1M Tris(hydroxymethyl)aminomethane - 0,15 M NaCl buffer pH 7,4 (4.3.2.1.2). 0,5 ml of each sample of the pooled fractions was dried, hydrolysed with 6,0M HCl and submitted for amino acid analysis (4.2.2).

4.1.6 THE GENERATION OF THE FORMIC ACID PEPTIDES OF TMV PROTEIN

4.1.6.1 CLEAVAGE OF THE ASP-PRO PEPTIDE BONDS OF TMV PROTEIN AND THE 1-PEPTIDE (RESIDUES 1-41) BY THE FORMIC ACID METHOD OF MIKI AND KNIGHT (1965)

10 mg/ml aqueous solutions of TMV protein and the 1-peptide (residues 1-41) were made 66,6% with concentrated formic acid (98-100%) and were incubated at 37°C for 20 hours in closed containers. The formic acid was then removed by evaporation in a vacuum dessicator containing NaOH pellets and P_2O_5.

The dried samples were resuspended in distilled water and in both cases an insoluble precipitate was formed. After centrifugation at 60000 g for 30 minutes the supernatants were tested for the presence of proteinaceous material by the ninhydrin reaction after alkaline hydrolysis for 2 hours in 2,5M NaOH at 110°C (Benson and Patterson, 1971). Only the supernatant of the sample containing degraded TMV protein showed colour development in excess of the background.

Portions of the whole digest, the washed precipitate and the supernatant of the degraded TMV protein were lyophilized and used for SDS-polyacrylamide gel electrophoresis (4.1.6.3).

4.1.6.2 SEPHADEX G50 EXCLUSION CHROMATOGRAPHY OF THE FORMIC ACID PEPTIDES OF TMV PROTEIN IN A SOLUTION OF 50% (V/V) GLACIAL ACETIC ACID IN WATER

The formic acid digest of TMV protein was evaporated to dryness (in a vacuum dessicator containing NaOH pellets and P_2O_5) and redissolved in a solution of 50% (v/v) glacial acetic acid in water so that the final concentration was 10 mg/ml. 5,0 ml of this solution was applied to a 80x1,6 cm column of Sephadex G50 fine equilibrated and run with a solution of 50% (v/v) glacial acetic acid in water. The flow rate was maintained at 30-40 ml/hr and 3,0 ml fractions were collected and monitored for their absorbance at 280 nm against a solution of 50% (v/v) glacial acetic acid in water as the blank (fig. 2.5).

The fractions were pooled according to the peaks of the elution pattern, dried and resuspended in water before lyophilization and SDS-polyacrylamide gel electrophoresis (4.1.6.3).

4.1.6.3 SDS - POLYACRYLAMIDE GEL ELECTROPHORESIS OF THE FORMIC ACID PEPTIDES OF TMV PROTEIN

This was done according to the method of Weber and Osborn (1975), using 15% (w/v) acrylamide gels containing 1,0% (w/v) sodium dodecyl sulphate (SDS).

14,625 g of acrylamide and 0,375 g of methylene bisacrylamide were dissolved in each 100 ml volume of 1,0% (w/v) SDS 0,1M sodium phosphate buffer pH7,0. The solution was degassed in ice before 1,0 ml of fresh 10,0% (w/v) ammonium persulphate solution and 100 µl N,N,N,N-tetramethylethylene-diamine (TEMED) were added. This solution was immediately pipetted into glass tubes (10x0,5 cm) so that they were filled to within 1,0 cm of the top of each tube. The tubes had been previously sealed with Parafilm (American Can Co.) at one end. Water was carefully layered over the gels and approximately 45 min was allowed for polymerization at room temperature. The Parafilm was removed and the gels were pre-electrophoresed in a Shandon disc. gel electrophoresis apparatus using 1,0% (w/v) SDS 0,1M sodium phosphate buffer pH 7,0 in the trays, at 2,0 mA per tube for 2-3 hrs until the voltage remained constant.

After pre-electrophoresis the tray buffer was changed and 10 µl of each sample was applied to the gels.

The samples consisting of 4,0 mg of each of three marker proteins: TMV protein (17500 daltons), lysozyme (14000 daltons) and insulin (6000 daltons), as well as 4,0 mg of the lyophilized formic acid TMV protein digest, its precipitate and its supernatant, were dissolved in 1,0 ml of the 1,0% (w/v) SDS 0,1M sodium phosphate buffer pH 7,0 containing 50 µl

2-mercaptoethanol. The samples were incubated at 100°C for two minutes and one drop of 0,05% (w/v) bromophenol blue and one drop of glycerol were added before application of the samples to the gels. The samples were electrophoresed at 6,6 mA per tube until the band of bromophenol blue had moved more than 3/4 of the way down the gel.

The gels were removed from the tubes, by forcing buffer between the gel and the glass with a syringe, and stained with a 0,25% (w/v) solution of Coomassie blue in methanol, glacial acetic acid and water (9:2:9) for an hour at 35°C. Destaining was accomplished by incubating the gels at 35°C in a solution of methanol, glacial acetic acid and water (9:2:9) until the bands were clear. The gels were stored in a 7% (v/v) solution of glacial acetic acid in water in stoppered tubes.

The pooled fractions from the Sephadex G50 column were electrophoresed in the same manner, using the same marker proteins.

4.2 CHARACTERIZATION OF TMV PROTEIN, THE PROTEINS OF THE TMV MUTANTS AND THEIR PEPTIDES

4.2.1 PEPTIDE MAPPING OF THE pH 4,5 SOLUBLE TRYPTIC PEPTIDES

A 40x35 cm sheet of Whatman 3 Chroma paper was prepared for electrophoresis by marking the origin in pencil in the middle of the length of the sheet and 5 cm from the edge, and by soaking the paper in electrophoretic buffer, which contained 200 ml acetone, 80 ml pyridine and 720 ml water. The pH of the buffer was adjusted to 6,4 by the addition of glacial acetic acid.

1 mg of the pH 4,5 soluble tryptic digest (4.1.4.1) was dissolved in

10 µl of water (a micro-drop of 25% (w/v) ammonia solution (Merck) was added if there was difficulty with solubilization) and applied with a Hamilton syringe to the origin of the damp paper. The digest was electrophoresed at approximately 1000 V and 45 mA in a Hormuth-Vetter Pherograph (model 64) for 2,5 hrs at +4°C, using wicks, and dried by evaporation at room temperature.

Subsequently, the dried paper was subjected to ascending chromatography with a pyridine:iso-amylalcohol:water as the phase, which was prepared by mixing the constituents in the ratio 5:4:4. A Shandon 500 Chromotank was used for the chromatography.

