The Self-assembly Of Potexvirus Proteins

Kerry Frances Ready

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LA THÈSE A ÉTÉ MICROFILMÉE TELLE QUE NOUS L'AVONS REÇUE
THE SELF-ASSEMBLY OF
POTEXVIRUS PROTEINS

by

Kerry Frances Mullin Ready
Department of Plant Sciences

Submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
London, Ontario
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ABSTRACT

The potexvirus family consists of flexuous helical plant viruses that are architecturally similar (Richardson et al., 1981). The behaviour of the coat proteins of several of these viruses (barrel cactus virus (BCV), foxtail mosaic virus (FTV), viola mottle virus (VMV), potato virus X (PVX) and tulip virus X (TVX)) is compared to determine whether related helical viruses assemble by a common pathway.

The assembly of the coat proteins from the various viruses was examined in the absence of RNA under various conditions of pH, temperature, ionic strength and protein concentration in solution and in the presence of different concentrations of (NH₄)₂SO₄ in hanging drops. The structure of the products was determined using optical diffraction.

The formation of small aggregates of the potexvirus proteins examined here appears to involve the formation of a hexamer/heptamer, in most cases from a trimer. No disks were observed. In this respect, the sub-assembly processes of the five proteins examined differ from those for papaya mosaic virus (PMV) and clover yellow mosaic virus (CYMV) (both potexviruses) and unrelated helical viruses. The formation of trimers and hexamers may be common to all potexviruses but disk formation may not be a requirement for reconstitution. The potexviruses do not share a common sub-assembly pathway nor can such a pathway be predicted from the structure of the final product.

The tubular polymers of BCV and FTV proteins are virus-like and form over a wide range of conditions, indicating that protein-protein
interactions are important for the assembly of these viruses. The proteins of VMV, PVX and TVX do not form virus-like helices. VMV protein forms paired stacked disks in 15% saturated (NH₄)₂SO₄ at pH 6.0. Five-start helices and particles composed of 9 parallel helices, each with 8 3/4 subunits/turn, form slowly in 0-15% saturated (NH₄)₂SO₄ at pH 6.0 and 8.0. PVX protein polymerizes into stacked rings, of 8 or 9 subunits each, at high concentrations of (NH₄)₂SO₄. TVX protein forms particles composed of 3 parallel helices with 8 1/2 subunits/turn at pH 5.0. The assembly of BCV and FTV as well as that of PMV and CYMV (Erickson and Bancroft, 1978; Bancroft et al., 1979) is directed primarily by protein-protein interactions, since their proteins form virus-like particles in the absence of RNA. The proteins of VMV, PVX and TVX do not. Therefore protein-RNA interactions are more important than protein-protein interactions in directing the assembly of their capsids.

Carboxyl-carboxylate interactions, like those that regulate the assembly of tobacco mosaic virus (TMV) protein and possibly that of PMV protein, do not seem to be necessary for all helical viruses. BCV protein polymerization probably involves an interaction between histidine and a basic amino acid residue. A second interaction may occur between a carboxylic acid side chain and a basic amino acid. The latter interaction probably also regulates the polymerization of FTV protein. Thus, the nature of the protein-protein interactions controlling capsid assembly is not the same for all helical viruses or even for all potexviruses.
ACKNOWLEDGEMENTS

I express my deepest gratitude to my supervisor, Dr. John B. Bancroft for his continued advice and guidance, his time and infinite patience during the course of my research. Working with him has been a rewarding experience.

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I dedicate this thesis to my husband Bill, for his continual love and encouragement, and his special way of seeing things.
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<td>alfalfa mosaic virus</td>
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<td>BCV</td>
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<tr>
<td>BMV</td>
<td>brome (grass) mosaic virus</td>
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<tr>
<td>BSMV</td>
<td>barley stripe mosaic virus</td>
</tr>
<tr>
<td>C</td>
<td>strain designation for tobacco rattle virus</td>
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<td>CAM</td>
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<td>CCMV</td>
<td>cowpea chlorotic mottle virus</td>
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<td>CYMV</td>
<td>clover yellow mosaic virus</td>
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<td>EDTA</td>
<td>ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>FTV</td>
<td>foxtail mosaic virus</td>
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<td>MES</td>
<td>2-(N-morpholino) ethane sulfonate</td>
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<td>NMV</td>
<td>narcissus mosaic virus</td>
</tr>
<tr>
<td>PMV</td>
<td>papaya mosaic virus</td>
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<td>PVX</td>
<td>potato virus X</td>
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<td>PVY</td>
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<td>pI</td>
<td>isoelectric pH</td>
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<tr>
<td>RNA</td>
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<td>/</td>
<td>Svedberg unit ($10^{-13}$ sec)</td>
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<td>SBMV</td>
<td>southern bean mosaic virus</td>
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<td>TBSV</td>
<td>tomato bushy stunt virus</td>
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<td>TMV</td>
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TRV  tobacco rattle virus
TVX  tulip virus X
VMV  viola mottle virus
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Radial projections of stacked disk particles with disks 35 Å apart and a true repeat of 2

Optical diffraction patterns of particles of VMV coat protein

Radial projections of different particle types: single helix; 2-start helix; 2 parallel helices

Optical reconstruction of a VMV protein particle
Histogram of $2\pi R_n$ values for the 1/36 Å reflection from VMV protein particles

The $2\pi R_n$ values, for the 1/36 Å reflection from VMV protein particles, against the change in cumulative variance ($\Delta s'$) for $2\pi R_n$ values listed in increasing order of magnitude

Terminology for describing a multi-helical particle

Radial projection of the most suitable model for Type 1 VMV protein particles

Transforms from radial projections of models for the structure of VMV protein particles

Radial projection of the most suitable model for Type 2 VMV protein particles

Radial projection of a model for Type 2 VMV protein particles

Optical diffraction pattern of TVX protein particles formed in 0.01 M citrate pH 5.0 at 4°C

Optical reconstruction of a TVX protein particle

Transforms from radial projections of models for the structure of TVX protein particles at pH 5.0
Radial projection of a model for the structure of TVX protein particles at pH 5.0, with 2 apparent starts

Transforms from radial projections of models for the structure of TVX protein particles at pH 5.0

Radial projection for the apparent 3-start model for the structure of TVX protein particles at pH 5.0

Transforms from radial projections of models for the structure of TVX protein particles at pH 5.0

Electron micrograph of ropes of VMV protein formed in 15% saturated $(NH_4)_2SO_4$ at pH 6.0

Optical diffraction pattern of VMV protein rope formed in 15% saturated $(NH_4)_2SO_4$ at pH 6.0

Optical reconstructions of VMV protein ropes

Diagrams showing the structure of VMV protein ropes

Electron micrographs of FTV protein ropes

Optical diffraction of FTV protein ropes formed in 0.01 M glycine, pH 9.0
CHAPTER 1
INTRODUCTION

1.1 Self-assembly processes

Large structures in many biological systems are built of many separate parts, which may be identical, which self-assemble in vivo. These include microtubules, ribosomes, flagella, muscle fibers and viruses (reviewed by Kushner, 1969). The main advantages of self-assembly systems lie in the economy of genetic information required and the low cost of error.

Studies to determine the pathways by which self-assembly processes occur and the nature of the forces that control them are most easily performed in vitro. One or both of two approaches may be taken: (1) Controlled degradation, using enzymes, detergents or extremes of temperature, ionic strength or pH, provides information on the nature of the forces stabilizing the intact particle but not on the pathway to its formation; (2) Reassembly provides information as to the forces, intermediates and equilibria involved in the assembly pathway. The conditions that may be manipulated include the environmental parameters (e.g. pH, temperature, ionic strength), the components present and their stoichiometry. These approaches are reviewed by Kaper (1975).

1.2 Stabilizing forces

1.2.1 Entropy

Entropy, or the degree of disorder, is one of the main driving
forces in spontaneous self-assembly, which is usually endothermic. For a spontaneous reaction, there must be an overall decrease in the free energy of the system \((\Delta G_{sys} < 0)\), which is expressed as

\[
\Delta G = \Delta H - T\Delta S
\]

If the reaction is endothermic, then \(\Delta H > 0\). If \(\Delta G < 0\), then \(\Delta S\), the change in entropy, must be sufficiently positive to overcome the increase in enthalpy \((\Delta H)\). It seems paradoxical that the system becomes more disordered to produce a highly ordered particle such as a virus. This can be accomplished by the release of bound water, which contributes to the overall entropy of the system, from the virus protein subunits as they polymerize. Lauffer (1975) provides an excellent discussion of this topic. Ionic interactions, which also exert a stabilizing effect in assembly processes, involve a certain electrostatic energy and an entropy increase.

In addition to the release of water, entropy may be increased by the release of counterions or fragments of molecules that might be cleaved during an assembly process (Lewin, 1974).

1.2.2 Enthalpy

Enthalpy may also be a stabilizing force, in hydrogen bonding for example. This could occur if there were sufficient difference between the bond energy of the hydrogen bond and the sum of the bond energies of the individual groups interacting with water.

Hydrogen bonding may be involved in self-assembly reactions in the form of protein-protein interactions and, as in the case of viruses,
protein-nucleic acid interactions.

One example of an enthalpy-driven reaction is the assembly of tobacco rattle virus (TRV) which, unlike most other helical viruses, has a low temperature requirement for its in vitro assembly (AbouHaidar et al, 1973).

1.3 Plant virus assembly

Plant viruses, in general, present excellent systems for the in vitro study of assembly processes, for several reasons. They are often structurally very simple, being composed of a single nucleic acid and many copies of a single protein. Also, they can be produced in large quantities and their components are relatively easy to isolate in pure, functional form. In contrast, many animal and bacterial viruses are structurally quite complex. They may contain several different structural proteins as well as non-structural proteins (e.g. replicases). In addition, some animal virus capsids contain glycoproteins and may have a lipid envelope, which is often host-coded. Although some bacteriophages can easily be produced in large quantities, this is difficult with most animal viruses. The assembly of some of the complex animal and bacterial viruses has been reviewed by Casjens and King (1975).

1.3.1 Architecture

Crick and Watson (1956) proposed that all viruses are composed of multiple copies of subunits which can be arranged in one of
two ways: in tubes with helical symmetry or in polyhedra with cubic symmetry. Klug and Caspar (1960) proposed that the isometric viruses have icosahedral symmetry. These two symmetries provide for a great variety in virus structure and at the same time an efficient means of protecting viral nucleic acid with a single type of protein.

1.3.2 Quasi-equivalence

The quasi-equivalence theory was proposed by Caspar and Klug (1962), who suggested that variations in virus structure from the basic T=1 icosahedron containing 60 asymmetric units, could be accommodated if the 20 triangular faces were divided into a greater number of near-equilateral faces so that the intersubunit bonds were not all strictly equivalent. Icosahedral symmetry provides less bond distortion than the other forms of cubic symmetry and the variations in this basic structure can be seen even in the complex bacteriophages such as the T-even phages.

An exception to the quasi-equivalence theory has recently been demonstrated in the structure of the polyomavirus (Py) capsid, which is an icosahedron with T=7 symmetry. Rayment et al (1982) suggested that the Py capsid was composed completely of pentameric morphological units (rather than a combination of pentameric and hexameric units) which could be either pentavalent or hexavalent in their bonding pattern. This has been supported by Baker et al (1983) in their examination of two types of tubular variant of Py, which all appear to be composed of pentamers. Quasi-equivalent bonding appears to be maintained in icosahedra of low triangulation number (e.g. tomato
bushy stunt virus (TBSV) and southern bean mosaic virus (SBMV) (Abad-Zapatero et al, 1980; Harrison et al, 1978)) but variations appear more common in larger shells (Klug, 1983). Whether the non-equivalent pentameric construction of Py occurs in other T=7 capsids remains to be seen.

Equivalent bonding is maintained in helical viruses, except at the ends. The bonding pattern in the sinuous viruses is quasi-equivalent (excepting the ends), distortions in the bonding producing the flexibility.

1.3.3 Physical-chemistry of assembly

This aspect of assembly is far less well understood than the architectural principles governing assembly. Much of what is known is due to the extensive research than has been done on tobacco mosaic virus (TMV).

Virus assembly occurs as a result of several types of non-covalent interactions between protein subunits and between the protein and the nucleic acid. The former may consist of polar interactions such as hydrogen bonds and ionic or salt linkages, as well as non-polar hydrophobic bonds. The latter consist of ionic linkages.

The conditions under which the assembly of some viruses occurs are regulated to a large degree by specific amino acid interactions. The most well-known example is TMV. Its assembly is regulated by anomalously titrating carboxyl-carboxylate pairs. Their existence was first proposed by Caspar (1963) and was demonstrated by Stubbs
et al (1977). These co-operative hydrogen bonds are also involved in the assembly of cowpea chlorotic mottle virus (CCMV) (Bancroft et al., 1967) and perhaps in that of papaya mosaic virus (PMV) (Durham and Bancroft, 1979).

Carboxyl-carboxylate interactions are not the only specific amino acid interactions involved in regulating virus assembly. The results of McDonald and Bancroft (1977; McDonald et al., 1976) with potato virus Y (PVY) protein indicate that histidine may be important in the assembly of the PVY capsid. As will be shown here, it also appears to be involved in the assembly of barrel cactus virus (BCV).

1.3.4 Mechanics of assembly

The mechanics of helical virus assembly have not been extensively described apart from TMV, and to a lesser extent, PMV.

The initiation of TMV assembly is internal (Lebeurier et al., 1977, Otsuki et al., 1977) and begins with the nucleation step: the binding of a bilayer disk to a specific sequence about 1000 nucleotides from the 3'-end of the RNA (Zimmern and Butler, 1977; Zimmern, 1977). From here, a disk-to-protohelix transition is assumed to occur so that helical growth may proceed (Butler and Durham, 1977). Mechanistically, this is probably the most efficient method of starting helical growth. However, it has yet to be conclusively demonstrated for certain other helical viruses. AbouHaidar et al (1973) found that the 36S disk of TRV protein is required to initiate the assembly of TRV. Erickson and Bancroft (1978) have shown that PMV protein forms a 14S aggregate,
which appears to be an 18-subunit disk (Erickson et al., 1983). However, they were unable to show an absolute requirement for the disk in PMV assembly. Similarly, clover yellow mosaic virus (CYMV) protein forms a 14S aggregate (Bancroft et al., 1979), but here the mechanics of the assembly process have not been defined.

One of the difficulties in elucidating the mechanics of helical virus assembly is that intermediates may be transient or occur in low concentrations. For example, in a two-layer disk aggregate for potexvirus assembly, the aggregate would theoretically not have to represent more than 1/75 of the protein present (Erickson and Bancroft, 1978). However, it seems probable that the concentration of an important sub-assembly aggregate would be higher than the absolute minimum required by stoichiometry in order for the nucleation step to proceed at a reasonable rate.

The protein of alfalfa mosaic virus (AMV), a bacilliform multipartite plant virus, has been shown to form two species. A 3S species which consists of dimers forms at pH 3-9 at low ionic strength (Driedonks et al., 1976). At pH 4-9 and higher ionic strength, a 30S species forms from the 3S material in an entropy-driven reaction (Driedonks et al., 1976, 1977). This species is a hollow sphere, composed of 30 dimers, which structurally resembles the cylindrical portion of the bacilliform virions converted into an icosahedron (Driedonks et al., 1977). Driedonks et al. (1976, 1977) have proposed that the dimer is the main assembling unit in the polymerization, since no intermediates have been reproducibly observed.
The assembly of the Bromoviruses and their proteins has been reviewed by Bancroft (1970). Several spherical plant viruses, including CCMV and brome mosaic virus (BMV) have been reassembled from their components (Bancroft et al., 1967; Bancroft and Hiebert, 1967; Hiebert et al., 1968). The proteins of CCMV and BMV have been assembled into RNA-free capsids at pH 6.0 or lower. It appears that under conditions where virus assembly occurs, the proteins of these viruses exist as dimers (Pfeiffer and Hirth, 1974; Adolph and Butler, 1976, 1977), as does AMV protein.

In contrast to the results obtained with the helical viruses, such as TMV and TRV, the spherical viruses such as CCMV and BMV appear to have very small protein aggregates as their assembling units. This indicates that although disk formation may make helix nucleation mechanically more favourable, it is not a requirement on the basis of the minimum size of protein aggregate that can be involved in initiation.

1.3.5 Specificity

The importance of specificity in plant virus assembly is not always demonstrated. Some reconstitution reactions are specific while others are not (e.g. spherical plant viruses such as CCMV (Bancroft, 1970)). With some viruses a certain lack of specificity has even been demonstrated in vivo. The mixed assembly, in vivo and in vitro of plant viruses has been reviewed by Okada and Ohno (1976).

The specificity of TMV assembly has been clearly demonstrated
(for a review, see Atabekov, 1972). The reconstitution of PMV (Erickson et al, 1978) is also specific for the homologous or related RNA, while the reconstitutions of barley stripe mosaic virus (BSMV), potato virus X (PVX) (Atabekov et al, 1970) and CYMV (Bancroft et al, 1979) are not specific.

The need for specificity in the assembly of helical plant viruses is suggested by the homogeneity of RNA extracted from the purified viruses and the lack or rarity of pseudovirions.

The specificity of TMV assembly, and perhaps also PMV, appears to be linked to the interaction of the bilayer disk aggregate with a certain sequence on the RNA. This aspect of specificity in assembly (specific recognition between the coat protein and a particular nucleotide sequence) has been reviewed by Atabekov (1972).

The assembly of helical plant viruses is polar (i.e. assembly occurs mainly in a particular direction, growth in the second direction being limited or absent). With TMV, initiation occurs internally, near the 3' end of the RNA (Zimmern, 1976) but in slightly different locations in different strains (Fukuda et al, 1980). TRV assembly appears to begin at or near the 5' end of the RNA (AbouHaidar and Hirth, 1977). AbouHaidar and Bancroft (1980) have shown the PMV protein can begin assembly on both PMV-RNA and TMV-RNA at or near the 5' end. CYMV assembly also begins at the 5' terminus of the RNA (AbouHaidar, 1981).

Whether polarity of assembly results from a highly specific interaction or not is unclear. However, the polarity of reconstitution reactions that have been investigated suggests that some degree of
specificity may be involved.

Another argument for specificity has been discussed by Atabekov (1972) who felt that it may be required to ensure that assembly occurs in phase on the nucleic acid, so that the nucleic acid is completely coated and no gaps occur. The validity of this argument is unclear.

It may be argued that initiation might occur at a particular site as a result of the secondary structure of the nucleic acid although this would not seem to be the case with TMV-RNA in view of the results obtained using PMV protein (AbouHaidar and Bancroft, 1980). However, the secondary structure of the RNA might be a factor, under conditions that produce specific reconstitution, which directs initiation to a particular site.

The precise reasons why assembly is usually specific are not known. Although it is an important requirement for the efficient production of virus, it may not always result from a specific protein-RNA interaction. It may simply occur, in vivo, because no other RNA is present at the assembly site.

1.4 Helical plant virus assembly

The helical plant viruses fall into two groups: the rigid helical plant viruses, including TMV, TRV and BSMV, and the flexuous helical plant viruses, which include the potexviruses (the family under investigation in this thesis), the potyviruses, the carlaviruses and the clavdiviruses.
The first virus to be successfully reassembled from its purified coat protein and nucleic acid was TMV (Fraenkel-Conrat and Williams, 1955). This reconstitution was considered successful because the particles produced were infective in the presence of a nuclease. TRV, on the other hand, has not been properly reassembled. The particles were uninfected (Semancik and Reynolds, 1969) or the encapsidation was incomplete (AbouHaidar et al, 1973). The reconstitution of BSMV produced virus-like particles but they were not infective (Atabekov et al, 1970).

Among the flexuous helical plant viruses, the reconstitution of PMV (Erickson and Bancroft, 1978) and CYMV (Bancroft et al, 1979) both produced infective virus-like particles. PVX has been reassembled to produce virus-like particles, with low and variable infectivity (Goodman et al, 1975, 1976), although the significance of the infectivity assays is unclear because they were not compared to that of purified RNA. Narcissus mosaic virus (NMV) has been reassembled into virus-like particles but infectivity was not examined (Bancroft et al, 1980). The reconstitution of PVY did not produce infective particles (McDonald and Bancroft, 1977).

The proteins of some helical plant viruses have been assembled into nucleic acid-free rod-shaped particles indicating that, for some viruses, protein-protein interactions are important to capsid formation. They are obviously most important to those viruses whose proteins can form virus-like particles in the absence of the nucleic acid. These include the proteins of TMV (Schramm, 1947; Franklin, 1955), TRV (Morris and Semancik, 1973; Fritsch et al, 1978), BSMV (Atabekov
et al., 1968), PMV (Erickson et al., 1976) and CYMV (Bancroft et al., 1979; Tollin et al., 1981).

The proteins of some helical plant viruses have not been assembled into virus-like helices, most notably those of PVX (Goodman et al., 1975, 1976; Kaftanova et al., 1975) and PVV (Goodman et al., 1976; McDonald et al., 1976), which aggregate into stacked rings. NMV protein has been shown to form stacked rings and 2-start helices (Bancroft et al., 1980). These results indicate that for these viruses, protein-RNA interactions are involved to some extent in directing the formation of a structurally correct capsid.

Some of the proteins that are able to form virus-like helices also form structures with abnormal geometry, under appropriate conditions. PMV protein forms rods which have a deviation in subunit position in the virus-like particle, so that morphological subunits can be seen (Erickson et al., 1982). CYMV protein forms stacked-ring tubes and, under the same conditions that result in virus-like helices, also forms wide particles with 3-4 extra subunits/turn of the helix (Bancroft et al., 1979). BSMV protein forms a double-helical structure and stacked disks (Kiselev et al., 1969), in addition to virus-like particles. TMV protein has been observed in stacked disks (Franklin and Commoner, 1955; Finch and Klug, 1971). The occurrence of tubular polymers in addition to virus-like helices points to the significance of the environmental conditions in promoting the correct protein-protein interactions for capsid formation. Although the information for this process may be contained within the subunit, other interactions may occur, depending on the environment. The specific nature of the
protein-protein interactions may be probed by manipulating the environmental conditions. In addition, these manipulations may reveal something of the relative importance of protein-protein interactions and protein-RNA interactions in forming and stabilizing a virus particle (Boatman, 1972).

1.4.1 Environmental conditions

The important interactions (protein-protein or protein-RNA) for virus assembly and protein polymerization are established by experimentally defining the optimal conditions for these processes. Although this thesis is primarily concerned with the polymerization of coat proteins, the effect of the environmental conditions on virus reconstitution will also be outlined here.

The reconstitution of TMV from its components occurs most efficiently at pH 7.25 in 0.1 M pyrophosphate buffer at 30°C (Fraenkel-Conrat and Williams, 1955; Fraenkel-Conrat and Singer, 1959). The "C" strain of TRV also assembles best near pH 7 (pH 7.5-8.0) in 0.25 M glycine (Morris and Semancik, 1973) but the CAM strain assembles most effectively at pH 4.7 in 0.5 M phosphate buffer (AbouHaidar et al., 1973). Both strains have a low temperature requirement (1-4°C) for their assembly in contrast to all other helical viruses which assemble in endothermic and therefore apparently, entropy-driven reactions. BSMV assembles best near pH 7.0, 30°C but requires 0.01 M tris-HCl (Atabekov et al., 1970), a lower ionic strength than that optimal for TMV and TRV assembly. The reconstitution of PVX is optimal at or slightly below neutrality (pH 6.0-6.2 (Goodman et al., 1975) or pH 6.5-7.0 (Kaftanova et al., 1975))
at low ionic strength (0.001-0.01 M buffer) and 20°C. PMV assembly is most efficient in 0.01 M tris, pH 8.0 at 25°C (Erickson and Bancroft, 1978) and that of CYMV at pH 7.0-7.5 (0.01 M tris) at 25°C. The reconstitution of NMV has been performed at pH 7 (0.01 M tris) (Bancroft et al, 1980). PVY assembles optimally at pH 7 to 8 at 20°C and low ionic strength (0.01 M MES) into short particles (McDonald and Bancroft, 1977).

In summary, while TMV and TRV assemble best at moderate to high ionic strength, BSMV and all of the potexviruses thus far examined require low ionic strength to assemble. Apart from the CAM strain of TRV, all other helical viruses assemble optimally at pH 7 ± 1. With the exception of TRV, reconstitution occurs most efficiently at 20-30°C, in what appears to be an entropy-driven process.

The coat proteins of the helical viruses that have been studied do not polymerize under conditions optimal for virus reconstitution. TMV protein assembles at acidic pH levels (at or below pH 6.5) (Läuffer and Stevens, 1968; Durham et al, 1971; Durham and Klug, 1971), unlike the virus, although both require moderate temperatures and ionic strength. TRV ("C" strain) protein polymerization requires higher temperature and lower ionic strength (0.001 to 0.1) than does reconstitution (Semancik and Reynolds, 1969; Morris and Semancik, 1973). Both processes occur at slightly alkaline pH levels. BSMV protein assembles upon storage at low temperature at pH 7.5-9.0, in low ionic strength buffer that also contains divalent cations up to a concentration of 0.1 M (Atabekov et al, 1968). PVY protein polymerizes at pH 6.0-9.0.
(Goodman et al., 1976, McDonald et al., 1976) at 4°C, in contrast to the reconstitution reaction which occurs at 20°C (McDonald and Bancroft, 1977). CPMV protein assembles at pH 4 (0.01 M citrate) at 20°C (Erickson et al., 1976), compared to virus assembly which occurs best at pH 8, 25°C (Erickson and Bancroft, 1978). CYMV protein polymerizes at pH 5.0-5.5 and pH 8, whereas reconstitution occurs at pH 7.0-7.5 (Bancroft et al., 1979). The temperature and ionic strength were similar for both reactions. PVX protein has been observed to form stacked disks by Kaftanova et al. (1975) but only at ionic strength (0.2 M buffer) higher than that suitable for reconstitution (0.001 to 0.01 M buffer). With NMMV protein in 0.1 M citrate, 0.1 M (NH₄)₂SO₄, pH 5, 2-start helices form. The pH is lower and the ionic strength higher than that necessary for reassembly of the virus (Bancroft et al., 1980).

The conditions for the reconstitution of helical viruses vary considerably with respect to their temperature and ionic strength requirements. However, the greatest variations occur with respect to the conditions for the polymerization of their proteins. This reflects the differences in the relative importance of the two major types of interaction (protein-protein and protein-RNA) and the exact nature of these interactions.

1.4.2 Sub-assembly aggregates

The behaviour of viral coat proteins in the absence of nucleic acid
is examined in attempts to determine the types of sub-assembly aggregates that may be involved, not only in the assembly of the protein helices but also in the assembly of the viruses. Much has already been elucidated about the construction of two helical viruses in terms of the mechanics and the specificity of protein-RNA interactions (see sections 1.3.4 and 1.3.5).

The assembly of TMV begins with the interaction between a two-layered protein disk and a particular nucleotide sequence about 1000 bases from the 3' end of the RNA (Zimmern, 1976). This initiation step occurs only with the double disk, whereas elongation can proceed with smaller, less ordered protein aggregates (Okada, 1975).

The initiation of TRV assembly requires the formation of the 36S protein disk aggregate (AbouHaidar et al., 1973; AbouHaidar, 1976). The question of similar initiation for BSMV has not been resolved, but its protein forms disks at pH levels above 6.3 (Gumpf and Hamilton, 1968), including the range (pH 7.2-7.5) where reconstitution occurs (Atabekov et al., 1970).

Since the entire RNA sequence of a virus could not confer specificity on assembly, the initiation step is clearly very important in this regard. What must be considered is that this specificity depends on two things: the nucleotide sequence involved and the protein aggregate that it must recognize.

The importance of the RNA sequence has been clearly demonstrated. The most recent example is in the initiation of PMV assembly. AbouHaidar (AbouHaidar and Erickson, 1983) has sequenced the first 40 nucleotides
after the 5'-terminal CAP of PMV-RNA. They consist of 8 consecutive repeating pentamers of N(C or A)AAA, presumably such that each pentamer binds to one protein subunit in each of the two rings in the 14S protein aggregate (Erickson et al, 1983). This double disk is one of the predominant aggregates and is implicated in the initiation process for both PMV and CYMV, although its requirement has not been demonstrated.

It has been argued (for example, see AbouHaidar and Erickson, 1983) that a disk provides the energetically most efficient way to start a helix, based on the number of protein-RNA and protein-protein bonds in the initiation complex. However, as mentioned in section 1.3.4, results obtained with the proteins of AMV (Driedonks et al, 1976, 1977) indicate that the sub-assembly aggregate for their capsid assembly is a protein dimer. This implies that a stable initiation interaction can occur between a nucleotide sequence and a protein aggregate much smaller than a disk. The results to be presented in this thesis will support this implication since not all of the potexviruses appear to form disk-like aggregates.

1.4.3 Non-equilibrium protein polymerization

Most of the experiments that are conducted in protein and virus assembly studies are done with equilibrium mixtures, primarily so that reproducible results may be obtained. The behaviour of TMV protein is affected not only by the environmental conditions but also by the rate of their change and the history of the protein sample. This accounts for hysteresis because the products formed after a pH or temperature change are not necessarily the equilibrium products of
the final state.

Long helical rods of TMV protein, at equilibrium, form only at acidic pH levels. At alkaline pH levels, small aggregates (4S) form, which probably consist mainly of trimers. At or near neutrality, the protein exists as a mixture of 4S and 20S (disks) species (Klug, 1972). For example, at pH 7.1, 20°C, the equilibrium mixture consists of 32% as 4S and 68% as 20S material (Schuster et al., 1979).

If a mixture of 4S and 20S TMV protein at pH 7.1, 20°C, is subjected to a rapid pH drop to pH 6.4-6.7, long rods form, which sediment at 100-130S (Schuster et al., 1979). These slowly depolymerize. This phenomenon of rapid polymerization, which was followed by gradual depolymerization, was termed overshoot polymerization (Scheele and Schuster, 1975). The rods depolymerized to 4S material, which then equilibrated with the 20S species. The final products are short rods that consist of 3-7 turns (Schuster et al., 1979).

The magnitude of the overshoot depends on the extent of the pH perturbation, the initial concentration of 20S material and the thermal history of the sample (Schuster et al., 1979), mainly because this affects the proportions of 4S and 20S protein.

Overshoot polymerization also occurs upon rapid heating from 0°C to 20°C (in 1-3 minutes) at pH 6.5. The magnitude of the overshoot depends on the magnitude of the temperature change (Schuster et al., 1979).

If the heating rate is slower (0.003-0.1 deg/min), then the formation of a metastable 30-35S species occurs (Shire et al., 1979). It is the overshoot product of a 24S species, which corresponds to 3 turns and
probably represents the smallest helical aggregate of TMV protein.

Metastability occurs in the 20S disk at 6.5°C. Depolymerization into 4S material occurs with first-order kinetics and a half-time of 12 days (Shire et al., 1979). Metastability of the 20S species is also implicated at pH 7.0, 20°C upon dilution. Overshoot polymerization is due solely to the low reaction rate of the conversion of 4S material into 20S disks. Since this reaction represents nucleation for helical rod assembly, the extreme overshoot results because the rate of rod elongation, with 4S aggregates, is much faster than the rate of 20S nucleus formation.

The existence of a metastable form of the PMV protein disk (14S) has been suggested on the basis of hysteresis in the pH effects on PMV protein rods (AbouHaidar and Erickson, 1983). Hysteresis in the pH-induced depolymerization of TMV protein rods also occurs (Scheele and Schuster, 1975).

The metastability effects in both TMV and PMV proteins may be altered, depending on the history of the sample. This must be considered whenever reproducibility is difficult and sometimes, even when it is not.

1.5 Optical diffraction

Optical diffraction has been used to determine the structure of the tubular polymers of potexvirus proteins in this thesis. The principles of diffraction by helical structures will be outlined here. For a more detailed description, Sherwood (1976, Chapter 16) provides
an excellent discussion of the topic and has been used as the basis for this introduction.

1.5.1 Diffraction

Diffraction occurs whenever a wave front encounters an object and some of the incident waves scatter from the object. Each scattered wave has a characteristic amplitude and phase, which depend upon the properties of the scattering object and the direction in which the wave is scattered.

A diffraction pattern results from the superposition of waves scattered in a given direction from all points on the scattering object. At large distances, the amplitude and phase in the diffraction pattern represents the Fourier transform of the scattering object.

In optical diffraction the scattering object, which here is an electron micrograph negative of a tubular potexvirus protein polymer, is illuminated with the coherent beam of light from a laser. The optical diffraction pattern, or Fourier transform of the scattering object, appears in the back focal plane of a lens. The relationship of the maxima or reflections in the diffraction pattern to periodic structure in the scattering object make this a very useful technique for the analysis of regular structures in electron micrographs, even when noise resulting from irregular structure in the micrograph is also present.

The Fourier transform or diffraction pattern exists in reciprocal space with respect to the scattering object. This means that there
is a reciprocal relationship between distances in the periodic structure of the object and the spacing of strong maxima or reflections in the transform (i.e. the larger the distance in the object, the smaller the distance in the diffraction pattern).

1.5.2 The Fourier transform of a continuous helix

Figure 1A shows a continuous helix which may be described using Cartesian coordinates, \((x,y,z)\), or more usefully in polar coordinates \((r,\theta,z)\) (Fig. 2). The helix may be defined in terms of its radius, \(r\), and its pitch, \(P\) (the change in \(z\) for one complete turn, \(2\pi\), of the helix).

The theory of diffraction by helical structures was first published by Cochran et al (1952) and was independently worked out by Stokes (unpublished; cited by Klug et al, 1958).

An infinite continuous helix is the convolution of a single helical turn, with a radius \(r\) and a pitch \(P\), and in infinite set of planes spaced at intervals of \(P\). The Fourier transform of such a helix is a convolution of the Fourier transform of the single turn and the Fourier transform of the set of planes. The result is that maxima occur only where the Fourier transform has non-zero values: only on layer lines at spacings of \(1/P\).

The cylindrical-polar coordinates in reciprocal space are \((R,\psi,\zeta)\) (see Figure 2), and

\[ \zeta = \pi/P \]

This simply expresses the fact that the transform has non-zero values
Figure 1.  (A) A continuous helix.  (B) A discontinuous helix.
Figure 2. Cylindrical-polar coordinates used to describe a point on a helix. (A) $(r, \theta, z)$ in real space. (B) $(R, \psi, z)$ in reciprocal space. Modified from Cochran et al (1952).
only at multiples of $1/P$ (i.e. $n/P$).

The Fourier transform of an infinite continuous helix may be written

$$F(R, \psi, n/P) = r_0 J_n(2\pi r_0 R_n) \exp[i\pi(\psi + \pi/2 - \theta_0)]$$

For a derivation, see Cochran et al (1952) or Sherwood (1976; Ch. 16).