The paper was then thoroughly dried by evaporation and the different peptide spots were developed by staining with the following reagents: (1) 0,2% (w/v) ninhydrin in ethanol containing 5% (v/v) collidine; (2) Ehrlichs reagent - 10% (w/v) p-dimethylaminobenzaldehyde in acetone (Merck "pro-analysi") containing 20% (v/v) concentrated (D=1.19 g/ml) HCl (Jepson, 1963); or (3) 0,02% (w/v) phenanthrenequinone in ethanol mixed with an equal volume of 10% (w/v) NaOH in a solution of 60% (v/v) ethanol in water (Yamada and Itano, 1966). The ninhydrin reagent stains all the pH 4,5 soluble tryptic peptides of TMV protein, except peptide 12 (residues 142-158), and imparts a distinctive yellow colour to peptide 11 (residues 135-141) and a grey colour to peptide 7 (residues 91-92). The two tryptophan containing peptides, peptide 3 (residues 47-61) and peptide 12 (residues 142-158) are stained with the Ehrlichs reagent, while the phenanthrenequinone method causes all the peptides except peptide 4 (residues 62-68) and 12 (residues 142-158) which do not contain arginine, to fluoresce in ultraviolet light with a wavelength of 266 nm.

4.2.2 AMINO ACID ANALYSIS

Dried samples of proteins (1,0 mg) and peptides (0,5 ml of each peptide solution was evaporated) for amino acid analysis were dissolved in 500 µl of twice distilled, constant boiling, HCl, containing 2 drops of thioglycollic acid, and hydrolysed for 20 hrs at 105°C in sealed tubes flushed with nitrogen and evacuated to 0,02 mm Hg (Hare, 1975). Mr. J. de A. Rodrigues, of the Department of Biochemistry of the University of Cape Town, assisted in the identification of the free amino acids on a Beckman Amino Acid Analyser model 119. An internal standard of norleucine was used with the neutral and acidic amino acids while α-amino β-guanidinopropionic acid (AGP) was used with the basic amino acids, and quantitation was done by means of A/D converters between the 440 and 570 mµ channels of the analyser and a Hewlett Packard computer (Laboratory Data System 3352B).

No corrections were made for hydrolytic losses or incomplete cleavage.

4.2.3 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF TMV PROTEIN AND THE TRYPTIC 1-PEPTIDES FROM TMV, Ni 118 AND Ni 568.

Samples (ranging from 5-50µl) of solutions of TMV protein and the tryptic 1-peptides from TMV, Ni 118 and Ni 568, were electrophoresed in SDS-polyacrylamide slab gels containing 18% (w/v) acrylamide according to the method of Thomas and Kornberg (1975), which is a modification of the method of Laemmli (1970). The electrophoresis was performed by Miss S. L.U. Schwager of the Department of Biochemistry of the University of Cape Town. The gels were stained in a 0,1% (w/v) solution of Coomassie brilliant blue in methanol:acetic acid:water (5:1:5), and destained by diffusion at 37°C in 5% (w/v) methanol mixed with 7,5% (v/v) glacial acetic acid in water. The results were recorded as densitometer traces

(Vitatron T L D 100 densitometer) using a 615 nm filter.

4.2.4 ULTRACENTRIFUGAL ANALYSIS OF TMV PROTEIN

The sedimentation coefficient (S) of TMV protein preparations was determined by sedimentation velocity experiments in a Beckman model E analytical ultracentrifuge (Chervenka, 1969). An AnD rotor with a standard 12 mm single sector centrepiece was used. The samples were centrifuged at 40000 r.p.m. and 56000 r.p.m. and photographs were taken at various intervals with the schlieren optical system. The logarithm of the distance of the peak from the axis of rotation at various time intervals was plotted against time and the S value was calculated from the resulting graph.

4.3 IMMUNOCHEMICAL METHODS

4.3.1 ANTISERA

4.3.1.1 PREPARATION OF ANTISERA

Rabbits were immunized by a series of intramuscular injections consisting of 2,0-4,0 mg immunogen in 1,0 ml sterile water (TMV protein), or 0,05M sodium phosphate buffer pH 7,5 (TMV), mixed with 1,0 ml of Freunds incomplete adjuvant, at intervals of 2-3 weeks. The immunogens were: TMV, formalinized TMV, TMV protein, formalinized TMV protein, bovine serum albumin, human serum albumin and H3 histone. The formalinized virus and virus protein were prepared by dialysis against 0,2-0,5% formaldehyde (final concentration w/v) solutions (van Regenmortel and Lelarge, 1973). Antiserum was collected from the animals at various intervals over a period of several months by bleeding from the ear vein followed by clotting of the blood at room temperature and extraction of the serum by centrifugation at 12000 g for 10 min. Normal rabbit serum from unimmunized animals was collected in the same manner. Often a number of

subsequent bleedings from the same animal were pooled.

Goat anti-rabbit serum was prepared by immunizing a goat with 4,0 mg of rabbit IgG (prepared by the method of Reif (1969) in 1,0 ml sterile 0,15M NaCl mixed with 1,0 ml of incomplete Freunds adjuvant, injected intramuscularly. Injections of immunogen were repeated weekly, as were the bleedings from the jugular vein. The serum was extracted from the blood by the same method that was used for the rabbit antisera. Every two subsequent bleedings were pooled.

All antisera were prepared under the supervision of Dr. M.B. von Wechmar of the Department of Microbiology of the University of Cape Town and subsequently stored at -20^{o}C.

4.3.1.2 CROSS-ABSORPTION OF SERA

4.3.1.2.1 ABSORPTION OF TMV PROTEIN ANTISERA WITH TMV

The virus specific antibodies were absorbed from TMV protein antisera by the incubation of the sera with formalinized TMV, which dissociates less than the untreated virus (van Regenmortel and Lelarge, 1973).

Antisera intended for absorption with TMV were titered by a tube precipitin test with TMV as the antigen, to ascertain optimal proportions for the reaction of the TMV specific antibodies in the serum with the intact virus. The amount of TMV required to absorb a specific volume of serum was calculated,and formalinized TMV in excess of this amount was mixed with the serum and incubated at 37^{o}C for 2 hrs before being left at +4^{o}C overnight. The TMV-antibody precipitate was removed by centrifugation at 17400 g for 15 min and the supernatant, which contained the TMV protein specific antibodies, was centrifuged at 167500 g

for 1 hr to remove any remaining unprecipitated virus.

This procedure was repeated until the TMV protein antisera were shown to be free of TMV specific antibodies by tube precipitin and Ouchterlony tests. The sera were concentrated to their original volume by evaporation and stored at -20°C.

4.3.1.2.2 ABSORPTION OF TMV ANTISERA WITH TMV PROTEIN

Lyophilized TMV protein was dissolved in distilled water at +4°C and diluted with an equal volume of pH 8,0 borate buffered saline (prepared by dissolving 78,1g NaCl, 70,0g H_3BO_3 and 50,5g $Na_2B_4O_7.10H_2O$ in 2,0 litre distilled water and adjusting the pH of a five-fold dilution of this solution to 8,0 with 1,0M HCl). This solution was centrifuged at 167500 g for 1 hr, to remove protein aggregates, and spectrophotometrically quantitated using an extinction coefficient of $E^{0,1\%}_{282\ nm}$ = 1,27 (Fraenkel-Conrat, 1957).

TMV antisera, intended for absorption with TMV protein, were adjusted to pH 8,0 with 0,1M NaOH and were titred by a tube precipitin test with TMV protein as the antigen, to ascertain optimal proportions for the reaction of the TMV protein specific antibodies in the serum with the disaggregated protein. The amount of TMV protein required to absorb a specific volume of serum was calculated, and an exactly equivalent volume of the pH 8,0 TMV protein solution was mixed with the serum and incubated overnight at +4°C. The TMV protein-antibody precipitate was removed by centrifugation at 17400 g for 10 min and the supernatant, which contained the TMV specific antibodies, was kept.