The exponential term represents the phase component in the Fourier transform and $r_0 J_n(2\pi r_0 R_n)$ represents the amplitude component. The square of the amplitude, the intensity, is the quantity observed in the diffraction pattern.

The amplitude, therefore, is dependent upon the Bessel function of $X$ of order $n$, $J_n(X)$ where $X = 2\pi r_0 R_n$, so that the Bessel function may be written $J_n(2\pi r_0 R_n)$.

The Bessel functions are illustrated in Figure 3 and consist of a series of maxima and minima. As the order, $n$, increases, the first maximum occurs at increasingly greater values of $X$ or $2\pi r_0 R_n$ and with decreasing amplitude.

On a diffraction pattern (refer to Fig. 4), this means that maxima contributed by lower order Bessel functions will appear at low values of $R_n$ (i.e. close to the meridian) and will have high intensity. As $R_n$ and therefore $2\pi r_0 R_n$ or $X$ increases, the first maximum will appear further from the meridian and have lower amplitude (and intensity). Only the zero-order Bessel function, $J_0(X)$ or $J_0(2\pi r_0 R_n)$, has a non-zero value at $2\pi r_0 R_n = 0$ (i.e. on the meridian). Moody (1967) lists the positions of the first maxima for Bessel functions of order 0 to 14, for helical structures.
Figure 3. Illustration of Bessel functions, $J_n(x)$ or $J_n\left(2\pi R_n r\right)$. From Sherwood (1976).
Figure 4. Typical appearance of the optical diffraction pattern of a helical structure. From Richardson et al (1981).
Since maxima are restricted to layer lines where $\zeta = \pi/P$ and on a given layer line the intensity distribution is determined by the Bessel functions, the diffraction pattern of an infinite continuous helix will have a characteristic X-shape, which is illustrated in Figure 5, and be symmetric about the equator. Although an infinite number of layer lines are theoretically possible, only a few would actually be observed.

1.5.3 The Fourier transform of a discontinuous helix

A discontinuous helix consists of a set of scattering points at equi-distant spacings on a continuous helix (Fig. 1B). The discontinuous helix represents the convolution of a continuous helix and a set of planes spaced at intervals of $p$, which corresponds to the distance along the $z$ axis between successive points on the helix (i.e. the discontinuous helix represents the intersection of the two) (see Fig. 6). Consequently the Fourier transform of a discontinuous helix is a convolution of the Fourier transform of a continuous helix and the Fourier transform of the set of planes spaced at intervals of $p$.

The Fourier transform of the discontinuous helix exists in reciprocal space with respect to the original helix and, hence, distances in the helix and its transform have a reciprocal relationship. The spacing, $p$, between points on the helix is less than the pitch, $P$, since there is more than one point in one turn of the helix. Consequently, on the transform $1/P < 1/p$.

The diffraction pattern of a discontinuous helix has a multiple X
Figure 5. The diffraction pattern of an infinite continuous helix. The layer lines are spaced at intervals of 1/P, the reciprocal of the pitch. From Sherwood (1976).
Figure 6. A discontinuous helix. (A) A discontinuous helix consists of a series of points spaced at intervals of p. The discontinuous helix is a convolution of (B) and (C). (B) A continuous helix with the same pitch, P, as the discontinuous helix. (C) A set of planes spaced at intervals of p. From Sherwood (1976).
pattern (i.e. it represents a convolution of the X-shape from the transform of a continuous helix and a set of planes spaced at intervals of 1/p). The X-shapes therefore occur at spacings of 1/p on the transform of a discontinuous helix (Fig. 7) and the layer lines are spaced at intervals of 1/P. Successive X-patterns are spaced at intervals of m/p, where m is an integer.

If 1/p is small, then the X-patterns from successive origins (m=-1, m=0, m=+1, etc.) will overlap (see Fig. 8A). Once again, the intensity decreases as the order of Bessel function increases. It should be noted that, in practice, only a small portion of the central region of this pattern would actually be observed.

If the discontinuous helix repeats in one turn (i.e. has an integral number of points or units/turn), then the layer lines are spaced at intervals of 1/P, as in Figure 8, and maxima may occur on every layer line. Successive origins are spaced at 1/p.

If the discontinuous helix does not repeat in one turn (i.e. has an integral number of points in a distance, c, where c is the true repeat characteristic of the helix), then the layer lines are spaced at intervals of 1/c (see Fig. 9). For a given origin, m, there will be c layer lines between maxima or reflections. Maxima will not occur on every layer line. The number of layer lines between successive origins will equal the number of points, u, in the true repeat (u layer lines in 1/p). Since there are c layer lines between successive maxima from a given origin, reflections from successive origins will coincide at spacings of u/p. (Note that they coincide for every layer line when c=P; Fig. 8).
Figure 7. The diffraction pattern of an infinite discontinuous helix. Successive origins are spaced at intervals of 1/p. Layer lines are spaced at intervals of 1/P. From Sherwood (1976).
Figure 8. Diffraction pattern of a discontinuous helix with 9 points in 1 turn.
ORDER OF BESSEL FUNCTION, $J_n(x)$
Figure 9. Diffraction pattern of a discontinuous helix with 26 points in 3 turns.
ORDER OF BESSEL FUNCTION $J_n(x)$
1.5.4 Helical selection rule

Any discontinuous helix is said to have a constant true repeat (c), which is characteristic of that helix. The true repeat is the vertical distance which contains an integral number of units. The true repeat distance contains \( u \) subunits in \( t \) turns. The spacing of layer lines on the diffraction patterns is \( 1/c \) and layer lines may be indexed by a layer line number, \( z \), which has units \( 1/c \). The integer representing the origin is \( m \) and \( n \) is the order of the Bessel function \( (J_{n}2\pi r_{0}R_{n}) \).

- The selection rule for a helical structure is

\[
z = tn + um
\]

(Cochran et al, 1952; Franklin and Klug, 1955; Klug et al, 1958). This selection rule relates the layer line number, \( z \), to the order, \( n \), of the Bessel function, which are the coordinates of the axes on the diffraction pattern, to predict allowable maxima for a given structure, where that structure is defined in terms of the helix with the smallest pitch within it.

1.5.5 The diffraction pattern of a helical biological structure

A helical biological structure, such as a helical plant virus, is more complex than a discontinuous helix which simply consists of atoms arranged in a helix. If a structure such as a virus consists of repeating monomers arranged in a helix, then every atom in a subunit is related to the equivalent atom in all other subunits by helical symmetry. The entire polymer is therefore the sum of many discontinuous helices. Its diffraction pattern is therefore the sum of many superposed
transforms originating from different sites in the monomers. The characteristics, such as pitch, true repeat, layer line spacings, of all these helices are identical. Therefore the only difference in the X-shaped patterns from these helices is in the precise position of the maxima. This varies because the radii of the helices are not the same. Since the variation in \( r \) for each atom will be very small, this has very little effect on the appearance of the diffraction pattern, just making the individual maxima somewhat smeared.

1.5.6 Analysis of the optical diffraction pattern from a helical object

The typical appearance of the optical diffraction pattern from a helical structure is shown in Figure 4. Information is obtained by measuring \( \xi \) and \( R \) for each reflection or maximum visible on the transform. A single helix produces a strong reflection near the meridian with \( \xi_1 = 1/P \) and another near the equator with \( \xi_n = 1/c \). Therefore, the number of turns in the true repeat of the helix equals \( \xi_1/\xi_n \). The order, \( n \), of the Bessel function contributing to each reflection may be determined from \( R \) using Moody's tables (Moody, 1967), if the particle radius is known. Moody's tables take into account different types of distortion (e.g. flattening) which may occur when the stain dries. For a single helix, the near-meridional reflection can be shown to originate from a \( J_1 \) Bessel function (i.e. 1-fold rotation axis exists). The near-equatorial reflection originates from a higher order Bessel function, where \( n \) equals the integral number of subunits/turn. The precise fractional value can be determined using the selection rule, if \( m \) is known. In our case, \( m \) will be either +1 or -1. It can be determined from a one-sided transform (i.e. a transform which contains only one pair of each reflection, unlike that represented in Figure 4, which contains 2 pairs of each reflection). The difference results from the
negative stain penetrating either one or both sides of the particle, respectively. Hence, the term one-sided is used. If the near-meridional and near equatorial reflections occur on the same side of the meridian, then \( m = -1 \). If they occur on opposite sides of the meridian, then \( m = +1 \) (see Richardson et al., 1981). The sign of \( m \) indicates whether the fractional number of subunits/turn lies above or below the integral value.

1.5.7 Multiple helices

A multiple helix has a parallel N-fold rotation axis. An example, 2 parallel helices or a 2-fold rotation axis, is shown in Figure 10A. The characteristics of each helix (e.g. pitch, true repeat, layer line spacing) are identical. Therefore, the layer lines on which reflections or maxima occur are the same as they would be for a single helix with the same characteristics. The N-fold rotation axis affects the order, \( n \), of the Bessel functions contributing to those maxima and, as a result, the maxima occur further from the meridian than they would for a single helix with the same characteristics. For example, a single helix would show a \( J_1 \) reflection at \( \frac{\pi}{2N} \), whereas a triple helix with the same characteristics would produce a \( J_3 \) reflection on the same layer line.

The selection rule for a multiple helix is

\[
\ell = \pi nN + \pi m,
\]

where the order of the Bessel function contributing to the maximum on the \( \ell \)th layer line is \( \pi mN \) (rather than \( n \)). The use of this selection rule must be based upon the helix with the smallest pitch.
Figure 10. Radial projection and \((\eta, \lambda)\) plot. (A) Radial projection of a 2-start helix. There are 2 parallel helices in register and a 2-fold rotation axis. There are 8 1/2 subunits/turn. The \((\eta, \lambda)\) coordinates, determined according to the method of Klug et al (1958) (see Section 1.5.10), are marked on the diagram for several sets of grooves. (B) The \((\eta, \lambda)\) plot or Fourier transform of (A).
1.5.8 Calibration

Quantitative measurements of the scale of periodic structure in the scattering object can only be made from the diffraction patterns if some method is available for converting distances on the pattern (in reciprocal space) into real distances on the object. This is usually done by diffracting an electron micrograph (at the same magnification) of a catalase crystal (Luftig, 1967; Misell, 1978; Wrigley, 1968) as a standard.

1.5.9 The object and the mask

The object in optical diffraction consists of an electron micrograph negative, for example of a viral protein polymer that has been stained with a heavy metal stain such as uranyl acetate. The particle has its own periodic structure which produces regular grooves in its surface. The negative stain settles in between the protein subunits, in the grooves, and the periodic surface structure is repeated in the stain distribution.

The stain can penetrate both the upper and lower surfaces of the particle. Therefore, depending on the distribution of stain, a diffraction pattern may contain information from one or both sides of the particle and is referred to as either a one-sided or two-sided pattern (see section 1.5.6).

When the stain dries on the particle surface, it usually shrinks to some extent. If this does not occur uniformly, then it will produce asymmetry in the position of reflections on the diffraction pattern.
The background on the electron micrograph produces random noise on the transform. In order to keep the signal-to-noise ratio at a maximum, the background around the particle to be diffracted is masked with opaque tape. The diffraction pattern of this mask shows high intensity on the equator and the meridian, near the origin (see Fig. 4). Periodic structure on the particle produces additional strong reflections, in comparison to the background noise.

1.5.10 The use of radial projections of models

If the object being diffracted is fairly simple, then its structure may be determined by enumerating $m$ and the $(n,\xi)$ coordinates and using the helical selection rule. With more complex helical structures, it is sometimes easier to work with models of potential structures. The $(n,\xi)$ coordinates of each reflection on the experimental diffraction pattern are compared to those that will arise from the proposed models, and the most compatible model is selected. This technique is especially useful since there is usually a certain variation in $n$ for each reflection, which makes deduction of the structure difficult.

The arrangement of subunits on the surface of a helical particle is called the helix lattice or net. This lattice is represented on the radial projection, which is a diagram of an unrolled particle.

Klug et al (1958) presented a simple method for determining the $(n,\xi)$ coordinates of possible diffraction maxima, from a radial projection of a model. First, the crystallographic repeat or basic rectangle (the smallest repeating arrangement of subunits) must be
determined. Within this basic rectangle, several sets of grooves or parallel lines are apparent. The number of times that a given set of grooves intercepts the horizontal edge of the basic rectangle equals the \( n \) coordinate of the reflection produced by that set of grooves. Similarly, the number of times the vertical edge of the basic rectangle is intercepted by the grooves equals their \( \varepsilon \) coordinate. For an example, see Figure 10A.

The transform of the model may then be represented by plotting \( n \) versus \( \varepsilon \) (Fig. 10B) or, more accurately, by plotting \( 2\pi r_n \) versus \( \varepsilon \). The \( 2\pi r_n \) values can be obtained, for the appropriate orders of \( n \), from Moody's table (Moody, 1967). At this point, the model can easily be compared to the optical diffraction pattern of the object with unknown structure. This technique is employed in Chapter 5.

1.6 The Potexviruses

The potexviruses represent a family of plant viruses that have many physical characteristics in common. They consist of flexuous rod-shaped particles containing a monopartite single-stranded RNA genome. The particles are 470-580 nm long, 14 nm in diameter with helical symmetry. The helix has a pitch of 3.5 nm, with slightly less than 9 subunits/turn (Richardson et al, 1981).

The nucleic acids of these viruses are about \( 2.0 \times 10^6 \) d in weight. The viral capsids are all composed of a single type of protein, each subunit weighing between 18,000 and 27,000 d (Koenig and Lesemann, 1978). Serological relationships between these viruses are quite distant and
their host ranges also differ fairly extensively (Lisa and Dellavalle, 1977; Koenig and Lesemann, 1978).

PVX is the type virus of the family. The definitive members include viola mottle virus (VMV) (Lisa and Dellavalle, 1977; Koenig and Lesemann, 1978) and tulip virus X (TVX) (Radwan et al., 1981). BCV (Attatham et al., 1978) and foxtail mosaic virus (FTV) (Paulsen and Niblett, 1977) are putative members of the potexvirus family (Richardson et al., 1981).

1.7 Goals of the research

The goal of this research is to determine whether the potexviruses, which are architecturally very similar (Richardson et al., 1981), share a common assembly pathway. The behaviour of the coat proteins of several potexviruses (BCV, FTV, VMV, PVX and TVX) is examined to determine the nature of the sub-assembly processes and the stabilizing interactions in assembly, as well as the size of the sub-assembly products. The structure of the tubular polymers of these proteins is also determined.
CHAPTER 2
MATERIALS AND METHODS

2.1 Growth of viruses

Stocks of desiccated leaf tissue infected with BCV, VMV, FTV and TVX were kindly provided by L. G. Weathers, Vittoria Lisa, C. L. Niblett and Brian Harrison, respectively. BCV, VMV and TVX were grown in Chenopodium quinoa. FTV was grown in Hordeum vulgare and PVX was grown in Nicotiana glutinosa.

Infected leaf tissue, fresh or desiccated, was ground in water and rubbed on leaves that has first been sprayed with aluminum oxide. The plants were then watered liberally. The leaves were harvested 2-3 weeks after inoculation. They were frozen at -20°C, if not required immediately.

2.2 Purification of BCV

Chilled fresh or frozen leaves were homogenized in a Waring blender at 4°C, with 2 volumes (w:vol) of 0.02 M borate buffer, pH 8.0, containing either 0.01 M ascorbic acid or 0.1% (w:vol) sodium sulfite. The mixture was emulsified with two volumes of a 1:1 (vol:vol) butanol: chloroform mixture. The mixture was then centrifuged at 9500 rpm for 20 minutes in a GSA rotor (Sorval). The upper aqueous phase (above the solid layer) was retained. Polyethylene glycol 6000 was added to 3% (w:vol), to precipitate the virus. The mixture was stirred gently in the cold for 1 hour. Centrifugation at 9500 rpm for 20 minutes was repeated. The pellets, well drained, were resuspended overnight in cold 0.01 M tris, 1 mM EDTA pH 8.0. The pellets were combined and stirred

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gently in the cold for 1 hour to complete resuspension. The vir
solution was centrifuged at 10,000 rpm for 10 minutes in an SS-34 rotor
(Sorvall) to remove cellular debris and the supernatant liquid decanted.
The pellets were resuspended and the above centrifugation was repeated.
The combined supernatant liquid was layered onto a cushion of 30%
(w:vol) sucrose in 0.01 M tris, 1 mM EDTA, pH 8.0 and centrifuged at
27,000 rpm for 4-5 hours in a pre-cooled Type 30 rotor (Beckman). The
pellets were resuspended in 0.01 M tris, 1 mM EDTA pH 8.0 and the above
cycle of centrifugation was repeated. The final pellets were reuspended
in a small volume of 0.01 M tris, 1 mM EDTA, pH 8.0 to a concentration of
approximately 10 mg/ml. The concentration was determined spectrophotomet-
ically using \( \varepsilon_{260nm}^{0.1\%} = 2.0 \). This was determined from dry weight measure-
ments. Routine yields of 0.5-1.0 mg of BCV/g of fresh tissue were obtain-
ed. Sodium azide (1 mM) was added for storage of the virus at 4°C.

2.3 Purification of VMV

VMV was purified by the butanol:chloroform method used for BCV.
Precipitation with polyethylene glycol 6000 was omitted since irrevers-
ible precipitation occurred with this virus, which substantially reduced
the yield. In between the first and second low-speed centrifugations,
the supernatant liquid was left at 4°C overnight. This produced much
cleaner virus pellets than if storage was omitted. Yields of 0.5-1.0
mg of VMV/g of fresh tissue were obtained. The concentration was de-
termined spectrophotometrically using \( \varepsilon_{260nm}^{0.1\%} = 3 \). Sodium azide (1 mM)
was added for storage of the virus at 4°C.
2.4 Purification of FTV and TVX

FTV and TVX were purified by the butanol:chloroform method used for BCV. The precipitation with polyethylene glycol 6000 was omitted. The pellets were resuspended in 0.01 M glycine-NaOH, 1 mM EDTA, pH 10.0 for FTV and 0.01 M tris, 1 mM EDTA, pH 8.0 for TVX. Yields of 0.5 mg of FTV and TVX/g of fresh tissue were obtained. The virus concentrations were determined spectrophotometrically using $\text{E}_{260nm}^{0.1\%} = 3$. Sodium azide (1 mM) was added for storage of the viruses at 4°C.

2.5 Purification of PVX

The chilled leaves were homogenized in a Waring blender with 2 volumes (w:vol) of 0.05 M sodium phosphate buffer, 0.01 M EDTA, pH 7.6. The homogenate was squeezed through 2 layers of cheesecloth and the liquid was retained. The liquid was centrifuged at 15,000 rpm for 20 minutes in an SS-34 rotor (Sorvall) and the supernatant liquid was collected. With stirring in the cold, triton X-100 (Baker Chemicals) (0.5% vol:vol) was added. The mixture was stirred at 4°C for 1 hour and then layered onto a 30% (vol:vol) sucrose: 0.05 M sodium phosphate, 1 mM EDTA, pH 7.6 cushion. It was centrifuged at 27,000 rpm in a precooled Type 30 rotor (Beckman) for 4-5 hours. The pellets were resuspended overnight at 4°C in 0.05 M phosphate buffer-1 mM EDTA, pH 7.6 and then the cycle of differential centrifugation was repeated as before. Final resuspension of the pellets gave a virus concentration of about 10 mg/ml. Yields were about 0.2 mg of PVX/g of fresh tissue. Virus concentration was determined spectrophotometrically using $\text{E}_{260nm}^{0.1\%} = 2.97$. 
2.6 Isolation of viral protein

Equal volumes of virus solution, at a concentration of not more than 10 mg/ml, and 4.0 M LiCl-0.01 M EDTA, using the buffers mentioned in the purification procedures, were combined. The tubes were immediately sealed with Parafilm, shaken to mix, and stored at -20°C for at least 48 hours. The samples were thawed on ice for 1 hour and checked for precipitation of the RNA and lack of birefringence, which indicate that the virus has dissociated. The precipitated RNA was removed by centrifugation at 10,000 rpm for 10 minutes in an SS-34 rotor (Sorvall). The supernatant liquid was decanted and centrifuged at 27,000 rpm for 4-5 hours in a pre-cooled Type 30 rotor (Beckman). This removed undissociated virus particles. The quality of the protein was then checked by scanning it in a UV spectrophotometer (Unicam 1800) from 220-340 nm. The LiCl was removed by dialysis against a large volume of distilled deionized water at 4°C. After removal of the LiCl, the UV spectrum of the protein was again checked.

This method of protein isolation was used, rather than the acetic acid method (Fraenkel-Conrat, 1957), since it gave satisfactory results for all proteins. When acetic acid was used to isolate BCV protein, the protein sometimes precipitated more easily in subsequent experiments than if it was isolated with LiCl. The quality of TVX protein, judged from its UV spectrum, was better when LiCl was used in the isolation then when acetic acid was used.

Protein concentrations were determined spectrophotometrically using $E_{280nm}^{0.1%} = 0.8$ for BCV protein, 1.0 for VMV, FTV, PVX and TVX proteins.
2.7 Concentration of viral protein

Viral protein was reversibly precipitated by the addition of saturated \((\text{NH}_4)_2\text{SO}_4\) to a concentration 10% higher than the point where the solution first became cloudy. The saturated \((\text{NH}_4)_2\text{SO}_4\) was added drop-wise and the solution mixed after each addition. The solution was kept on ice for 1 hour while the protein precipitated. It was then centrifuged at 4°C at 7,000 rpm for 7 minutes in an SS-34 rotor (Sorvall). The pellet was resuspended in a small volume of distilled deionized water. The protein solution was dialyzed against a large volume of water at 4°C.

The protein was further concentrated, when required, by placing the dialysis tubing, containing the protein solution, on a bed of crushed Carbowax (polyethylene glycol 20,000). The tubing was also covered with the crushed Carbowax. The volume was checked frequently and the sac was removed when the volume had decreased sufficiently. Sometimes the protein precipitated with the volume decrease and temperature increase, but this was reversible upon cooling.

2.8 The pH change of protein solutions

The pH of the protein samples was adjusted by one of two methods, termed "fast" and "slow" pH change.

For the fast experiments, FTV and PVX proteins were prepared in distilled water, in which they do not polymerize. VMV protein was prepared by dialysis against distilled water or \(10^{-4}\) M tris pH 8.0. It sometimes precipitated in water but this could be reversed by dialysis.
against $10^{-3}$ M tris pH 8.0 followed by $10^{-4}$ M tris pH 8.0. Alternatively, it could be prevented by dialysis against $10^{-4}$ M tris pH 8.0. VMV protein did not polymerize under these conditions. BCV protein was prepared first in distilled water and then dialyzed against $10^{-4}$ M glycine-HCl pH 3.5 at 4°C to depolymerize any rods that formed in the water.

The pH of the samples was adjusted to the desired pH by the addition of a small volume of 1 M buffer to the cold sample, followed by titration. The samples were loaded into the analytical ultracentrifuge immediately. Usually about 20-30 minutes elapsed between the pH change and full rotor speed, which gave the samples sufficient time to reach 20°C if required.

For the slow experiments, FTV and PVX proteins were prepared in distilled water and VMV protein was prepared in distilled water or $10^{-4}$ M tris pH 8.0. BCV protein was prepared by dialysis against 0.01 M glycine-HCl pH 3.5 at 4°C for 12-24 hours. Then the dialysis fluid was changed to the appropriate 0.01 M buffer for the final pH change. The samples were dialyzed overnight at 4°C and then for 48 hours at either 4°C or room temperature (24°C).

After this dialysis period, the samples were loaded into the analytical ultracentrifuge at the appropriate temperature.

2.9 Analytical ultracentrifugation

A Beckman Model E analytical ultracentrifuge equipped with Schlieren optics was used to analyze the protein products after various treatments. Sedimentation coefficients are expressed in Svedberg units, $S$, corrected
to water at 20°C. Photographs were taken on Kodak metallographic glass plates.

Cold runs were done using pre-cooled cells, rotor and chamber.

2.10 Preparation of hanging drops

Viral coat proteins were prepared as follows: BCV protein was in 0.01 M glycine-HCl, pH 3.5 at a concentration of 38 mg/ml; FTV, PVX and VMV proteins were in distilled water at concentrations of 7.0, 10.1 and 7.6 mg/ml, respectively.

Solutions of (NH₄)₂SO₄ at concentrations ranging from 0-50% saturated, in increments of 5%, were prepared as the mother liquor in 0.01 M MES at pH 6.0 and pH 7.0 (BCV only) or 0.01 M tris at pH 7.0. All solutions contained 1 mM sodium azide and the solutions of mother liquor were filtered sterilized.

The wells were filled with 1 ml of mother liquor. Equal volumes (5 ul) of protein solution and mother liquor were placed on coverslips that had been dipped in 1% dimethyl dichlorosilane : toluene (vol:vol), and air dried. Nonwettable micropipets were used. The upper surface of the tray around each well was lined with high-vacuum silicone grease. The coverslip was inverted over the well and pressed down on the grease to make the well air-tight. The tray of wells was stored in a temperature-controlled incubator.

Using this method, the concentration of (NH₄)₂SO₄ in the drop is initially half that of the mother liquor. As vapour diffusion proceeds,
the concentration of \((\text{NH}_4)_2\text{SO}_4\) in the drop gradually increases. It reaches equilibrium at some point just below the concentration of \((\text{NH}_4)_2\text{SO}_4\) in the mother liquor.

All samples are referred to in the text by the concentration of \((\text{NH}_4)_2\text{SO}_4\) in the mother liquor.

Before the wells were opened, the drops were examined for birefringence, using polarized light microscopy. The wells were usually opened after 2-3 weeks incubation, once crystals had appeared. A portion of the drop was removed and the crystals were crushed with a small glass rod. Mother liquor was used to dilute the samples for electron microscopy.

2.11 Optical diffraction

Electron micrographs, taken on a Philips EM201 electron microscope, of particles stained with 1% uranyl acetate, had magnifications of about 43,000 and 65,000. The pictures on the negatives were used as objects for optical diffraction. The diffractometer was described by Tollin et al (1979) and Richardson (1981). Straight particles were masked with opaque tape and diffraction patterns were observed on a television screen. Photographs of suitable patterns were recorded on Polaroid film. The size of the prints was suitable for use as masks for optical reconstructions. Reconstructions were recorded on the same type of film.
CHAPTER 3

THE BEHAVIOUR OF POTEXVIRUS COAT PROTEINS

3.1 Introduction

The behaviour of the coat proteins of several potexviruses (BCV, FTV, VMV and PVX) was compared in the absence of RNA to determine their assembly properties. The role of protein-protein interactions in the assembly and sub-assembly processes and the forces that regulate them were examined in two principal types of experiment: 1) incubation of the protein under various conditions of pH, temperature, protein concentration and ionic strength and the examination of soluble products using analytical ultracentrifugation and electron microscopy, and 2) incubation of the protein in hanging drops in vapour diffusion with various concentrations of \((\text{NH}_4)_2\text{SO}_4\) and the examination of the insoluble products using polarized light microscopy and electron microscopy. Structural analysis of large polymers was done with optical diffraction of electron micrographs.

The results will show that the potexvirus proteins do not appear to share a common assembly pathway since their subassembly products differ.

3.1.1 Experimental rationale

3.1.1.1 The formation of soluble products

Analytical ultracentrifugation was used in conjunction with electron microscopy to study the formation of soluble protein polymers.

The effect of pH on the state of aggregation of the coat proteins was examined to determine if pH-mediated steps were involved in the
assembly or sub-assembly processes. The pH range over which such events occur may give some indication of the amino acids involved based on their pKₐ values.

Two methods were used to change the pH in the experiments presented in this chapter. First, the pH was changed by the addition of a small volume of 1 M buffer to a final concentration of 0.01 M and titration (usually 0.1 pH units) to the desired pH level. This took about 30 seconds and was designated "fast pH change". It produces a sudden pH change and the products formed have had less time to reflect the effect of intermediate pH levels encountered in dialysis experiments. This method does not necessarily produce an equilibrium mixture. For example, non-equilibrium polymerization occurred with TMV protein after rapid changes in pH or temperature (Scheele and Schuster, 1975). It is referred to as "overshoot" polymerization because long metastable rods formed which slowly depolymerized as the mixture reached equilibrium (Schuster et al, 1979). This type of reaction will be discussed in Section 3.3.3.

The second type of experiment was designated "slow pH change". The pH changed gradually during dialysis against 0.01 M buffer at the appropriate pH. The entire pH change takes about 30 minutes and proceeds through intermediate pH levels before reaching the final pH. After 2-3 days of dialysis, the mixture reached equilibrium. The products may or may not be the same as those from a fast change. If the sub-assembly products are the same for both types of pH change, then the products will be the same. Depending on the kinetics of the reaction, the proportions of the products may differ. The sub-assembly products may differ with the rate of pH change. The final products will be minimum energy states and may or may not be the same for different intermediates at a given pH.
If the intermediate product represents an energy minimum, then polymerization will proceed no further.

The effect of temperature was examined by comparing the behaviour of each protein at 20°C and 4°C. These experiments may show whether or not the assembly or sub-assembly reactions are entropy-driven, as with TMV and PMV. Temperature will also affect the rate constants of the reactions. If this effect is significant, then experiments at low temperature may reveal sub-assembly products.

The effect of protein concentration on the sedimentation behaviour of the coat proteins was examined to determine whether the products observed in the pH experiments were involved in concentration-dependent equilibria.

The effect of ionic strength was examined using KCl. A high ionic strength generally stabilizes hydrophobic interactions and destabilizes salt or ionic interactions.

3.1.1.2 The formation of insoluble products

The formation of insoluble protein products was examined using vapour diffusion in hanging drops. Various concentrations of (NH₄)₂SO₄ were used. The increased ionic strength and dehydration will tend to stabilize hydrophobic interactions and destabilize ionic interactions. The hanging drops were incubated for several weeks and checked for the formation of birefringent products using polarized light microscopy. Samples were examined using electron microscopy.

The structure of any large products, soluble or insoluble, observed
using electron microscopy was determined with optical diffraction. The results show if the structure of the products is consistent with that of the viral capsids. The results of structural analysis are presented in the final chapter of the results.

3.2 The behaviour of BCV coat protein

3.2.1 Dissociation of BCV protein

The virus was degraded into protein and RNA in 2 M LiCl at -20°C. The precipitated RNA was sedimented by centrifugation and the LiCl was removed by dialysis against water at 4°C. The protein polymerized into rods during the 24 hr dialysis period. For experiments on the effect of different environmental conditions on rod formation, the starting material must not contain large aggregates. Since the protein polymerized into rods in water, experiments were done to determine whether it could be dissociated at high or low pH.

After dialysis against 0.01 M glycine - NaOH, pH 10.0 or 0.01 M glycine - HCl, pH 3.5, at 4°C overnight, the rods dissociated into slowly sedimenting aggregates (Fig. 11A), as they did after a fast pH change to 10^-3 or 10^-4 M buffers at these pH levels. This was confirmed using electron microscopy (Fig. 12).

After dissociation of the rods, the pH was changed to 7.0 or 8.0 by dialysis. Figure 11 (B and C) shows that polymerization occurred. In both cases, the protein polymerized into two species that sedimented at -100S and -200S. Dissociation at pH 10.0 resulted in slightly more heterogeneous peaks, especially at pH 8.0 (Fig. 11B, upper), than
Figure 11. Schlieren diagram showing the effect of dissociation pH on polymerization of BCV protein. The protein was first prepared in water.

(A) Upper: 7S product of protein that was dialyzed against 0.01 M glycine-NaOH, pH 10.0, at 4°C for 2 days; lower: 4S product of protein that was dialyzed against 0.01 M glycine-HCl, pH 3.5, at 5°C for 2 days.

(B) Upper: BCV protein was dialyzed against 0.01 glycine-NaOH, pH 10.0, at 4°C, overnight. It was then dialyzed against 0.01 M tris, pH 8.0 for 3 days at 24°C. The products are 102S, 123S and 234S, along with slowly sedimenting material; lower: BCV protein was dialyzed against 0.01 M glycine-HCl, pH 3.5, at 4°C overnight. It was then treated the same as the upper sample. The products are 119S and 182S, along with slowly sedimenting material.

(C) Upper: treated as in upper (B) except that the final dialysis was against 0.01 M MES, pH 7.0. Products are 110S and 199S; lower: treated as in lower (B) except the final dialysis was against 0.01 M MES, pH 7.0. Products are 111S and 180S.

Runs were done at about 20°C at a speed of 24,530 rpm for B and C, and 50,740 for A. Protein concentration was 2-3 mg/ml. Photographs were taken 9 minutes after reaching speed, with the exception of A which was after 19 minutes. Sedimentation is to the right.
Figure 12. Electron microscopy of BCV protein (A) stored in water and then (B) titrated to $10^{-4}$ glycine, pH 3.5. Bar represents 100 nm.
dissociation at pH 3.5. Thus, the protein could be depolymerized at either pH 3.5 or 10.0 and retain its ability to reassemble.

3.2.2 Effect of pH

The effect of pH on the polymerization of BCV coat protein was examined with fast and slow pH changes, as described in the Materials and Methods. The protein was usually dissociated at pH 3.5. The pH was then adjusted over the range of pH 3.5 to 10.0. The results were determined using analytical ultracentrifugation and electron microscopy.

3.2.3 Effect of a fast pH change

BCV coat protein in water was quickly adjusted to 10^{-4}M glycine-HCl, pH 3.5 at 4°C and then quickly adjusted to the desired final pH in 0.01 M buffer, at 20°C. The results of analytical ultracentrifugation are shown in Fig. 13 (A-D) and Table 1. The protein was unpolymerized (i.e. contained no large aggregates) at pH 3.5 and 10.0. Most extensive polymerization occurred at pH 6.0 to 9.0. The two major species present at these pH levels sedimented at ~100S and ~200S. All the protein polymerized at pH 6.0. Some unpolymerized material remained at pH 7.0 to 9.0, the amount being less at the lower pH. At pH 4.0 and 5.0, small amounts of unpolymerized protein were present but most of the protein was present as a species which sedimented at 86S. No ~100S or ~200S material formed at these pH levels.

Electron microscopy showed that rod-shaped particles formed at pH 4.0 to 9.0 but were longest at pH 6.0 and 7.0 (Fig. 14).
Figure 13. Schlieren patterns showing the effect of pH and temperature on the fast polymerization of BCV protein. The protein, prepared in water, was first depolymerized at 4°C by the addition of \(10^{-2} \text{M}\) glycine-\(\text{HCl}\) to a final concentration of \(10^{-4} \text{M}\) pH 3.5. The protein was then quickly adjusted to the desired final pH by the addition of 1 M buffer to a final concentration of 0.01 M. The final pH was adjusted at either 24°C (patterns on the left) or 4°C (patterns on the right).