This procedure was repeated until the TMV antisera were shown to be

free of TMV protein specific antibodies by tube precipitin and Ouchterlony tests. The resultant diluted antiserum supernatant was adjusted to pH 5,0 with 0,1M HCl to encourage the formation of RNA-free helices of any unprecipitated TMV protein remaining in the serum, and left for 2 days at room temperature. After centrifugation of the serum at 167500 g for 1 hr it was adjusted back to pH 7,5 with 0,1M NaOH, and concentrated to its original volume by evaporation. The absence of free TMV protein in the absorbed sera was demonstrated by tube precipitin and Ouchterlony tests with a good anti-TMV protein serum. Absorbed sera were stored at -20°C.

4.3.1.3 THE ISOLATION OF THE GLOBULIN FRACTION FROM WHOLE SERA

Anti-TMV and anti-TMV protein globulins were prepared from whole and absorbed immune sera by precipitation in 50% (w/v) saturated ammonium sulphate by the method advocated by Campbell *et al.* (1970).

Each serum was mixed with an equal volume of 4,0M $(NH_4)_2SO_4$, that was added dropwise with a burette, so that an insoluble precipitate was formed. The pH of the suspension was adjusted to pH 7,4 with 2,0M NaOH and left stirring for 30 min at room temperature before centrifugation at maximum revolutions (1000 g) in a Roto-Uni bench top centrifuge for 30 min. The supernatant was discarded and the precipitate was dissolved in 0,15M NaCl made 0,001M with phosphate buffer and pH adjusted to 7,4 with 1,0M NaOH. This crude globulin preparation was precipitated two more times in the same manner, to purify it. The final solution of globulins in pH 7,4 saline was exhaustively dialysed against repeated changes of borate buffered saline pH 8,0 (4.3.1.2.2) until no more sulphate ions could be detected in the dialysate, outside the membrane, with barium chloride. After dialysis the globulin prep-

aration was centrifuged at 27000 g for 60 min and the volume of the supernatant was adjusted to that of the original whole serum.

The concentration of the globulin preparations was determined spectrophotometrically using an extinction coefficient of $E^{0,1\%}_{280\ nm} = 1,43$ as a compromise for that suggested by Little and Eisen (1968) on the advice of Dr. J.D. Young (personal communication). The globulin preparations were stored at -20°C.

4.3.2 COMPLEMENT FIXATION ASSAYS

The microcomplement fixation method of Levine (Wasserman and Levine, 1961; Levine and van Vunakis, 1968) was used with minor modifications as described below.

4.3.2.1 EXPERIMENTAL PROCEDURE

4.3.2.1.1 EQUIPMENT

Sorvall glass centrifuge tubes with the dimensions 120x17 mm were employed as reaction tubes and adaptors were used to fit the tubes into the swinging-buckets of the Sorvall HS-4 rotor. All low speed centrifugation was carried out in a refrigerated Sorvall RC2-B centrifuge.

Pipetting was done with serological pipettes or Cornwall type, 2 ml and 5 ml, automatic pipetting outfits (Becton, Dickinson and Co.).

The complement fixation assays were incubated in a large thermoregulated waterbath (Labotec Pty. Ltd.) and in a cold room regulated to +4°C.

All optical density determinations were made with a Pye Unicam SP 1700 digital read-out spectrophotometer.

4.3.2.1.2 DILUENTS

A stock solution of TRIS-NaCl buffer was prepared by dissolving 81,6g NaCl and 12,1g Tris(hydroxymethyl)aminomethane in approximately 800 ml of distilled water. A further 6,6 ml of concentrated HCl was added, the solution was mixed, and 33,0 ml of 0,15M $MgSO_4$ and 15,0 ml of 0,1M $CaCl_2$ were added to the solution. The pH was adjusted to 7,4 with concentrated HCl and the volume was made up to 1,0 litre. Ten-fold dilutions of this stock buffer were made, adjusted to pH 7,4 and used in the assays. Both stock buffer and the diluted TRIS-NaCl buffer were stored at +4°C.

A separate 0,1% (w/v) solution of ovalbumin in TRIS-NaCl diluent was also prepared and stored at +4°C. No preservatives, such as sodium azide, were used in the buffers or sera, as these compounds have an adverse effect on sheep erythrocytes.

4.3.2.1.3 SHEEP ERYTHROCYTES

Blood taken from sheep was mixed with an equal volume of Alsevers solution and stored at +4°C for a week before use so that the erythrocytes were uniformly susceptible to immune haemolysis (Levine and van Vunakis, 1968). It was found that sheep erythrocytes remained stable at this temperature for at least a month.

A 5% suspension of sheep erythrocytes was prepared from the whole blood immediately preparatory to its use in an assay: The blood was washed three times in the TRIS-NaCl diluent at 500 g for 10 min, or

until the supernatant was colourless. A $^{1}/_{20}$ dilution of packed sheep erythrocytes was made in TRIS-NaCl diluent and quantitated by lysing 1,0 ml of this suspension with 14,0 ml of fresh 0,1% (w/v) Na_2CO_3 solution. The absorbance of the lysed cells was read against distilled water at a wavelength of 541 nm. The volume of the $^{1}/_{20}$ suspension was adjusted to make it 5% with respect to sheep erythrocytes according to the following formula:-

$$\text{volume of SRBC solution in ml} \times \frac{\text{absorbance at 541 nm}}{0{,}680} = \text{volume of diluent to be added in ml.}$$

1,0 ml of the 5% suspension of sheep erythrocytes lysed in 14,0 ml of 0,1% (w/v) Na_2CO_3 solution had an absorbance of 0,680 at 541 nm (Levine and van Vunakis, 1968).

4.3.2.1.4 SENSITIZATION OF SHEEP ERYTHROCYTES

Rabbit haemolytic serum for sheep red cells (haemolysin) was obtained from Wellcome Reagents Ltd. and was found to have a consistent titre of $^{1}/_{100}$. If, however, the haemolytic antibody is to be titered for optimum sensitization of cells, the method of Osler et al. (1952) should be used. All haemolysin was stored at +4°C without preservatives.

For sensitization of the sheep erythrocytes, an equal volume of a $^{1}/_{100}$ dilution of haemolysin in TRIS-NaCl diluent was added slowly to the 5% suspension of erythrocytes (4.3.2.1.3) in an Erlenmeyer flask with constant swirling of the contents. After incubation at 37°C for 15 min to allow for maximum sensitization, the resultant 2,5% suspension of the sensitized erythrocytes was diluted with TRIS-NaCl diluent to ensure a final concentration of 0,25% sheep erythrocytes. This suspension of sensitized erythrocytes was kept on ice at 0°C, for a maximum of 8 hrs, until it was used.

4.3.2.1.5 COMPLEMENT

Lyophilized guinea pig serum (Behring Institut) was reconstituted with the supplied diluent and stored at +4°C.