(A) Upper: 7S species in 0.01 M glycine-\(\text{NaOH}\), pH 10.0; lower: 8S, 128S and 270S species in 0.01 M borate, pH 9.0. (B) Upper: 8S, 108S and 255S species in 0.01 M tris, pH 8.1; lower: 3S, 105S and 252S species in 0.01 M MES, pH 7.1. (C) Upper: 97S and 190S species in 0.01 M phosphate pH 6.0; lower: 3S and 87S species in 0.01 M citrate, pH 5.0. (D) Upper: 3S and 86S species in 0.01 M citrate, pH 4.0; lower: 4S species with a small amount of 7S material in 0.01 glycine-\(\text{HCl}\), pH 3.5. (E) Upper: 5S species in 0.01 M glycine-\(\text{NaOH}\), pH 10.0; lower: 6S species in 0.01 M borate, pH 9.0. (F) Upper: 6S and 111S species, and a small amount of 250S material, which flattened out after 8 minutes, in 0.01 M tris, pH 8.0; lower: 6S, 107S and 250S species in 0.01 M MES, pH 7.1. (G) Upper: 3S and 108S species in 0.01 M phosphate, pH 6.0; lower: 3S and 85S species in 0.01 M citrate, pH 5.0. (H) Upper: 3S and 79S species in 0.01 M citrate, pH 4.0; lower: 2S species in 0.01 M glycine-\(\text{HCl}\), pH 3.5.

Runs were done at temperatures of about 20°C (patterns on the left) or about 4°C (patterns on the right). Protein concentration was 2-3 mg/ml. Rotor speed was 24,630 rpm. Photographs were taken between 9 and 11 minutes after reaching speed, except (E), which was taken 17 minutes after reaching speed. Sedimentation is to the right.
Table 1. The effect of pH and temperature on the sedimentation of BCV coat protein

<table>
<thead>
<tr>
<th>pH Change</th>
<th>Fast</th>
<th>Slow</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>4°C</td>
</tr>
<tr>
<td>Temperature</td>
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<tr>
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<td>10</td>
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</tr>
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<td>9</td>
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<td>7</td>
</tr>
<tr>
<td>8</td>
<td>7,108,255</td>
<td>7,111,250</td>
</tr>
<tr>
<td>7</td>
<td>4,105,252</td>
<td>7,107,250</td>
</tr>
<tr>
<td>6</td>
<td>97,190</td>
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<td>4,86</td>
<td>4,79</td>
</tr>
<tr>
<td>3.5</td>
<td>4.7</td>
<td>2</td>
</tr>
</tbody>
</table>

* Species sedimenting at 6, 7 and 8S are considered one species and have been grouped as 7S. Similarly, 3, 4 and 5S species have been grouped together as 4S.

** pH may not be exact for fast pH change. See Figure 13 for the precise values.
Figure 14. Electron microscopy of BCV coat protein in 0.01 M phosphate, pH 6.0 after a fast pH change at 24°C. Bar represents 100 nm.
3.2.4 Effect of a slow pH change

BCV coat protein in water was first dialyzed against 0.01 M glycine - HCl pH 3.5 at 4°C and slowly adjusted to the desired pH levels at 24°C (see Materials and Methods). The results of analytical ultracentrifugation are shown in Figure 15A-D and Table 1. The protein was unpolymerized (7S) at pH 3.5, 9.0 and 10.0. At pH 8.0 most of the protein was unpolymerized (8S) but a small amount was present which sedimented at -100S. Sometimes, most of it polymerized into -100S and -200S species (Fig. 11B). At pH 7.0 some unpolymerized protein was present. The rest of the protein was present as two species which sedimented at -100S and -200S. At pH 6.0 most of the protein sedimented at -100S. No -200S material was present. At pH 5.0 a single species formed which sedimented at 32S, while at pH 4.0 two species were present which sedimented at 6S and 82S. However, in other runs two species were observed which sedimented at 6S and 46S at pH 5.0 and 4S and 47S at pH 4.0, indicating that the behaviour of the protein at these two pH levels was the same.

Electron microscopy showed long rods at pH 6.0 to 8.0 (Fig. 16). Short rods were observed at pH 4.0 and 5.0 and at pH 3.5, 9.0 and 10.0 no rods were seen.

The rate of pH change did not affect the protein at pH 7.0, where most of the protein polymerized into -100S and -200S material. At pH 6.0, 8.0 and 9.0, polymerization was less extensive after a slow pH change than after a fast one. This was most noticeable at pH 9.0 (Fig. 17). At pH 4.0 and 5.0, the species present were also dependent on the rate of pH change. A fast pH change results in most of the protein sedimenting at 87S, whereas after a slow pH change it sedimented at 30 - 50S. Electron
Figure 15. Schüeren patterns of BCV protein polymerized under various conditions of pH and temperature. All samples were dialyzed overnight, against 0.01 M glycine-HCl, pH 3.5, at 4°C. The samples were subsequently dialyzed against 0.01 M buffers at the listed pH levels for 3 days, at either 24°C (diagrams on the left) or 4°C (diagrams on the right).

(A) Upper: 7S species in 0.01 M glycine-NaOH, pH 10.0; lower: 7S species in 0.01 M borate, pH 9.0. (B) Upper: 8S and 102S species in 0.01 M tris, pH 8.0; lower: 7S, 106S and 214S species in 0.01 M MES, pH 7.0. (C) Upper: 7S and 97S species in 0.01 M phosphate, pH 6.0; lower: 32S species in 0.01 M citrate, pH 5.0. (D) Upper: 6S and 82S species in 0.01 M citrate, pH 4.0; lower: approximately 6S species in 0.01 M glycine-HCl, pH 3.5. (E) Upper: 3S species in 0.01 M glycine-NaOH, pH 10.0; lower: 3S species in 0.01 M borate, pH 9.0. (F) Upper: 7S species in 0.01 M tris, pH 8.0; lower: 7S and 90S species in 0.01 M MES, pH 7.0. (G) Upper: 7S and 70S species in 0.01 M phosphate, pH 6.0; lower: 24S species in 0.01 M citrate, pH 5.0. (H) Upper: 5S and 36S species in 0.01 M citrate, pH 4.0; lower: 2S species in 0.01 M glycine-HCl, pH 3.5. Runs were done at temperatures of 20°C, for the patterns on the left, and about 4°C, for the patterns on the right. Protein concentration was about 3 mg/ml. Photographs were taken between 9 and 14 minutes after reach-speed, with the exception of (E), which was after 24 minutes. (A), (E) and (H) were run at 50,740 rpm and all others were at 24,630 rpm. Sedimentation is to the right.
Figure 16. Electron microscopy showing the effect of pH on the polymerization of BCV protein, after a slow pH change at 24°C. (A) The longest rods were present at pH 7.0. (B) No rods formed at pH 3.5. (C) Short rods formed at pH 4.0. Bar represents 200 nm.
Figure 17. Electron micrographs showing the effect of the rate of pH change at pH 9.0. (A) After a fast pH change. (B) After a slow pH change. Bar represents 200 nm for (A) and 100 nm for (B).
microscopy showed that longer rods were present after a fast pH change than after a slow pH change (Fig. 18).

3.2.5 Rate of polymerization

Since the rate of pH change appeared to have no effect at pH 7.0, where polymerization at 20°C was most extensive, the effect of time and temperature after a slow pH change to pH 7.0 was examined. The protein was dialyzed for 1, 3 or 7 days after the pH change. The results are shown in Figure 19 and Table 2. At 24°C, -100S material was present after 1 day but not all of the protein had polymerized. After 3 days, all of the protein had polymerized into -100S and -200S material. The sedimentation pattern was unchanged after 7 days.

Electron microscopy revealed that long rods were present after 1 day at 24°C but there were also more short rods and end-ons than after 3 days.

At 4°C, the -200S species did not form even after 7 days. The proportion of un polymerized material was greater than at 24°C. A species which sedimented at -100S was present after 3 and 7 days but a species which sedimented at 70 - 80 S was present after 1, 7 and possibly 3 days. This probably represents a population of rods shorter than those at 24°C.

Electron microscopy revealed that very few long rods were present after 1 day at 4°C. Most of the protein existed as short rods, end-ons and some non-specific aggregates. After 3 days at 4°C, many long rods were present but the proportion of short rods and end-ons was higher than after 3 days at 24°C. The results show that at pH 7.0, BCV protein
Figure 18. Electron micrographs showing the effect of the rate of pH change at pH 5.0. BCV protein in 0.01 M citrate, pH 5.0 at 20°C (A) after a fast pH change and (B) after a slow pH change. Bar represents 100 nm.
Figure 19. Schlieren patterns showing the effect of temperature and time on the polymerization of BCV protein. The protein was first dialyzed against 0.01 M glycine-HCl, pH 2.5, at 4°C overnight, and subsequently against 0.01 M MES, pH 7.0 at either 24°C (upper patterns) or 4°C (lower patterns) for 7, 3 or 1 days (A, B, C, respectively).

(A) Upper: 111S and 238S products after 7 days at 24°C. A small amount of 3S material was seen at higher speed; lower: 7S, 72S and 108S products after 7 days at 4°C. (B) Upper: 120S and 201S products after 3 days at 24°C; lower: 7S and 108S products after 3 days at 4°C. (C) Upper: 8S and 111S products after 1 day at 24°C; lower: 8S and 78S products after 1 day at 4°C. Runs were done at 20°C at a speed of 24,630 rpm. Protein concentration was 2 mg/ml. Photographs were taken between 8 and 10 minutes after reaching speed. Sedimentation is to the right.
Table 2. The effect of temperature and time on the behaviour of BCV protein after a slow pH change from pH 3.5 to pH 7.0

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24°C</td>
</tr>
<tr>
<td>1</td>
<td>7, 111</td>
</tr>
<tr>
<td>3</td>
<td>120, 201</td>
</tr>
<tr>
<td>7</td>
<td>111, 283</td>
</tr>
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</table>
reaches equilibrium within 3 days at 24°C. More time was required at 4°C.

3.2.6 Effect of temperature

To investigate the effects of low temperature more thoroughly, experiments were done over the range of pH 3.5 to 10.0 with both fast and slow pH changes at 4°C. The results are shown in Figures 13, 15 (E-H), and Tables 1 and 2.

After a fast pH change, polymerization leading to small aggregates occurred at pH 3.5 and 10.0. The aggregates were probably smaller than those present at 24°C, since the protein sedimented more slowly. In contrast to the results at 24°C; rod formation did not occur at pH 9.0, 4°C. Rods which sedimented at -100S were present at pH 6.0 to 8.0 at 4°C. Their aggregates, which sedimented at -200S, were present only at pH 7.0 and 8.0. The proportion of -200S material was lower and that of the small aggregates was higher at 4°C than at 24°C. After a fast pH change at pH 5.0 the sedimentation coefficient of the main species varied from 59S to 85S at 4°C, indicating that the rods were probably shorter than at 24°C. This was confirmed using electron microscopy. Polymerization after a fast pH change at pH 4.0 was not affected by temperature.

The effect of low temperature was greater after a slow pH change than after a fast pH change. At pH 3.5, 9.0 and 10.0, after a slow pH change at 4°C, the size of the small aggregates was apparently reduced by low temperature. In contrast to the results after a fast pH change, no large rods formed at pH 8.0, 4°C after a slow pH change, although a
few short rods were observed using electron microscopy (Fig. 20). No
-200S material formed at any pH level. Long rods, which sedimented at
-100S, were present at pH 6.0 and 7.0 but in lower proportion than at
24°C. At pH 5.0, 4°C the protein was present as a single species which
sedimented at 24S, indicating that the rods were shorter than at 24°C.
This was confirmed using electron microscopy (Fig. 21). After a slow
pH change at pH 4.0, 4°C, most of the protein sedimented at 4S and some
sedimented at about 36S, indicating that the rods were shorter at 4°C than
at 24°C. The results of electron microscopy showed that the proportion
of short rods and end-ons was higher at 4°C than at 24°C.

The results show that polymerization was inhibited by low temperature.
It increased the proportion of small aggregates and reduced their size.
It decreased the proportion, length and rate of formation of rods. This
means that the polymerization reaction was shifted toward the small agg-
regates at low temperature and may be entropy-driven. The aggregation
of rods was reduced by low temperature, perhaps because rod formation was
slower and less extensive at 4°C than at 24°C.

3.2.7 Effect of KCl

The effect of KCl on the polymerization of BCV protein at pH 7.0
was investigated using a slow pH change. The results of analytical
ultracentrifugation are shown in Figure 22A and show that the presence
of 0.2 M KCl did not affect polymerization, suggesting that the process
was probably not ionic.
Figure 20. Electron microscopy of BCV coat protein in 0.01 M tris, pH 8.0 after 3 days (A) at 24°C and (B) at 4°C. Bar represents 100 nm.
Figure 21. Electron micrographs showing the effect of temperature at pH 5.0. BCV protein after 3 days in 0.01 M citrate, pH 5.0. (A) at 24°C and (B) at 4°C. Bar represents 100 nm.
Figure 22. Schlieren patterns showing the effect of KCl and protein concentration.

(A) Schlieren diagram showing the effect of KCl on BCV protein at pH 7.0 during a slow pH change at 24°C. Upper: 112S and 232S products, with 0.2 M KCl in the final buffer; lower: 112S and 268S products; with no KCl. Run was done at 20°C at a speed of 24,630 rpm, using a 12 mm cell. Protein concentration was 2 mg/ml. Photograph was taken 5 minutes after reaching speed. Sedimentation is to the right. (B) Schlieren diagram showing the effect of protein concentration on BCV protein at pH 7.0 with a slow pH change at 24°C. Upper: T05S product at 0.5 mg/ml; lower: 104S and 249S products at 3 mg/ml. Run was done at 20°C at a speed of 24,630 rpm, using a 30 mm cell. Photograph was taken 9 minutes after reaching speed. Sedimentation is to the right.
3.2.8 Effect of protein concentration

The effect of protein concentration was examined with a slow pH change to 0.01 M MES pH 7.0, using protein concentrations of 3 mg/ml and 0.5 mg/ml. The results of analytical ultracentrifugation are shown in Figure 22B. At both concentrations, all of the protein polymerized and 100S rods formed. The 200S species only formed at the higher of the two concentrations, indicating that it exists in a concentration-dependent equilibrium with the 100S rods.

3.3 Discussion
3.3.1 The identity of the species observed

The size of the polymerization products can be calculated from their sedimentation coefficients and the calculated partial specific volume of the protein, as has been done for TMV (Caspar, 1963), and PMV (Erickson et al, 1983). The calculations indicate that the 2-3S species is probably composed of monomers and/or dimers and the 6-7S species probably consists of pentamers and/or hexamers.

The rods which formed at pH 6.0 to 9.0 and sedimented at about 100S are calculated to be about 200 nm long. The sedimentation coefficient of this species varied from 90S to 128S. In this range, a small change in sedimentation coefficient may reflect a significant change in rod length. Although the rods would usually be about 200 nm long, they may vary from 150 nm to 400 nm in length. Particle length measurements of rods formed after a slow pH change to pH 7.0 indicate that the mean particle length was 223 nm (σ = 154 nm). This is consistent with the calculations. Very long rods (>1000 nm) were observed
only at pH 6.0 after a fast pH change (Fig. 14).

The identity of the 200S species, which formed at pH 6.0 to 9.0 after a fast pH change and at pH 7.0 after a slow pH change, presents two possibilities: 1) a single rod or end-to-end aggregate of rods approximately 1800 nm long; 2) three 200 nm rods aggregated side-by-side. These are the extreme possibilities.

Individual rods long enough to sediment at 200S (1800 nm) would be very fragile. End-to-end aggregation would also be highly unlikely. The surface area of contact would be very small and effective collisions would be relatively rare.

With side-by-side aggregation, effective collisions would be more frequent, involve a greater surface area for interparticle bonding and produce less fragile aggregates. It would not be unreasonable for side-by-side aggregation to occur between BCV protein rods since some of the viruses in the PVX family aggregate in this manner (Tollin et al., 1967; Radwan et al., 1981). BCV sediments as two species at 128S and 246S. The latter probably consists of virus particles aggregated side-by-side. The 200S species of BCV protein probably consists of 3 rods, each about 200 nm long, aggregated side-by-side since 1800 nm rods were rare at pH 6.0 after a fast pH change and absent under other conditions. In cases where the peak has a sedimentation coefficient of about 250S, the aggregates would probably consist of 4 rods.

3.3.2 Effect of pH

The results of the pH series (Figs. 13, 15; Table 1) show that the
polymerization of BCV protein was a pH-dependent process. Less polymerization occurred at pH 5.0 than at pH 6.0, which indicates that carboxyl-carboxylate pairs which regulate the assembly of TMV and CCMV (Caspar, 1963; Klug, 1972; Bancroft, 1970), are not likely to control the polymerization of BCV protein. Longer rods formed at pH 6.0 than at pH 5.0. The transition between pH 5 and 6 probably involves hydrogen bonding of the imidazole group of histidine, which has a $pK_a$ value of 6.0. When polymerization occurs (pH>6) this group could behave as an uncharged proton acceptor and interact with a proton donor (a group with a higher $pK_a$ value). There are 2 histidine residues in each BCV protein subunit (Margaret Short, unpublished data).

The second group (proton donor) must have a $pK_a$ value near 9 because fairly extensive polymerization can occur at pH 9.0. If this is very close to the $pK_a$ of a group involved in the pH-mediated interaction, then a small error in titration of the samples could produce variable results.

Interaction between the imidazole group of histidine and a group with a $pK_a$ near 9 could regulate polymerization at both ends of the pH range. The only amino acid R-group with a $pK_a$ near 9 is the sulfhydryl group of cysteine, which has a $pK_a = 8.3$. The number of cysteine residues in BCV protein is 2 (Margaret Short, unpublished data). The interaction between histidine and cysteine would not be ionic, but would be a proton transfer complex. Such bonding has been described by Lindemann and Zundel (1978) and Rastogi et al (1981) between histidine and aspartic and glutamic acid. The interaction would not occur until deprotonation of the imidazole group occurred, near pH 6.0 (Fig. 23A). A hydrogen bond could then occur between the uncharged imidazole nitrogen and the
Figure 23. Models for amino acid interactions. Interactions between the R groups of histidine and (A) cysteine, (B) lysine and (C) tyrosine.
sulfhydryl group. When the latter became ionized, the bond would break.

The existence of the hydrogen bond would make ionization of the sulfhydryl group less favourable (i.e. make it a weaker acid) and its $pK_a$ value would be higher than 8:3 (Laskowski and Scheraga, 1954). Similarly, the presence of the bond would lower the $pK_a$ of the histidyl imidazole group. Therefore, the actual transition point for the formation of long BCV protein rods may lie somewhere between pH 5 and 6 and the transition point for not forming them around pH 9.0. This is assuming that the $pK_a$ values of the two groups are not further affected by other neighbouring groups.

Groups with a high intrinsic $pK_a$ (e.g. the phenolic hydroxyl of tyrosine, $pK_a = 10.1$; the $\epsilon$-amino of lysine, $pK_a = 10.5$) might have an abnormally low $pK_a$ value as a result of acidic neighbours and regulate polymerization near pH 9. These interactions would be proton transfer hydrogen bonds, as shown in Figure 23B,C. They are of the same type as those between histidine and cysteine, except that below pH 6 the histidine and lysine would actually exhibit charge repulsion rather than simply not interact, as with histidine and cysteine. The $pK_a$ values of the R-groups of tyrosine and lysine would be increased by the presence of the hydrogen bond because ionization (proton donation) would be less favourable. This effect would have to be countered by the effect of the local environment in lowering the $pK_a$ of the lysine or tyrosine residues to about 9. Above this pH there would be no hydrogen bond and no polymerization.

Of the three proposed interactions, that involving tyrosine would probably be the most stable in a polar environment (in the presence of
water) (Kristof and Zundel, 1980; Rastogi et al., 1981) and be the strongest through the electronegativity of the H... donor group. Its hydrogen bonded or proton transfer complex would be stabilized more by resonance than complexes of histidine...lysine or histidine...cysteine, and the interaction with tyrosine would probably produce the greatest amount of water displacement. However, the $pK_a$ value of cysteine is the most suitable for the regulation of BCV protein polymerization.

Intersubunit interactions between histidine and either tyrosine or lysine may be involved in the polymerization of PVY protein (McDonald and Bancroft, 1977), which also occurs at pH 6.0 to 9.0.

Whether or not a second interaction involving a carboxyl side chain is involved in the polymerization of BCV protein is unclear. Very little equilibrium polymerization occurred at pH 4.0 and 5.0, indicating perhaps no regulation by the interaction of a carboxyl group with a neutral or basic amino acid residue. The low level of polymerization that does occur at acid pH may simply result from histidine-basic residue interactions still occurring, although at a relatively low frequency, at low pH.

Alternatively, the interaction between histidine and a basic residue may predominate but be complemented by another interaction between a de-protonated carboxyl side chain (of aspartic or glutamic acid) and a more basic amino acid, such as lysine. This interaction would raise the $pK_a$ of the acidic residue and lower that of the basic residue, and would be strongest near neutrality, where the polymerization of BCV protein is most extensive. At low pH, which would be unfavourable for the hydrogen bond involving histidine, polymerization may still occur as a result of
the second interaction but be limited without the stabilizing effect of the histidine-containing hydrogen bond.

Procedures for the chemical modification of specific amino acids could be used to determine which residues are involved in the polymerization of BCV protein. Histidine can be most specifically modified by dye-sensitized photooxidation with rose bengal (Glazer, 1970; Westhead, 1965). The most useful method for modifying tyrosine is nitration with tetranitromethane (TNM) (Cohen, 1968; Stärk, 1970; Riordan and Vallee, 1972a). Modification of cysteine may also occur with the use of rose bengal and TNM, but these residues can be reversibly blocked with mercuric chloride or tetrahionate (Glazer, 1970). The most specific reagent for modifying lysine is trinitrobenzenesulfonic acid (TNBS), which trinitrophenylates the amino groups (Means and Feeney, 1971), although acid anhydrides could also be used (Cohen, 1968; Means and Feeney, 1971; Riordan and Vallee, 1972b). The most selective method for the modification of cysteine is dye-sensitized photooxidation with crystal violet (Jori et al, 1969). The most useful method for the modification of carboxyl sidechains is amide formation promoted with water-soluble diimides (e.g. 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide). The reaction components may be varied for different experimental situations and the ionic character of the products may also be varied. Sulfhydryl groups and the phenolic hydroxyl group of tyrosine may also participate in this reaction (Means and Feeney, 1971). All of the above methods can be used under conditions where BCV polymerization is limited and generate products that are stable under conditions for protein polymerization and amino acid determinations.
3.3.3 Effect of the rate of pH change

The effect of the rate of pH change can be examined by comparing the results of the fast and slow pH series at 24°C (Figs. 13A-D, 15A-D, Table 1) with respect to the formation of small aggregates, individual rods and their aggregates.

The Schlieren patterns and the data listed in Table 1 show that the rate of pH change did not affect polymerization leading to small aggregates.

Rod formation was, however, affected by the rate of pH change. After a fast pH change rods formed from pH 4.0 to 9.0. The longest and most rapidly sedimenting ones formed from pH 6.0 to 8.0. The sedimentation coefficient of the rods was not affected by the rate of pH change nor was the proportion of the protein present as individual rods. However, if the rods present in the side-by-side aggregates are also considered, then the rods represented a smaller fraction of the total protein at pH 6.0 and 8.0 after a slow pH change than after a fast pH change.

At pH 9.0, rod formation occurred only after a fast pH change, not after a slow one. This discrepancy may be due to small errors in titration, if the groups regulating polymerization titrate near pH 9.

At pH 4.0 and 5.0, the reaction was affected by the rate of pH change. After a fast pH change, material which sedimented at 86S was present. After a slow pH change at pH 4.0, the protein sedimented at 86S in some samples and as slowly as 47S in other samples. After a slow pH change at pH 5.0, the protein sedimented at 32S and 46S. It is evident that the sedimentation coefficient was lower after a slow pH change than after
a fast one, indicating that the rods were shorter. This was confirmed using electron microscopy (Figs. 18, 24).

With TMV protein, non-equilibrium polymerization has been shown to occur after rapid changes in pH or temperature (Schuster et al., 1979). Long rods formed if a mixture of small aggregates (4S) and 20S disks at pH 7.0 was subjected to a rapid pH drop to pH 6.4 - 6.7 or if the 4S species at pH 6.5, 0°C was rapidly heated to 20°C, in 1-3 minutes. The long rods, which sedimented at 100-130S, slowly depolymerized into short rods consisting of 3-7 turns. This phenomenon of rapid polymerization, which is followed by gradual depolymerization, was termed overshoot polymerization as mentioned previously. The magnitude of the overshoot was greatest if the pH or temperature change was large and if the concentration of 20S disks or the ratio of 20S:4S species in the starting material was low. The 20S disks nucleate helical rod assembly and form from the small 4S aggregates. Their formation has a half-time of about 4 hours at pH 7.0, 20°C (Durham and Klug, 1972). The rate of rod elongation with 4S material is much higher than the rate of 20S disk formation. At first, the 4S material polymerizes onto the 20S disks that are present. This rapidly produces long rods (overshoot). These gradually depolymerize into 4S material again, which slowly produces 20S disks. As new disks form, elongation can occur on them. The final products at equilibrium are shorter than the original overshoot rods.

It is conceivable that this type of overshoot polymerization occurred with BCV protein after a fast pH change to pH 4.0 and 5.0 in which case the 86S rods would slowly depolymerize into shorter more slowly-sedimenting rods. Experiments have not yet been done to confirm this.
Figure 24. Electron micrographs showing the effect of the rate of pH change at pH 4.0. BCV protein in 0.01 M citrate, pH 4.0 (A) after a slow pH change at 24°C; (B) after a fast pH change at 24°C. Bar represents 100 nm.
The idea of non-equilibrium mixtures being present after a fast pH change is not unreasonable. The results after different dialysis times at pH 7.0 showed that equilibrium was not established during the first day, even though over half of the protein formed rods in that time. The proportion of side-by-side aggregates present after a fast pH change at pH 7.0 was lower than after a slow pH change, indicating that the fast pH change did not produce an equilibrium mixture. In fact, the aggregated rods, which occurred at pH 6.0 to 9.0 after a fast pH change, were equilibrium products only at pH 7.0.

The effect of the rate of pH change can be examined by comparing the results of the fast and slow pH series at 4°C (Figs. 13, 15, Table 1). The effects are similar to but more drastic than those observed at 24°C. Polymerization leading to small aggregates was unaffected by the rate of pH change. However, the proportion of the total protein present as rods and their sedimentation coefficients were lower after a slow pH change than after a fast pH change. Side-by-side aggregation of rods was detected by analytical ultracentrifugation after a fast pH change to pH 7.0 and 8.0 at 4°C, but not after a slow pH change. The results indicate that overshoot or non-equilibrium polymerization occurred after a fast pH change at 4°C from pH 4.0 - 8.0, i.e. over a wider pH range than at 24°C.

3.3.4 Effect of temperature

The effect of temperature on the behaviour of BCV protein was analyzed by comparing the results from 20°C and 4°C after fast pH changes, slow pH changes and different dialysis times at pH 7.0 (Figs. 13, 15, 19; Tables 1, 2).
Small aggregates were more prevalent at 4°C than at 20°C. This was particularly striking at pH 9.0 after a fast pH change and at pH 8.0 after a slow pH change. The sedimentation coefficient of the small aggregates was often reduced at low temperature. The peaks of slowly-sedimenting (7S) material were not always symmetrical at 20°C. They tailed off on the upper side. This indicates that the 7S material may be in equilibrium with smaller species. This equilibrium was shifted from the 7S species (pentamers and/or hexamers) towards 2-4S species (monomers and/or dimers) at 4°C.

The proportion of protein present as rods (100S and 200S) was higher at 20°C than at 4°C. This indicates that rod formation was stabilized by hydrophobic interactions and may have been entropy-driven. This is supported by the fact that 0.2 M KCl did not affect polymerization at pH 7.0.

The sedimentation coefficient, corrected to 20°C, of the rods was lower at 4°C than at 24°C after a slow pH change (pH 4.0-7.0), indicating that rod length was affected by temperature. The results of electron microscopy confirmed this, especially at pH 5.0. The weight average of particle lengths at pH 5.0 after a slow pH change was 166 nm at 20°C and 23 nm at 4°C. Rod formation was least sensitive to temperature at pH 7.0, where polymerization was most favourable at either temperature. Since the amino acids regulating the polymerization of BCV protein must have pK_a values of about 6 and 9, their interactions are probably strongest at or slightly above pH 7. If entropy is a driving force in rod formation, as the results at 4°C suggest, its role may be proportionally smaller at pH 7, where the pH-mediated amino acid interactions play their
greatest role, than at other pH levels.

Side-by-side aggregates (200S) of rods were more prevalent at 20°C than at 4°C, which indicates that their formation was stabilized by hydrophobic interactions and may have been entropy-driven. The results in Fig. 22A show that these aggregates existed in a concentration-dependent equilibrium with the 100S rods. However, the results at 4°C do not simply reflect a concentration effect. The concentration of 100S material at 4°C, pH 6.0 to 8.0 after a fast pH change was higher than at 20°C, yet little or no 200S material was present at 4°C. However, at pH 7.0 after a slow pH change, the absence of aggregated rods is almost certainly due to the low levels of 100S rods in the sample. Therefore, two factors contribute to the effect of temperature on the aggregation of BCV protein rods: 1) concentration-dependence of rod formation, which may be entropy-driven; 2) entropy as a driving force in the aggregation process.

3.4 The behaviour of PVX coat protein

3.4.1 Effect of pH

The effect of pH on the state of aggregation of PVX coat protein at 20°C and 4°C was studied using analytical ultracentrifugation and electron microscopy. The protein was prepared in water and adjusted either quickly or slowly to the desired pH (see Materials and Methods).

3.4.2 Effect of fast pH change

The results of analytical ultracentrifugation after a fast pH change
at approximately 20°C are shown in Figure 25A-C and Table 3. The predominant species, present at all pH levels from pH 4.0 to 9.0, except pH 5.0, sedimented at 4S. The asymmetry of the peaks indicates that this species was in equilibrium with a larger aggregate. At pH 5.0, the main species present sedimented at 10S and was in equilibrium with smaller material.

Electron microscopy revealed disordered aggregates at all pH levels.

3.4.3 Effect of slow pH change

The results of analytical ultracentrifugation after a slow pH change at 20°C are shown in Figure 26A-C and Table 3. Again, the predominant species sedimented at about 4S. It was present at pH 4.0 and 6.0 to 8.0. The asymmetry of the peaks indicates that the 4S species was in equilibrium with larger material. At pH 9.0, the major species present sedimented at 6S. A smaller shoulder sedimenting at 8-10S was present at pH 8.0 and 9.0. The protein precipitated at pH 5.0.

Electron microscopy revealed disordered aggregates at all pH levels.

3.4.4 Effect of temperature

The effect of temperature on PVX protein was examined after fast and slow pH changes at 4°C (see Materials and Methods). The results were determined using analytical ultracentrifugation and electron microscopy.

The results of analytical ultracentrifugation after a fast pH change
Figure 25. Schlieren patterns of PVX protein prepared quickly under various conditions of pH and temperature. All samples were prepared in water at 4°C and then adjusted to the desired pH by the addition of 1 M buffer to a final concentration of 0.01 M. The pH was adjusted at either 24°C (patterns on the left) or 4°C (patterns on the right).

(A) Upper: 4S species in 0.01 M citrate, pH 4.0; lower 10S in 0.01 M citrate, pH 5.0. (B) Upper: 4S species in 0.01 M MES, pH 6.0; lower: 4S species in 0.01 M MES, pH 7.0. (C) Upper: 4S species in 0.01 M tris, pH 8.0; lower: 4S species in 0.01 M glycine-NaOH, pH 9.0. (D) Upper: 4S species in 0.01 M citrate, pH 3.9; lower: 3S species with a 5S shoulder in 0.01 M citrate, pH 4.9. (E) Upper: 3S species in 0.01 M MES, pH 6.0; lower: 5S species in 0.01 M MES, pH 7.0. (F) Upper: 4S species in 0.01 M tris, pH 7.9; lower: 5S species in 0.01 M glycine-NaOH, pH 9.0.

Runs were done at temperatures of about 20°C (patterns on the left) or about 4°C (patterns on the right). Protein concentration was about 2 mg/ml. Photographs were taken between 17 and 22 minutes after reaching speed. Rotor speed was 50,740 rpm. Sedimentation is to the right.
Table 3. The effect of pH and temperature on the sedimentation of PVX coat protein

<table>
<thead>
<tr>
<th>pH Change</th>
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<th>Slow</th>
</tr>
</thead>
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<td>4°C</td>
</tr>
<tr>
<td>Temperature</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH **</td>
<td></td>
<td></td>
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<tr>
<td>4</td>
<td>4</td>
<td>4</td>
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<tr>
<td>5</td>
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<td>6</td>
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<td>7</td>
<td>4</td>
<td>4</td>
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<tr>
<td>8</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>9</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

* Species sedimenting at 3, 4 and 5S are considered one species and have been grouped as 4S

** pH may not be exact for fast pH changes. See Figure 25 for precise values

- precipitate

(.) minor species
Figure 26. Schlieren patterns of PVX protein prepared slowly under various conditions of pH and temperature. The protein, prepared in water, was dialyzed against the appropriate 0.01 M buffer at 4°C overnight and then for 48 hours at 24°C (patterns on the left) or 4°C (patterns on the right).

(A) Upper: 4S species and precipitate in 0.01 M citrate, pH 4.0; lower: precipitate in 0.01 M , pH 5.0. (B) Upper: 4S species in 0.01 M MES, pH 6.0; lower 4S species in 0.01 M MES, pH 7.0.
(C) Upper: 3S species with 10S shoulder in 0.01 M tris, pH 8.0; lower: 6S species with 10S shoulder in 0.01 M glycine-NaOH, pH 9.0.
(D) Upper: 8S species in 0.01 M citrate, pH 4.0; lower: 10S species in 0.01 M citrate pH 5.0. (E) Upper: 4S species in 0.01 M MES pH 6.0; lower: 5S species in 0.01 M MES, pH 7.0. (F) Upper: 5S species in 0.01 M tris, pH 8.0; lower: 5S species in 0.01 M glycine-NaOH, pH 9.0.

Runs were done at temperatures of about 20°C (patterns on the left) or about 4°C (patterns on the right). Protein concentration was about 2 mg/ml. Photographs were taken between 18 and 24 minutes after reaching speed. Rotor speed was 50,740 rpm. Sedimentation is to the right.
at 4°C are shown in Figure 25D-F and Table 3. At all pH levels from pH 4.0 to 9.0, a single species of protein was present which sedimented at 3-5S. The peaks were more symmetric than at 20°C. The results show that low temperature shifted the equilibrium toward the smaller aggregates.

Electron microscopy showed disordered aggregates in all samples.