The complement was titered by adding 1,0 ml of each of a series of complement dilutions corresponding to 1/100, 1/125, 1/150, 1/175,... .... 1/400, to 4,0 ml of TRIS-NaCl diluent and 1,0 ml of 0,1% (w/v) solution of ovalbumin in diluent in centrifuge tubes, in an ice bath. After incubation for 16-18 hrs at +4°C (to mimic the conditions of an assay), 1,0 ml of the 0,25% suspension of sensitized sheep erythrocytes (4.3.2.1.4) was added and haemolysis was allowed to proceed at 37°C for 60 min. Controls with an excess of complement, as well as no complement, were included in order to determine the extent of complete lysis and the background lysis of the sensitized blood cells respectively. After incubation the haemolysis reaction was stopped by immersing the tubes in an ice bath for 15 min preparatory to centrifugation at 770 g for 10 min. The absorbance of each supernatant was read at a wavelength of 413 nm against distilled water, and this value was plotted against the complement dilution as illustrated in fig. 4.3. The titre of the complement was taken as the highest dilution of complement that gave complete lysis.

Complement should be re-titrated at least every seven days as its activity decays in solution.

4.3.2.1.6 ANTISERA

Antisera specific for the various antigens (4.3.2.1.7) used in the complement fixation assays were raised in rabbits (4.3.1.1). Each serum was incubated at 56°C for 10 min to inactivate its own comple-

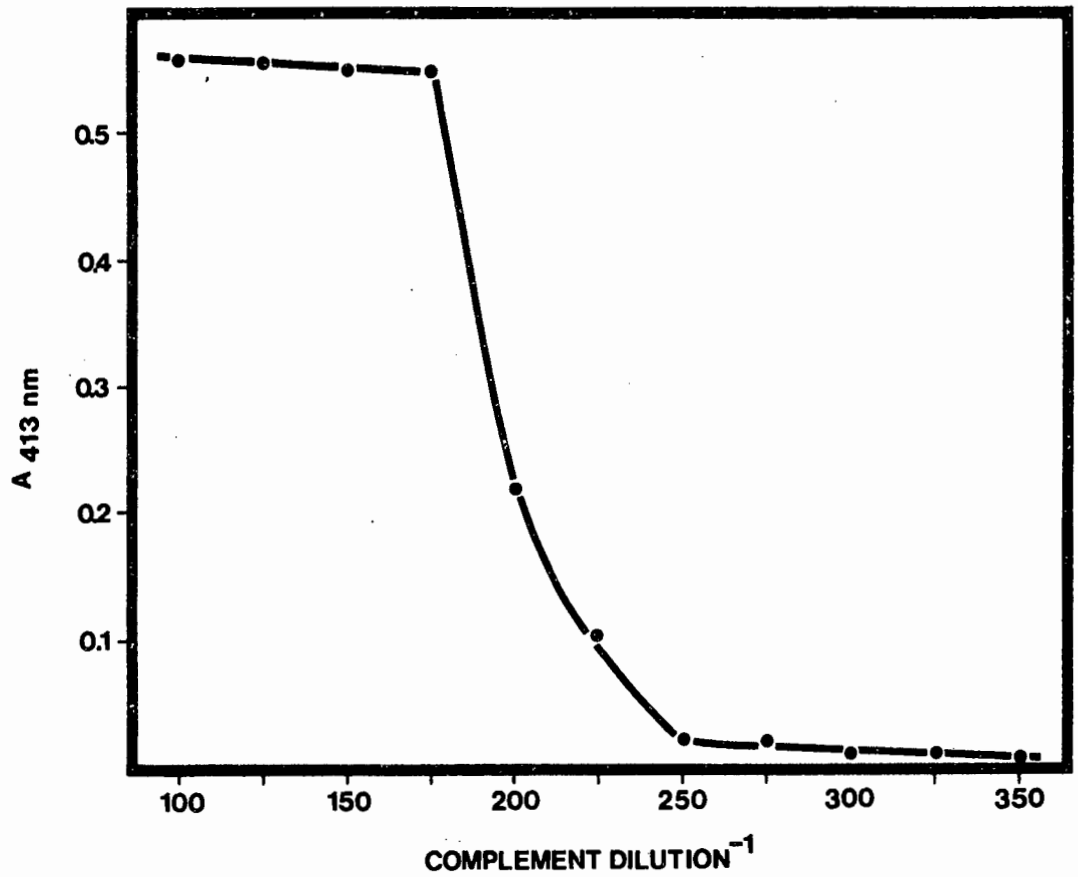

Fig. 4.3 : Titration of reconstituted lyophilized guinea pig complement for complement fixation tests. Increasing dilutions of complement were used to lyse a constant number of sheep erythrocytes sensitized with rabbit haemolysin with a titre of 1/100. The titre of the complement is 1/175. (4.3.1.1)

ment proteins before being diluted with TRIS-NaCl diluent to the desired extent for the particular complement fixation assay.

The titre of an antiserum was taken to be that dilution of the serum which gave 75% fixation at the peak of the complement fixation curve (Champion et al., 1974). Dilutions of antiserum were chosen with the intention of bracketing the titre so that the complement fixation curves ranged from approximately 20-80% fixation, but the most suitable dilutions had to be determined by trial and error for each antiserum.

4.3.2.1.7 ANTIGENS

The antigens used in complement fixation assays were prepared differently.

Prior to each assay, TMV was centrifuged at 87500 g for 20 min in a swinging-bucket rotor to select for long virus rods and to remove any dissociated protein. It was then resuspended in TRIS-NaCl diluent and the concentration of the solution was determined from its extinction at 260 nm. A correction for light-scattering was made by using the formula of Rayleigh and the following extinction coefficient:

$$A_{260nm\ (corrected)} = A_{260nm} - \left(A_{320nm} \times \frac{320^4}{260^4} \right) \quad \text{(Hendry, 1977)}$$

$$E^{0,1\%}_{260nm_{(corrected)}} = 3,0$$

Lyophilized TMV protein was dissolved in cold distilled water (+4°C), diluted with double strength TRIS-NaCl diluent and centrifuged at 167500 g for 1 hr immediately before use, to remove protein aggregates. The concentration of protein in the supernatant was determined from its

extinction at 282 nm with the extinction coefficient of $E^{0,1\%}_{282\ nm} = 1,27$ (Fraenkel-Conrat, 1957).

Salt-free, lyophilized H3 histone was obtained from Mr. D.R. Absolom of the Department of Microbiology, University of Cape Town. It was dissolved in TRIS-NaCl diluent to make a stock solution of known concentration.

The range of antigen concentrations required to achieve maximum fixation of complement was found to vary with different antisera and was selected by trial and error.

4.3.2.1.8 THE COMPLEMENT FIXATION ASSAY

Each complement fixation assay was usually performed with a range of antiserum dilutions which bracketed the serum titre. A series of centrifuge tubes was set aside for each antiserum dilution.