The results of analytical ultracentrifugation after a slow pH change at 4°C are shown in Figure 26D-F and Table 3. At pH 4.0 and 5.0, the protein sedimented as one species at 8-10S which appeared to be in equilibrium with smaller material. The apparent increase in the size of the aggregates is a result of increased protein concentration in the absence of precipitation. At pH 6.0 and 7.0, the protein was present as a single species which sedimented at 4-5S, as it did at 20°C. At pH 8.0 and 9.0, the size of the aggregates was reduced at low temperature. The protein was present as a single species which sedimented at 5S, compared to 8-10S at 20°C.

Electron microscopy showed disordered aggregates at all pH levels.

Low temperature shifted the equilibrium among the minor 8-10S aggregates toward the 4S species indicating an entropic factor.

3.4.5 Effect of KCl

The effect of KCl on the behaviour of PVX protein was examined after a slow pH change (see Materials and Methods) to 0.01 M MES pH 6.0, containing either 0 M, 0.1 M or 0.2 M KCl.
The results of analytical ultracentrifugation are shown in Figure 27A. In the absence of KCl (lower) the protein was present as a main 4S species and an 8S shoulder. In the presence of 0.1 M and 0.2 M KCl (upper), the protein was present as a single species which sedimented at 3-4S. Electron microscopy revealed disordered aggregates in all samples.

The results indicate that KCl inhibited the formation of the 8S aggregate, which is probably stabilized to some extent by ionic interactions in addition to the entropic factor indicated by the effect of temperature (section 3.4.4).

3.4.6 Effect of protein concentration

The effect of protein concentration on the behaviour of PVX protein was examined using analytical ultracentrifugation and electron microscopy after a slow pH change to 0.01 M MES pH 6.0 at 20°C.

The results of analytical ultracentrifugation are shown in Figure 27B. At a protein concentration of 0.25 mg/ml, the protein was present as a 2S species with a small 4S shoulder. At a concentration of 2 mg/ml, the main species sedimented at 4S with an 8S shoulder. This indicates that a concentration-dependent equilibrium existed between monomers, dimers and octamers.

3.4.7 Discussion

The results showed that in the range of pH 4.0 to 9.0, PVX protein
Figure 27. Schlieren patterns showing the effect of KCl and protein concentration on PVX protein.

(A) Schlieren patterns showing the effect of KCl on the behaviour of PVX protein at pH 6.0. The protein was dialyzed against 0.01 M MES pH 6.0, containing 0 M or 0.2 M KCl. Dialysis was at 4°C overnight and then at about 24°C for 48 hours. Upper: 4S species in 0.01 M MES pH 6.0, 0.2 M KCl; lower: 4S and 8S material in 0.01 M MES, pH 6.0. Run was done at 22°C. Protein concentration was about 2 mg/ml. Photograph was taken 27 minutes after reaching a rotor speed of 50,740 rpm. Sedimentation is to the right. (B) Schlieren patterns showing the effect of protein concentration on the behaviour of PVX coat protein at pH 6.0. The protein, at a concentration of either 2 mg/ml or 0.25 mg/ml, was dialyzed against 0.01 M MES pH 6.0, at 4°C overnight and then at about 24°C for 48 hours. Upper: 2S species with a small amount of 4S material, at a protein concentration of 0.25 mg/ml; lower: 4S species and 8S shoulder at a protein concentration of 2 mg/ml. Run was done at 24°C. Photograph was taken 29 minutes after reaching a rotor speed of 50,740 rpm. 30 mm cells were used. Sedimentation is to the right.
does not form rod-shaped particles. Therefore, protein-protein interactions are probably less important in the assembly of PVX than for viruses such as TMV, TRV, PMV and BCV, the proteins of which can form virus-like particles in the absence of the RNA (Durham et al., 1971; Morris and Semancik, 1973; Erickson et al., 1976).

PVX protein did not form disk aggregates, like those of the proteins of TMV (Butler and Klug, 1971), TRV (Morris and Semancik, 1973) and like the 14S species of PMV and CYMV proteins (Erickson and Bancroft, 1978; Bancroft et al., 1979). The main species of PVX protein sedimented at 4S and 8-10S. The formation of the larger species was sensitive to low temperature and the presence of KCl, indicating that entropic and ionic interactions were both involved to some extent in its formation. A concentration-dependent equilibrium existed between 2S, 4S and 8S species at pH 6.0. This probably involved monomers (2S), dimers and/or trimers (4S) and octamers (8S). The 10S species was probably composed of 9 subunits arranged in a ring, although such structures were not observed using electron microscopy. Similarly, the 14S aggregate of PMV protein is thought to consist of an 18-subunit disk, although it has not been observed with electron microscopy. Erickson et al. (1983) suggested that the disk is difficult to visualize as a result of extensive hydration.

Goodman (1975) has reported the presence of a 4S species of PVX protein in the presence of NaCl or KCl near pH 6, which is consistent with the results presented here. Two species of PVX protein have been reported, which sedimented at 3-5S and 10-15S (Kaftanova et al., 1975; Goodman et al., 1976). The latter sediments more rapidly than material that I observed (8-10S) and probably consisted of single rings and 2-
layer disks. Goodman et al (1976) suggested that the 10-15S species may consist of rings composed of 9 subunits and observed stacked disk structures in protein preparations in 0.1 M NaCl or KCl, or in 0.01 M MES, pH 6.2. Kaftanova et al (1975) observed single- and double-layered disks of protein in 0.2 M phosphate buffer at pH 7.0-7.5, 4-20°C after 24 hours. Stacks of 4-16 disks formed upon ageing of these preparations. Disk formation did not occur under conditions suitable for reconstitution of PVX (Kaftanova et al, 1975). However, this does not rule out the possibility of a disk aggregate being involved in the initiation of PVX assembly, as pointed out by Goodman et al (1976). Such an aggregate could be present; for initiation, in quantities small enough to go undetected by Schlieren optics (Erickson and Bancroft, 1978).

The optimal conditions for the reconstitution of PVX are a pH of 6.0-6.2 (Goodman et al, 1975) or pH 6.5-7.0 (Kaftanova et al, 1975) in low ionic strength buffers at 20-24°C. Reconstitution was inhibited by low temperature (Kaftanova et al, 1975; Goodman et al, 1975, 1976), indicating that entropy was a driving force. It was also inhibited by the presence of 0.1 M NaCl or KCl, although it did proceed in the presence of 0.2 M Bicine (Goodman, 1977). This indicates that electrostatic interactions, perhaps between the protein and the RNA, are involved in assembly, since the chaotropic action of the ions suggested by Goodman (1977) is unlikely in view of the concentrations of salts used (Erickson and Bancroft, 1978). The effect of low temperature and KCl on reconstitution may result from their effect on the protein, in shifting the equilibrium toward the 4S species.
Like the disk-type structures observed by Kaftanova et al (1975),
the 10S species of PVX protein did not form at pH 6-7, the optimal pH
for reconstitution. The role of this species in assembly is therefore
not clear. A species which sedimented at 8S (Table 3) formed after a
slow pH change to pH 6.0. This probably consists of octamers and may
be a subassembly product. Several such aggregates might form an initia-
tion complex upon their interaction with a certain region of the RNA
that would facilitate helical growth.

It is clear from the results that the assembly of PVX must begin
with a sub-assembly product of protein that is considerably smaller
than those involved in the assembly of TMV, TRV, PMV and CYMV (Butler,
1971, 1972; AbouHaidar et al, 1973; AbouHaidar, 1976; Erickson and
Bancroft, 1978; Bancroft et al, 1979), whether it is a single ring of
9 subunits or an even smaller aggregate.

3.5 The behaviour of FTV coat protein

3.5.1 Effect of pH

The effect of pH on the behaviour of FTV coat protein was examined
by adjusting the pH either quickly or slowly, as described in the
Materials and Methods. The results were determined using analytical
ultracentrifugation and electron microscopy, and are shown in Figures
28, 30 and Tables 4 and 5.

3.5.2 Effect of a fast pH change at 20°C

Protein samples were quickly adjusted to the desired pH levels at
about 20°C. The results of analytical ultracentrifugation are shown
Figure 28. Schlieren patterns showing the effect of pH and temperature on FTV protein after a fast pH change. All samples were prepared in water at 4°C and then adjusted to the desired pH by the addition of 1 M buffer to a final concentration of 0.01 M. The pH was adjusted at either 24°C (patterns on the left) or 4°C (patterns on the right).

(A) Upper: 4S species in 0.01 M citrate, pH 4.0; lower: 4S species in 0.01 M citrate, pH 5.0. (B) Upper: 6S species in 0.01 M MES, pH 6.0; lower: 7S species in 0.01 M MES, pH 6.9. (C) Upper: 6S species in 0.01 M tris, pH 8.0; lower: major 7S species with minor species of 5S and 6S in 0.01 M glycine-NaOH, pH 9.0. (D) Upper: 5S species in 0.01 M citrate, pH 4.0; lower: 7S species in 0.01 M citrate, pH 5.0. (E) Upper: 6S species in 0.01 M MES, pH 6.0; lower: 5S species in 0.01 M MES, pH 7.0. (F) Upper: 7S species in 0.01 M tris, pH 8.0; lower: 5S and 31S species in 0.01 M glycine-NaOH, pH 9.0.

Runs were done at temperatures of about 20°C (patterns on the left), or about 4°C (patterns on the right). Protein concentration was about 2 mg/ml. Photographs were taken between 24 and 27 minutes after reaching speed. Rotor speed was 50,740 rpm. Sedimentation is to the right.
Table 4. The effect of pH and temperature on the sedimentation of FTV coat protein

<table>
<thead>
<tr>
<th>Rate of pH Change</th>
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</tr>
<tr>
<td>8</td>
<td>6</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>9</td>
<td>(5,6)7</td>
<td>5,3</td>
<td>6 (8)</td>
</tr>
</tbody>
</table>

* pH may not be exact for fast pH changes. See Figure 28 for precise values.

Bracketed S values are minor species.
Table 5. The effect of pH and temperature on the morphology of FTV coat protein, as determined by electron microscopy

<table>
<thead>
<tr>
<th>pH Change</th>
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<th>SLOW</th>
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<td>Morphology:</td>
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<td></td>
</tr>
<tr>
<td>Paracrystals</td>
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<td></td>
<td>pH 5</td>
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<td>Ropes</td>
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<td></td>
<td>pH 5-9</td>
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<tr>
<td>Rods</td>
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<td>pH 5-9</td>
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in Figure 28A-C and Table 4. A single species which sedimented at 4S was present at pH 4.0 and 5.0. At pH 6.0 to 9.0, the protein sedimented at 5-7S. The major peaks on the Schlieren patterns were asymmetric, indicating that either an equilibrium existed or aggregates of different sizes were present.

The results of electron microscopy are listed in Table 5. Nonspecific aggregates were present at all pH levels. Rods and ropes (intertwined rods) (Fig. 29A,B) were present at pH 4.0 to 9.0, although many ropes were broken at pH 7.0 and 8.0. Paracrystals were present only at pH 4.0 and 5.0 (Fig. 29C).

3.5.3 Effect of slow pH change at 20°C

The results of analytical ultracentrifugation and electron microscopy after a slow pH change at about 20°C (see Materials and Methods) are shown in Figure 30A-C and Tables 4 and 5.

Most of the protein sedimented at 4-6S at pH 4.0 to 9.0. A small amount of protein was present at pH 7.0 and 9.0 which sedimented at 8S. Therefore, the formation of small aggregates was unaffected by pH or its rate of change. Asymmetry in the peaks again indicates that they contain more than one species or that an equilibrium may exist. Rapidly-sedimenting material was present at pH 5.0 to 9.0.

The results of electron microscopy are listed in Table 5. Disordered aggregates were present at all pH levels. No large aggregates formed at pH 4.0 and paracrystals formed only at pH 5.0. Rods and ropes were present from pH 5.0 to 9.0. Therefore, the paracrystals which
Figure 29. Electron micrographs showing the different forms of FTV protein. (A) Rope formed with a fast pH change to 0.01 M tris pH 8.0 at 24°C. (B) Rods formed after a fast pH change to 0.01 M citrate pH 5.0 at 24°C. (C) Paracrystals formed after a fast pH change to 0.01 M tris pH 8.0 at 24°C. Bar represents 100 nm.
Figure 30. Schlieren patterns of FTV protein under various conditions of pH and temperature after a slow pH change. All samples were prepared by dialysis against the desired 0.01 M buffer, at 4°C overnight and then for 48 hours at 24°C (patterns on the left) or 4°C (patterns on the right).

(A) Upper: 6S species in 0.01 M citrate, pH 4.0; lower: 4S species in 0.01 M citrate, pH 5.0. (B) Upper: 6S species in 0.01 M MES, pH 6.0; lower: 6S species with a minor 8S species in 0.01 M MES, pH 7.0. (C) Upper: 5S species with a minor peak also sedimenting at 5S in 0.01 M tris, pH 8.0; lower: 6S species with minor species of 8S in 0.01 M glycine-NaOH. (D) Upper: 6S species in 0.01 M citrate, pH 4.0; lower: 9S species in 0.01 M citrate, pH 5.0. (E) Upper: 7S species in 0.01 M MES, pH 6.0; lower 5S species in 0.01 M MES, pH 7.0. (F) Upper: 6S species in 0.01 M tris, pH 8.0; lower: 4S species in 0.01 M glycine-NaOH, pH 9.0

Runs were done at temperatures of about 20°C (patterns on the left) or about 4°C (patterns on the right). Protein concentration was about 3 mg ml. Photographs were taken between 17 and 23 minutes after reaching speed. Rotor speed was 50,740 rpm. Sedimentation is to the right.
formed after a fast pH change to pH 4.0, but not after a slow pH change, may not be equilibrium products. Paracrystal formation was strongly dependent on pH but the formation of rods and ropes was not.

3.5.4 The effect of temperature

The effect of temperature on FTV coat protein was examined under conditions of fast and slow pH change (see Materials and Methods) at 4°C. Analytical ultracentrifugation was done at about 4°C and grids of the samples were prepared for electron microscopy.

The results of analytical ultracentrifugation and electron microscopy after a fast pH change at 4°C are shown in Figure 28D-F and Tables 4 and 5. Protein sedimenting at 5-7S was present at pH 4.0 to 9.0. A species which sedimented at 31S was present in 0.01 M glycine-NaOH, pH 9.0. Rapidly-sedimenting material was present at pH 5.0 to 8.0.

Electron microscopy (Table 5) showed that rods of various lengths were present at pH 5.0 to 8.0, along with very short rods and end-ons. No ropes or paracrystals were present.

The presence of the 31S species at pH 9.0, the absence of ropes and paracrystals and the appearance of very short rods and end-ons at 4°C is a sign that the equilibrium shifted towards smaller products at the lower temperature. The 31S material may be a sub-assembly product for the formation of ropes, which were observed in the warm samples. Calculations indicate that the 31S product is probably 6 disks or turns of a helix with about 9 subunits/turn.

The results of analytical ultracentrifugation and electron
microscopy after a slow pH change at 4°C are shown in Figure 30 and Tables 4 and 5. The protein sedimented at 5-7S at pH 4.0 and pH 6.0 to 8.0. The sloping baseline at pH 8.0 (Fig. 30F, upper) indicates that a heterogeneous population of larger aggregates also existed. A single species was present which sedimented at 9S at pH 5.0 and 4S at pH 9.0. Rapidly-sedimenting material was present at pH 6.0 to 9.0. For this reason, the peaks shown in Figure 29D-F are larger at pH 4.0 and 5.0 than at pH 6.0 to 9.0, although the initial protein concentrations were the same.

Temperature had no effect on the small aggregates at pH 4.0 to 7.0. At pH 8.0 to 9.0, the sedimenting material was more heterogeneous and, therefore, the equilibrium appeared to involve a wider range of aggregates at 4°C than at 24°C.

The results of electron microscopy are summarized in Table 5. At pH 4.0 and 5.0, non-specific aggregation occurred. From pH 6.0 to 9.0 rods were observed with great variation in length. Some end-ons were present. No ropes or paracrystals were observed.

The results at 4°C show that the size of the small aggregates was not affected by temperature or pH. Calculations indicate that they consist mainly of pentamers and/or hexamers. The formation of ropes, paracrystals and, to a lesser extent, rods was sensitive to low temperature and may be entropy-driven. Rope formation may proceed from a 31S product with 6 disks or turns of a helix and about 9 subunits/turn.

3.5.5 Effect of KCl

The effect of KCl on the behaviour of FTV coat protein was examined
using a slow pH change (see Materials and Methods) to 0.01 M MES, pH 7.0 containing 0 M, 0.1 M or 0.2 M KCl.

The results of analytical ultracentrifugation are shown in Figure 31. In the absence of KCl, the protein was present as a major species which sedimented at 6-7S and a minor species which sedimented at 4S. In the presence of 0.1 M and 0.2 M KCl, the protein sedimented at 4S. The results indicate that KCl inhibits the formation of small aggregates (probably hexamers) of FTV protein, which may be stabilized mainly by ionic interactions.

Rapidly-sedimenting material was present in all samples. Its quantity and sedimentation rate were reduced in the presence of KCl. Electron microscopy showed that ropes formed in the absence of KCl, while only precipitate was observed in the samples that contained KCl. This indicates that the 7S aggregate may be a sub-assembly product for rope formation.

3.5.6 Effect of protein concentration

The effect of protein concentration on FTV coat protein was examined with a slow pH change to 0.01 M MES, pH 7.0 (see Materials and Methods). The results of analytical ultracentrifugation are shown in Figure 32. At a protein concentration of 0.25 mg/ml (upper), a single species was present which sedimented at 3S. At a concentration of 1.5 mg/ml, most of the protein sedimented at 7S but a small amount sedimented at 3S and a rapidly-sedimenting species was also present. Ropes formed only at the higher protein concentration.