To each series of centrifuge tubes, in an ice bath, was added in order, 1,0 ml diluted antiserum, 1,0 ml 0,1% (w/v) solution of ovalbumin in diluent, 2,0 ml of TRIS-NaCl diluent, 1,0 ml diluted complement, previously titred (4.3.2.1.5) and 1,0 ml antigen solution, serially diluted in twofold steps. Similtaneously, the following controls were prepared to assist the interpretation and quantitation of the assay: (1) tubes containing each antiserum dilution and complement, but no antigen, to test for anticomplementary activity by the serum; (2) a tube containing the highest concentration of antigen used in the assay and complement, but no antibody, to test for complement binding or destruction by the antigen; (3) two tubes, one with a single aliquot of complement and the other with double the amount, to

Summary of complement fixation assay.

A) Preparation:

1) TRIS-NaCl diluent
2) 0,1% (w/v) ovalbumin in TRIS-NaCl diluent
3) titrate complement (if necessary)
4) Ab dilutions
5) Ag dilutions
6) C' dilutions

B) Assay:

Test	Controls			
	Ab	Ag	100% lysis	background lysis
1,0 ml Ab dilution	1,0 ml	—	—	—
1,0 ml 0,1% ovalb.	1,0 ml	1,0 ml	1,0 ml	1,0 ml
2,0 ml TRIS-NaCl diluent	3,0 ml	3,0 ml	3,0 ml 4,0 ml	5,0 ml
1,0 ml C' dilution	1,0 ml	1,0 ml	2,0 ml 1,0 ml	—
1,0 ml Ag dilution	—	1,0 ml	—	—

16 - 18 hrs at +4°C

Wash SRBC
standardize SRBC
prepare haemolysin dilution
sensitize SRBC

1,0 ml sensitized SRBC to all tests and controls

1 hr at 37°C
15 min at 0°C (Ice bath)
770 g for 10 min
Read at A_{413} nm

C) Results:

$$\%CF = 100 \times \frac{(A_{413}\ 100\%\ \text{lysis} - A_{413}\ \text{bkgd.}^*) - (A_{413}\text{test} - A_{413}\text{bkgd.}^*)}{(A_{413}\ 100\%\ \text{lysis} - A_{413}\ \text{bkgd.}^*)}$$

(* bkgd. = background)

Plot %CF vs Ag conc.

determine the extent of total lysis; and (4) two tubes containing only diluent and ovalbumin solution, to determine the background lysis of the sheep erythrocytes. The total volume contained in each tube of the assay, as well as the controls, was 6,0 ml.

The contents of each tube were mixed and left to incubate at +4°C for 16-18 hrs while antigen binding and complement fixation occurred. Thereafter, 1,0 ml of a 2,5% suspension of sensitized sheep erythrocytes (4.3.2.1.4) was added to each tube and haemolysis was allowed to proceed for 60 min in a 37°C waterbath. After immersion in an ice bath for 15 min to stop the haemolytic reaction, the tubes were centrifuged at 770 g for 10 min and the absorbance of the supernatants was read at a wavelength of 413 nm.

The results, expressed as the percentage of complement fixed by the antibody-antigen complexes, were calculated as follows:

$$\%CF = \frac{(A_{413nm}\text{total lysis} - A_{413nm}\text{bkgd.*}) - (A_{413nm}\text{test} - A_{413nm}\text{bkgd.*})}{(A_{413nm}\text{total lysis} - A_{413nm}\text{bkgd.*})} \times 100$$

(* bkgd. = background)

The percentage complement fixed was plotted against the antigen concentration in µg/ml to give the characteristic complement fixation curve (figures 2.13 and 2.14).

4.3.2.1.9 INHIBITION AND ENHANCEMENT OF COMPLEMENT FIXATION BY PEPTIDES

The antiserum dilution was carefully chosen, so that the complement fixation curve formed a peak at approximately 75% fixation (the titre of the serum), for inhibition studies, while a peak of approximately 30% fixation was preferable for studies with peptides known to enhance complement fixation.

1,0 ml of diluted antiserum was pipetted into each centrifuge tube in the assay and to this was added 1,0 ml of the appropriate concentration of peptide dissolved in 0,1% (w/v) solution of ovalbumin in diluent. Identical peptide dilutions were added to each tube of a series comprising a complement fixation curve, and increasingly diluted peptide solutions were added to each subsequent series of tubes. The antiserum-peptide mixtures were incubated at 37°C for 15 min in a waterbath. After cooling in an ice bath the normal procedure for complement fixation (4.3.2.1.8) was continued, except that an additional control was prepared with peptide and complement only, to ensure that the peptide did not behave in a pro- or anti-complementary fashion. Normal complement fixation assays with the same antiserum dilution were performed in triplicate, simultaneously with the inhibition or enhancement assay, for the purpose of comparison.

The percentage inhibition of complement fixation caused by each concentration of peptide was calculated from the degree by which the peak of the normal complement fixation curve was depressed in the presence of the peptide. The following formula was used:

$$\%\ \text{Inhibition of complement fixation} = 100 - \left(\frac{\%\ \text{fixation}_{\text{peptide}}}{\%\ \text{fixation}_{\text{normal}}} \times 100 \right)$$

Percentage inhibition of complement fixation was plotted against the amount of peptide inhibitor (fig. 2.18).

In the case of a peptide enhancing the complement fixation, caused by the antigen and antibody, no attempt was made to calculate the extent of that enhancement.

4.3.3 BINDING ASSAYS WITH N-(^{3}H)ACETYL PEPTIDES

4.3.3.1 DETERMINATION OF RADIOACTIVITY

Radioactivity was determined using a Packard Tri-Carb model 3385 liquid scintillation counter. Samples, made up to 1,0 ml with distilled water or buffer, were counted in standard plastic scintillation vials (Packard) using 10,0 ml of Insta-Gel scintillation cocktail (Packard).

Counts per minute (cpm) were either used without correction, or were converted to disintegrations per minute (dpm) using Packard standard quenched samples. The quench correction curves were stored in a Wang 700 bench top computer program, which was written by Mr. E. Lee of the Department of Biochemistry, University of Cape Town.

4.3.3.2 LABELLING OF PEPTIDES WITH ^{3}H-ACETIC ANHYDRIDE

Tritiated acetic anhydride with a specific activity of approximately 4 Ci/m-mole was obtained from The Radiochemical Centre, Amersham, and used to label the four pH 4,5 soluble tryptic peptides of TMV protein utilized in this work. They were, peptide 4 (residues 62-68), peptide 8 (residues 93-112), peptide 11 (residues 135-141) and peptide 12 (residues (142-158). A synthetic peptide corresponding to residues 134-141 of the protein of the U2 strain of TMV was prepared by Dr. J.D. Young of the Space Sciences Laboratory of the University of California by the solid phase peptide synthesis method (Merrifield and Stewart, 1965), and it also was labelled with tritiated acetic anhydride. The method described by Benjamini *et al.* (1965) was used to acetylate the peptides.

500 n-moles of peptide in aqueous solution were pipetted into the

titration vessel and buffered with 100 µl of a 0,5 M sodium phosphate buffer pH 7,5. The volume of the peptide solution was increased to 1,0 ml with distilled water so that a small electrode from a pH meter could be immersed in it. The pH of the peptide solution was adjusted to 7,5 with 0,1M NaOH while stirring continued.

The tritiated acetic anhydride was dissolved in 1,0 ml of benzene (added to the frozen tritiated acetic anhydride in its vial) and this was added to the peptide solution at the rate of 100 µl every five minutes with constant stirring. The pH was maintained at 7,5 by the dropwise addition of 0,1M NaOH. After all the tritiated acetic anhydride solution had been added, the peptide solution was left stirring at room temperature for an hour, then the pH was again adjusted to 7,5. The acetylated peptide solution was sealed and left at +4^{o}C for 16-18 hrs before the benzene was evaporated off the solution.

The N-(^{3}H)acetyl peptide was separated from the unreacted acetate in the solution by exclusion chromatography using a 40x2,6 cm column of Sephadex G25 medium equilibrated with a solution of 50% (v/v) glacial acetic acid in water. The column was run with the same solution and a flow rate of 30 ml/hr was maintained while 2,0 ml fractions of the eluant were collected. 5 µl of each fraction, 1,0 ml of distilled water and 10,0 ml of Insta-Gel scintillation cocktail (Packard) were mixed in plastic scintillation vials and counted in a Packard Tri-Carb model 3385 liquid scintillation counter for 0,1 min ((4.3.3.1). Counts per 0,1 min were plotted against the fraction number to obtain the elution profile (fig. 4.4). The first peak in the elution profile was pooled and evaporated to dryness in a vacuum dessicator in the presence of phosphorous pentoxide and NaOH pellets, while the second peak

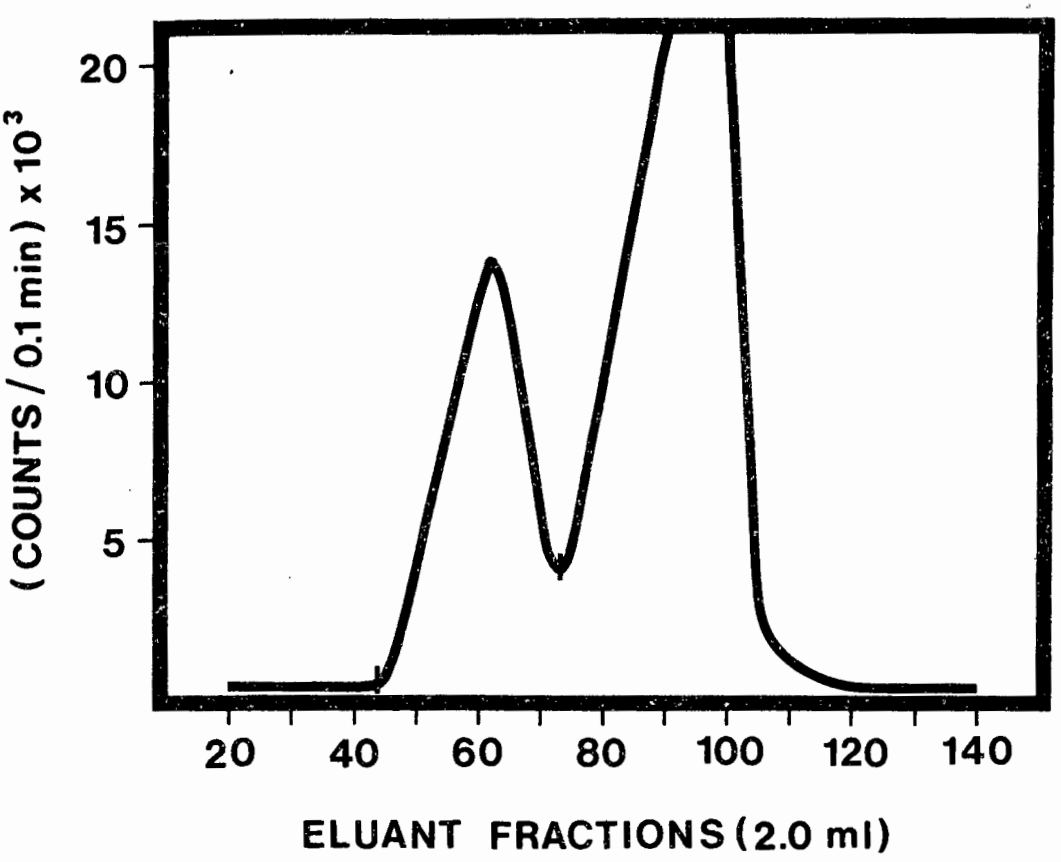

Fig. 4.4 : The separation of N-(^{3}H) acetyl tryptic peptide 4 (residues 62-68) from unreacted (^{3}H) acetate by exclusion chromatography on a 40x2,6 cm Sephadex G25 column equilibrated and run with a solution of 50% (v/v) glacial acetic acid in water. The flow rate was maintained at 30 ml/hr and 2,0 ml fractions were collected. The radioactivity in 5 µl of each sample was determined and plotted against the fraction number. The bars (▌) indicate the fractions that were pooled.

was discarded. The purified, dried N-(^{3}H) acetyl peptide was dissolved in distilled water to a calculated concentration of 100 n-moles/ml and a portion of this solution was submitted for amino acid analysis after the peptide had been hydrolysed (4.2.2).

4.3.3.3 EXPERIMENTAL PROCEDURE

The method, here described, is a modification of the Farr technique (Farr, 1958) which was used by Benjamini et al. (1965) in their work with tryptic peptide 8 (residues 93-112). Instead of precipitating the immune complexes with 50% (w/v) saturated $(NH_4)_2SO_4$, a double-antibody system (Coombs et al., 1945) was used.

4.3.3.3.1 EQUIPMENT

Falcon clear plastic tubes with the dimensions 75x10 mm were employed as reaction tubes and adaptors were used to fit the tubes into the swinging-buckets of a Sorvall HS-4 rotor. Centrifugation was carried out in a refrigetated Sorvall RC2-B centrifuge.

Pipetting was done with microliter Finnpipettes using Finntips of the appropriate sizes. Radioactivity determinations were carried out on a Packard Tri-Carb model 3385 liquid scintillation counter (4.3.3.1).

4.3.3.3.2 DILUENTS

A stock solution of borate buffered saline was prepared by dissolving 78,1g of NaCl, 70,0g of H_3BO_3 and 50,5g of $Na_2B_4O_7.10H_2O$ in distilled water and adjusting the volume of the solution to 1,0 litre. Five-fold dilutions of this stock buffer were made, adjusted to pH 7,5 with 1,0M HCl and used to dilute the globulin preparations. Both the stock buffer and the diluted borate buffered saline were stored at +4^{o}C.

A separate 1,0% (w/v) solution of ovalbumin in borate buffered saline pH 7,5, was also prepared and stored at +4°C. This was used to dilute the N-(^{3}H)acetyl peptide solutions.

4.3.3.3.3 SPECIFIC RABBIT GLOBULINS

Globulin preparations (4.3.1.3) of cross-absorbed (4.3.1.2) and whole rabbit antisera (4.3.1.1) were made and diluted to a final concentration of 5,0 mg/ml with borate buffered saline pH 7,5, for use in the direct binding assays. The diluted globulin preparations were stored at + 4°C with sodium azide 0,04% (w/v) as a preservative.

4.3.3.3.4 GOAT-ANTI RABBIT GLOBULINS

A globulin preparation (4.3.1.3) of a goat antiserum specific for rabbit IgG (4.3.1.1) was adjusted to serum strength with borate buffered saline pH 7,5, and titrated against increasing concentrations of rabbit IgG (prepared by the method of Reif (1969) by tube precipitin test to find the proportions of goat and rabbit antibodies, which gave complete precipitation. This was found to occur with 500 µl of goat-anti rabbit globulins and 0,05 mg of rabbit IgG.

The goat-anti rabbit globulins were stored at +4°C with sodium azide 0,04% (w/v) as a preservative.

4.3.3.3.5 N-(^{3}H)ACETYL PEPTIDES

The concentrations of the N-(^{3}H)acetyl peptide preparations (4.3.3.2) were calculated from the amino acid analyses (4.2.2) of the peptides and adjusted to a final concentration of 0,042 n-moles/100 µl with a 1% (w/v) solution of ovalbumin in borate buffered saline pH 7,5. The labelled peptide solutions were stored at -20°C.

4.3.3.3.6 THE DIRECT BINDING ASSAY

The binding assay consisted of mixing 0,05 mg (100 µl of a 0,5 mg/ml solution) of specific globulins with 0,042 n-moles (100 µl) of N-(^{3}H) acetyl peptide and incubating the mixture for 15 min at room temperature. Thereafter 500 µl of goat-anti rabbit globulins (4.3.3.3.4) were added and the mixture incubated for 17 hrs at +4^{o}C. The precipitates were centrifuged at 7000 g for 1 hr and the supernatants were discarded. The inner sides of each tube were wiped dry with a tissue, without disturbing the precipitate. The precipitates were dissolved in 1,0 ml 1,0M NaOH and 0,5 ml of the resuspended precipitate was transferred to counting vials, where a further 0,5 ml of 2,0M HCl and 10,0 ml Insta-Gel scintillation cocktail (Packard) were added. The vials were counted twice on a Packard Tri-Carb model 3385 liquid scintillation counter (4.3.3.1).

The extent of non-specific binding was determined by using globulins obtained from unimmunized rabbits and from rabbits immunized with human serum albumin. The specific binding was calculated by subtracting the average count of two non-specific binding tests from the binding observed with specific globulins:

$$\%\ \text{peptide specifically bound} = \left(\frac{\text{av. cpm/test} - \text{av. cpm/non-specific control}}{\text{specific activity of peptide in cpm/n-mole}} \right) \times \frac{100}{0{,}042}$$

The background cpm resulting from N-(^{3}H)acetyl peptide sticking to the walls of the tubes was determined by omitting the globulins from a set of controls, and then treating them identically to the rest of the assay.

4.3.3.3.7 INHIBITION OF THE SPECIFIC BINDING OF THE N-(^{3}H)ACETYL PEPTIDES BY UNLABELLED PEPTIDES

The direct binding assay was done as described in section 4.3.3.3.6,

except that increasing concentrations of the same, but unlabelled, peptide were incubated for 15 min at room temperature with a constant amount of specific globulins (0,05 mg) before 0,042 n-moles of the N-(^{3}H)acetyl peptide was added to each test. Controls containing no inhibitor were carried out simultaneously with the assay, in addition to the non-specific binding and background controls.

4.3.4 MICRO-PRECIPITIN ASSAYS WITH RADIOLABELLED ANTIGENS

4.3.4.1 PREPARATION OF RADIOLABELLED TMV AND TMV PROTEIN

4.3.4.1.1 INCORPORATION OF ^{3}H-NUCLEOSIDES INTO TMV

Tritiated nucleosides were incorporated into TMV in infected plants by the same basic method used by Singer (1971) to incorporate tritated amino acids into TMV protein.

Young plants at the 3-4 leaf stage of development were infected with TMV by rubbing onto their leaves a mixture of an 8,0 mg/ml solution of TMV and celite. This was washed off and the plants were left for 2-4 days while the infection became systemic (Hirai and Wildman, 1967). The stems of the plants were then cut off at soil level and immersed in a small volume of a solution of the tritiated nucleosides until it had been completely imbibed. The radioactive material was "washed" into the plants by further small aliquots of water, and then the plants were floated on Knopps solution (1,0g $Ca(NO_3)_2$, 0,25g KH_2PO_4, 0,25g $MgSO_4$ and 0,12g KCl dissolved in 1,0 litre distilled water) with constant lighting for a further 2-3 days, or until signs of wilting became evident.

The tritiated nucleosides that were used were [5,6-^{3}H] uridine and [2,5',8-^{3}H] adenosine, chosen for their high specific activities (∿50 Ci/m-mole) and obtained from The Radiochemical Centre, Amersham. These

tritiated nucleosides were used singly, and mixed, so that 1,0 mCi of the radioactive material was distributed amongst eight plants. The radioactive solutions were diluted with distilled water.

The radiolabelled TMV was extracted from the plant material by the same method as von Wechmar and van Regenmortel (1970). In some preparations polyethylene glycol was used to concentrate the virus, while in others the virus was concentrated by centrifugation at 65000 g for 1 hr after filtration through activated charcoal and celite (4.1.2).

The purity and concentration of the radiolabelled TMV preparations were assessed by ultraviolet spectrophotometry (2.1.1) and the specific activity was determined using a Packard Tri-Carb model 3385 liquid scintillation counter (4.3.3.1).

The radiolabelled virus, suspended in 0,1M Tris(hydroxymethyl)aminomethane-0,15M NaCl buffer pH 6,8 (4.3.4.2.2), was stored at +4^{o}C.

4.3.4.1.2 INCORPORATION OF ^{3}H-AMINO ACIDS INTO TMV PROTEIN

The method of Singer (1971) was used to incorporate the following tritiated amino acids into TMV protein in infected plants: L-[2,3-^{3}H] alanine, L-[4,5(n)-^{3}H] isoleucine, L-[4,5-^{3}H] leucine, L-[3-^{3}H] serine and L-[3,4(n)-^{3}H] valine. These radiolabelled amino acids, which were obtained from the The Radiochemical Centre, Amersham, were chosen for their high specific activities (10-40 mCi/m-mole). They were diluted with distilled water so that 2,5 mCi of radioactive material was distributed amongst ten infected plants. The plants were selected, infected with TMV, and prepared to imbibe the solution of the radioactive amino acids in the manner described in section 4.3.4.1.1.

TMV was extracted from the plant material by the method of von Wechmar and van Regenmortel (1970), using polyethylene glycol to concentrate the virus (4.1.2). The radiolabelled TMV protein was prepared from the virus by the ethanolamine-DEAE method (Durham, 1972) as described in section 4.1.3.2, except that it was stored at +4°C in aqueous solution and not lyophilized.

The purity and concentration of the radiolabelled TMV protein preparations were assesed by ultraviolet spectrophotometry (2.1.2) and the specific activity was determined using a Packard Tri-Carb model 3385 liquid scintillation counter (4.3.3.1).

4.3.4.2 EXPERIMENTAL PROCEDURE

4.3.4.2.1 EQUIPMENT

Standard glass Wasserman tubes, with the dimensions 75x11 mm, were employed as reaction tubes and adaptors were used to fit the tubes into the swinging-buckets of a Sorvall HS-4 rotor. Centrifugation was carried out in a refrigerated Sorvall RC2-B centrifuge.

Pipetting was done with serological pipettes, Drummond Microdispensers and Finnpipettes (using Finntips of the appropriate sizes). Radioactivity determinations were carried out on a Packard Tri-Carb model 3385 liquid scintillation counter (4.3.3.1).

4.3.4.2.2 DILUENTS

0,1M Tris(hydroxymethyl)aminomethane-0,15M NaCl buffers were prepared by the tenfold dilution of the TRIS-NaCl stock solution used in the complement fixation system (4.3.2.1.2). The pH of the buffers was adjusted to 6,8 , 7,4 or 8,0 with 1,0M HCl or 1,0M NaOH, as required for

a particular assay.

Separate solutions of 1,0% (w/v) ovalbumin in the TRIS-NaCl buffer were also adjusted to pH 6,8 , 7,4 or 8,0 with 1,0M NaOH as required.

All the buffers were stored at +4°C until use.

4.3.4.2.3 ANTISERA

Antisera specific for TMV and TMV protein were raised in rabbits (4.3.1.1). Before use, the antisera were diluted with a solution of 1,0% (w/v) ovalbumin in TRIS-NaCl buffer, which was adjusted to the pH at which the assay was to be performed.

4.3.4.2.4 RADIOLABELLED ANTIGENS

Radiolabelled TMV and TMV protein were prepared as described in sections 4.3.4.1.1 and 4.3.4.1.2.

Prior to each assay, the radiolabelled TMV was centrifuged at 87500 g for 20 min in a swinging-bucket rotor to select for intact virus rods and to remove any dissociated protein. It was then resuspended in the TRIS-NaCl buffer pH 6,8 and the concentration of the solution was determined from its extinction at 260 nm. A correction for light scattering was made by using the formula of Rayleigh and the following extinction coefficient:

$$A_{260nm\ (corrected)} = A_{260nm} - \left(A_{320nm} \times \frac{320^4}{260^4}\right) \qquad \text{(Hendry, 1977)}$$

$$E^{0,1\%}_{260\ nm\ (corrected)} = 3,0$$

The solution of radiolabelled TMV was adjusted to a final concentration of 25,0 µg/ml with TRIS-NaCl buffer pH 6.8.

The radiolabelled TMV protein solution, on the other hand, was diluted with double strength TRIS-NaCl buffer pH 7,4 at +4°C, and centrifuged at 167500 g for 1 hr immediately before use to remove protein aggregates. The concentration of the protein in the supernatant was determined from its extinction at 282 nm with an extinction coefficient of $E^{0,1\%}_{282\ nm} = 1,27$ (Fraenkel-Conrat, 1957). The solution of radiolabelled TMV protein was adjusted to a final concentration of 25,0 µg/ml with TRIS-NaCl buffer pH 7,4, for use in the assays.

4.3.4.2.5 TITRATION OF THE ANTISERA WITH THE RADIOLABELLED ANTIGENS

0,5 ml of each of a series of twofold dilutions of antiserum were added to 1,0 ml of TRIS-NaCl buffer (at the desired pH) in Wasserman tubes. To this was added 0,5 µg (20 µl) of the appropriate radiolabelled antigen and the mixture was incubated for 17 hrs at +4°C. The precipitates were centrifuged at 3079 g for 10 min and 1,0 ml of the supernatant was mixed with 10,0 ml of Insta-Gel scintillation cocktail (Packard) in plastic counting vials (Packard). The radioactivity in the vials was determined (4.3.3.1) and disintegrations per minute (dpm) were plotted against the reciprocal of the antiserum dilution. From this curve an antiserum dilution, which gave antibody:antigen proportions in the zone of antigen excess, near the equivalence point,was chosen for the inhibition studies (Fig.s 2.8 and 2.10).

Five minimum binding controls, containing the radiolabelled antigen, but with a solution of 1,0% (w/v) ovalbumin in TRIS-NaCl buffer replacing the antiserum, were included with each titration.

In the case of radiolabelled TMV protein, the initial mixing of

antigen and antiserum was carried out at +4°C to discourage protein aggregation (Klug and Durham, 1971).

4.3.4.2.6 INHIBITION OF THE PRECIPITIN REACTION OF RADIOLABELLED TMV AND TMV PROTEIN WITH SPECIFIC ANTISERA

The inhibition assays, with different inhibitors, were performed under various pH conditions. The inhibition of the radiolabelled TMV-anti TMV reaction by unlabelled virus protein was carried out at pH 6,8 and pH 8,0, while inhibition experiments with peptides in this system were done at pH 6,8. The inhibition of the radiolabelled TMV protein-anti TMV protein reaction by unlabelled virus protein and peptides was carried out at pH 7,4. Increasing amounts of the inhibitors were diluted with TRIS-NaCl buffers at the appropriate pH for the assay, prior to their use.

The antiserum was titrated (4.3.4.2.5) at the same pH as the inhibition assay was to be done, and the antiserum dilution providing the antibody:antigen proportions most suitable for inhibition studies was chosen. The serum was diluted with a solution of 1,0% (w/v) ovalbumin in TRIS-NaCl buffer at the appropriate pH.

The inhibition assay consisted of mixing 0,5 ml of the diluted antiserum in a solution of 1,0% (w/v) ovalbumin in TRIS-NaCl buffer with 1,0 ml aliquots of increasingly concentrated solutions of the inhibitor in TRIS-NaCl buffer at the appropriate pH. The mixture was incubated for 15 min at 37°C then left at +4°C for 17 hrs. 0,5 µg (20 µl) of the radiolabelled antigen was added and the mixture was incubated for a further 17 hrs at +4°C. The precipitates were centrifuged at 3079 g for 10 min and 1,0 ml of the supernatant was mixed with 10,0 ml

of Insta-Gel scintillation cocktail (Packard) in plastic counting vials (Packard). The radioactivity in the vials was determined (4.3.3.1) and disintegrations per minute (dpm) were calculated.

When radiolabelled TMV protein was the antigen, it was added to the mixture of antiserum and inhibitor at +4°C to discourage the formation of protein aggregates (Klug and Durham, 1971).

The following controls were included with each assay: Five tubes containing the radiolabelled antigen, but with a solution of 1,0% (w/v) ovalbumin in TRIS-NaCl buffer and TRIS-NaCl buffer substituted for the antiserum and inhibitor respectively. These provided the average minimum binding control. Five tubes containing antiserum and radiolabelled antigen, but no inhibitor, provided the average maximum binding control. All the controls were carried out at the same pH as the assay.

The average dpm in the minimum binding controls corresponds to 100% inhibition, and the average dpm in the maximum binding controls, to 0% inhibition. The percentage inhibition caused by each concentration of inhibitor is therefore calculated as follows:

$$\% \text{ inhibition} = \left(\frac{\text{test dpm} - \text{max binding av. dpm}}{\text{min binding av. dpm} - \text{max binding av. dpm}} \right) \times 100$$

The percentage inhibition was plotted against inhibitor concentration to give the inhibition curve.

Controls containing only 0,5 µg of the radiolabelled antigen were carried out simultaneously with the assay as a check on the amount of the radiolabelled antigen added to each test.

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