The results show that a concentration-dependent equilibrium existed
Figure 31. Schlieren patterns showing the effect of KCl on FTV protein at pH 7.0. The protein was prepared in distilled water and dialyzed against 0.01 M MES pH 7.0 containing the appropriate concentration of KCl. Dialysis was at 4°C overnight and then at about 24°C for 48 hours.

(A) Upper: 4S product in 0.2 M KCl; lower: 6S product with a small amount of 4S material with no KCl. (B) Upper: 4S product in 0.01 M KCl; lower: 6S product with 4S shoulder with no KCl. Sedimentation rates for the very small peaks in (A) and (B) upper could not be measured. Runs were done at 20°C at a speed of 50,740 rpm. Protein concentration was about 2 mg/ml. Photographs were taken between 17 and 20 minutes after reaching speed. Sedimentation is to the right.
Figure 32. Schlieren patterns showing the effect of protein concentration on FTV protein at pH 7.0. The protein, prepared in distilled water, was dialyzed against 0.01 M MES, pH 7.0 at 4°C overnight and then at about 24°C for 48 hours, at the required protein concentration. Upper: 3S product at 0.25 mg/ml; lower: minor 3S species and major 7S species with a 7S shoulder, at 1.5 mg/ml. The run was done at 20°C using 30 mm cells. Rotor speed was 50,740 rpm. The photograph was taken 38 minutes after reaching speed. Sedimentation is to the right.
between the 3S and 7S species. This probably involved monomers and/or dimers and pentamers and/or hexamers. Rope formation depended on protein concentration. Therefore, the 7S species may be a sub-assembly product for the formation of rods and, subsequently, ropes.

3.5.7 Discussion

The results showed that the formation of small aggregates of FTV protein is unaffected by pH in the range of pH 4.0 to 9.0 or the rate of its change, or temperature in the range of 4-20°C. The small aggregates probably consist mainly of hexamers at pH 7.0, but sizes may range from monomers or dimers up to heptamers or octamers.

A concentration-dependent equilibrium existed between the 3S species (monomers and/or dimers) and the 7S species (pentamers and/or hexamers) at pH 7.0. The equilibrium was sensitive to the presence of KCl, indicating that ionic interactions played an important role in the formation of the 7S aggregate.

The formation of paracrystals, which were equilibrium products only at pH 5.0, was sensitive to pH and temperature. It may involve carboxyl-carboxylate interactions on the surfaces of the particles composing the paracrystals or simply result from reduced charge repulsion between particles at low pH.

Rope and rod formation was relatively independent of pH. Rope formation and, to some extent, rod formation were inhibited by low temperature. This indicates that these processes may be entropy-driven. Their sensitivity to KCl and protein concentration implies that the 7S aggregate, the
formation of which is concentration-dependent and stabilized by ionic interactions, is required for rod and, subsequently, rope formation.

A 31S species formed at pH 9.0, 4°C after a fast pH change. It probably consists of 6 disks or helical turns. It is unlikely to represent a sub-assembly product. Six disks would probably be too large to start helical rod formation and make a disk-to-helix transition. The appearance of this species is coincident with the absence of long rods, which formed at 4°C at pH 5.0 to 8.0. This indicates that rod formation depends more on entropy at pH 9.0 than at lower pH levels. Perhaps other stabilizing forces for rod formation exist at pH 5-8 that are absent at pH 9.0 (e.g. specific amino acid interactions and ionic linkages).

No equilibrium polymerization occurred at pH 4.0. It appears that polymerization of FTV protein requires a pH-mediated interaction that may involve a carboxyl group of either aspartic acid or glutamic acid. At the upper end of the pH range for rod and rope formation, lysine, cysteine or tyrosine may be involved. Since rod and rope formation was extensive at pH 9.0, 20°C, cysteine is probably not involved. The pKₐ of its sulfhydryl group (pKₐ = 8.3) is rather low. Entropy may be a driving force for rod and rope formation, which are inhibited by low temperature. Therefore, tyrosine is probably a better candidate for the interaction than lysine, because the former is the more hydrophobic in character.

Carboxyl-carboxylate pairs control the polymerization of TMV protein (Caspar, 1963; Stubbs et al., 1977) and may control that of PMV protein. Both of these proteins polymerize into virus-like particles at low pH
levels (Durham et al., 1971; Durham and Klug, 1971; Erickson et al., 1976). Equilibrium polymerization of FTV protein results in the formation of virus-like particles at pH 5.0-9.0 (see sections 5.2, 5.7). Particle formation may be controlled by a carboxyl group of aspartic or glutamic acid at the lower end of this pH range. Even with abnormally titrating groups, carboxyl-carboxylate pairs like those involved with the polymerization of TMV protein could not be maintained to pH levels as high as pH 9. Therefore, carboxyl-carboxylate pairs could not regulate the polymerization of FTV protein, which is extensive at pH 9. If a carboxylic acid is involved, as the results at low pH suggest, a highly basic amino acid residue, such as lysine, tyrosine or cysteine, would have to interact with the carboxyl group. Such an interaction with lysine would be ionic and might explain the results in the presence of KCl: this type of interaction could be involved in helix formation and also in the formation of a sub-assembly product such as the 7S aggregate, which is sensitive to ionic strength.

Helix formation by FTV protein is inhibited by low temperature. An interaction between a carboxylic acid and lysine would be affected by temperature. Low temperature reduces the entropy contribution from water displacement and the dissociation of lysyl residues is affected by temperature.

The results show that the sub-assembly product for the polymerization of FTV protein is probably quite small. There is no evidence for the formation of a discrete disk-type aggregate in detectable quantity. If such aggregates exist, they must result from reactions that produce them in much smaller quantities than those that result in disk formation by
TMV protein, and in the production of the 14S species of PMV and CYMV proteins. The 7S aggregate, which probably consists of pentamers and/or hexamers, is the most likely sub-assembly product of FTV protein.

Protein-protein interactions are probably very important in controlling capsid formation by FTV protein during virus assembly. In fact, they seem to be of comparable strength to those of BCV protein. The interactions involved in the polymerization of BCV and FTV proteins are not the same because FTV protein polymerizes over a wider pH range than BCV protein, but the size of their sub-assembly products may be. The results with both proteins show that the involvement of carboxyl-carboxylate linkages (as opposed to linkages involving one carboxyl group and a basic residue) and the existence of disk-type sub-assembly products are not common factors in the formation of helical plant virus capsids.

3.6 The behaviour of VMV coat protein

3.6.1 Effect of pH

The effect of fast and slow pH changes (see Materials and Methods) on the behaviour of VMV coat protein at 20°C and 4°C was examined using analytical ultracentrifugation and electron microscopy.

3.6.2 Effect of a fast pH change

The results of analytical ultracentrifugation after a fast pH change at 20°C are shown in Figure 33A-C and Table 6. At pH 5.0 to 8.8, the protein sediments at about 7S indicating that the formation of the
Figure 33. Schlieren patterns of VMV protein after fast pH changes at 20°C or 4°C. All samples were prepared in water and then adjusted to the desired pH by the addition of 1 M buffer to a final concentration of 0.01 M. The pH was adjusted at either 24°C (patterns on the left) or 4°C (patterns to the right).

(A) Upper: 3-5S material in 0.01 M citrate, pH 4.0; lower: 7S species in 0.01 M citrate, pH 5.0. (B) Upper: 7S species in 0.01 M MES, pH 6.3; lower: 7S species in 0.01 M MES, pH 7.2. (C) Upper: major 7S species, 7S shoulder and minor 5S species in 0.01 M tris, pH 7.7; lower: major 7S species, 7S shoulder and minor 6S species in 0.01 M glycine-NaOH, pH 8.8. (D) Upper: 7S species in 0.01 M citrate, pH 4.0; lower: 7S species in 0.01 M citrate, pH 5.0. (E) Upper: 6S species in 0.01 M MES, pH 5.7; lower: 7S species in 0.01 M MES, pH 7.2. (F) Upper: 5S species in 0.01 M tris, pH 8.0; lower: 8S species in 0.01 M glycine-NaOH, pH 9.0.

Runs were done at temperatures of about 20°C (patterns on the left) or about 4°C (patterns on the right). Protein concentration was about 2 mg/ml. Photographs were taken between 17 and 19 minutes after reaching speed. Rotor speed was 50,740 rpm. Sedimentation is to the right.
Table 6. The effect of pH and temperature on the sedimentation of VMV coat protein

<table>
<thead>
<tr>
<th>pH Change</th>
<th>Fast</th>
<th>Slow</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20°C</td>
<td>4°C</td>
</tr>
<tr>
<td>Temperature</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH *</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4**</td>
<td>3-5</td>
<td>7</td>
</tr>
<tr>
<td>5</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>6</td>
<td>7</td>
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<td>7</td>
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<td>7</td>
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<tr>
<td>8</td>
<td>(5), 7</td>
<td>6</td>
</tr>
<tr>
<td>9</td>
<td>(5), 7</td>
<td>8</td>
</tr>
</tbody>
</table>

Bracketed values are minor species

* pH may not be exact for fast pH changes. See Figure 33 for precise pH values

** contained precipitate at 20°C
small aggregates was insensitive to changes in pH. Small quantities of protein which sedimented at 5S were also present at pH 7.7 and 8.8. At pH 4.0, most of the protein precipitated but a small amount was present which sedimented at 3-5S.

Electron microscopy showed disordered aggregation at all pH levels.

3.6.3 Effect of a slow pH change

The results of analytical ultracentrifugation after a slow pH change at 20°C are shown in Figure 34A-C and Table 6. They are the same as those from the fast pH change. At pH 4.0, where most of the protein precipitated, a small amount of protein was present which sedimented at 2S. This probably consisted of monomers. At pH 5.0 - 9.0, a single species was present which sedimented at about 8S. This probably consisted mainly of hexamers. The results indicate that the samples which contained a small amount of 5S material after a fast pH change to pH 7.7 and 8.8 had not reached equilibrium because the 5S species was absent after a slow pH change to pH 8.0 and 9.0.

Electron microscopy again showed no ordered aggregation.

3.6.4 Effect of temperature

The results of analytical ultracentrifugation after a fast pH change at 4°C are shown in Figure 33D-F and Table 6. At all pH levels, the protein was present as a single species which sedimented at 7S. This shows that the formation of small aggregates of VMV protein was
Figure 34. Schlieren patterns of VMV protein prepared slowly under various conditions of pH and temperature. All samples were prepared in water at 4°C and then adjusted to the desired pH by dialysis against the appropriate 0.01 M buffer, at 4°C overnight and then at about 24°C (patterns on the left) or 4°C (patterns on the right) for 48 hours.

(A) Upper: 2S species in 0.01 M citrate, pH 4.3; lower: 8S species in 0.01 M citrate, pH 5.2. (B) Upper: 9S species in 0.01 M MES, pH 6.0; lower: 8S species in 0.01 M MES, pH 7.0. (C) Upper: 8S species in 0.01 M tris, pH 8.0; lower: 7S species in 0.01 M glycine-NaOH, pH 9.0. (D) Upper: 9S species in 0.01 M citrate, pH 4.0; lower: 8S species in 0.01 M citrate, pH 5.0. (E) Upper: 7S species in 0.01 M MES, pH 6.0; lower: 7S species in 0.01 M MES, pH 7.0. (F) Upper: 7S species in 0.01 M tris, pH 8.0. Small peaks were present at pH 6.0, 7.0 and 8.0 which sedimented at about the same rate as the major peaks, but flattened out very quickly; lower: 6S species in 0.01 M glycine-NaOH, pH 9.0.

Runs were done at temperatures of about 20°C (patterns on the left) or about 4°C (patterns on the right). Protein concentration was 2-3 mg/ml. Photographs were taken between 17 and 21 minutes after reaching speed with the exception of (E), which was taken after 30 minutes. Rotor speed was 50,740 rpm. Sedimentation is to the right.
not affected by the usual changes in pH or temperature.

The results of a slow pH change at 4°C are shown in Figure 34D-F and Table 6. At all pH levels, the protein sedimented at 7S-8S. The protein concentration of these samples was higher than in the other pH series but was not different enough to cause a measurable difference in sedimentation rate.

Electron microscopy showed disordered aggregation in all samples.

3.6.5 Effect of KCl

The effect of 0.2 M KCl on the behaviour of VMV coat protein was examined at pH 8.0, 20°C with a slow pH change. The results of analytical ultracentrifugation are shown in Figure 35A. In both 0 M and 0.2 M KCl, the protein was present as a single species which sedimented at 7S. Therefore, 0.2 M KCl did not affect the formation of the VMV protein aggregates, which are presumably not stabilized by ionic interactions.

3.6.6 Effect of protein concentration

The behaviour of the protein at different concentrations indicates that a concentration-dependent equilibrium existed between the 4S and 7S material at pH 8.0.

The results of analytical ultracentrifugation after a slow pH change to pH 8.0 at 20°C are shown in Figure 35B. Protein at a concentration of 0.25 mg/ml (upper) and 2.5 mg/ml (lower) sedimented as a single species at 4S and 7S, respectively.
Figure 35. Schlieren patterns showing the effect of KCl and protein concentration on VMV protein at pH 8.0.

(A) Schlieren pattern showing the effect of 0.2 M KCl on VMV coat protein. The protein was prepared in water and then slowly adjusted to 0.01 M tris, pH 8.0 at 20°C M KCl (upper) or no KCl (lower). Upper: 7S product in 0.01 M tris, pH 8.0, 0.2 M KCl; lower: 6S product in 0.01 M tris, pH 8.0. Run was done at about 20°C. Protein concentration was 2 mg/ml. Photograph was taken 25 minutes after reaching a rotor speed of 50,740 rpm. Sedimentation is to the right. (B) Schlieren patterns showing the effect of protein concentration on VMV protein after a slow pH change in 0.01 M tris, pH 8.0 at 20°C. Upper: 4S product at 0.25 mg/ml; lower: 7S product at 2.5 mg/ml. Run was done at about 20°C at a rotor speed of 50,740 rpm. Photograph was taken 27 minutes after reaching speed. Sedimentation is to the right.
In summary, the results show that small aggregates of VMV coat protein existed as a species which sedimented at about 7S and probably consisted of pentamers and/or hexamers. It was not affected by pH, rate of pH change, temperature or 0.2 M KCl.

3.6.7 Discussion

The results showed that VMV protein sediments at approximately 7S, regardless of the pH, temperature or ionic strength. The formation of this aggregate must be stabilized by ionic and hydrophobic interactions since it is unaffected by temperature or ionic strength.

The 7S species, which probably consists mainly of pentamers and/or hexamers, exists in a concentration-dependent equilibrium with a 4S species, which probably consists mainly of dimers and/or trimers.

The results indicate that protein-protein interactions do not play a major role in directing capsid formation during VMV assembly. Therefore, this must be achieved by protein-RNA interactions.

As with PVX and FTV, it appears unlikely that a disk aggregate exists as a sub-assembly product for VMV assembly. The sub-assembly product is probably a hexamer, which sediments at approximately 7S. This product is considerably smaller than the protein disks that initiate the assembly of TMV and TRV (Butler and Klug, 1971; Butler, 1971, 1972; Morris and Semancik, 1973; AbouHaidar et al, 1973; AbouHaidar, 1976) and the 14S aggregates of PMV and CYMV proteins (Erickson and Bancroft, 1978; Bancroft et al, 1979).
3.7 The behaviour of TVX coat protein

3.7.1 Effect of pH

The effect of a slow pH change (see Materials and Methods) on the behaviour of TVX coat protein at 20°C and 4°C was examined using analytical ultracentrifugation and electron microscopy. The results are summarized in Table 7.

3.7.2 Effect of slow pH change at 20°C

At pH 4.0, much of the protein precipitated. The remainder sedimented at 3S. At pH 5.0 to 9.0, the main species present sedimented at 7S. Some precipitate was present at pH 5.0 and usually at pH 6.0. A typical Schlieren pattern is shown in Figure 36.

Electron microscopy revealed the formation of short rods at pH 5.0 which must have sedimented at low speed with the precipitate. Disordered aggregates were present at all other pH levels.

3.7.3 Effect of a slow pH change at 4°C

The behaviour of TVX protein was not affected by temperature. At 4°C after a slow pH change to pH 4.0, precipitation occurred. A single species was observed which sedimented at 4S. At pH 5.0 to 9.0, the protein sedimented at 7S. Some precipitate was present at pH 5.0. A typical Schlieren pattern is shown in Figure 36.

Electron microscopy again revealed short rods at pH 5.0 (Fig. 37) and disordered aggregates at all other pH levels.
Table 7. The effect of pH and temperature on the sedimentation of TVX protein after a slow pH change

<table>
<thead>
<tr>
<th>pH</th>
<th>20°C</th>
<th>4°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.0</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>5.0</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>6.0</td>
<td>7</td>
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<td>7.0</td>
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<td>8</td>
</tr>
<tr>
<td>8.0</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>9.0</td>
<td>6</td>
<td>7</td>
</tr>
</tbody>
</table>
Figure 36. Schlieren patterns of TVX protein after a slow pH change.

(A) Upper: 7S species in 0.01 M MES, pH 6.0 at 20°C; lower: 7S species in 0.01 M MES, pH 7.0 at 20°C. (B) Upper: 4S species in 0.01 M citrate, pH 4.0 at 4°C; lower: 6S species in 0.01 M citrate, pH 5.0 at 4°C. All runs were done at a protein concentration of 2-3 mg/ml and a rotor speed of 50,740 rpm. Runs were done at 20°C (A) or 4°C (B). Sedimentation is to the right.
Figure 37. Electron micrograph of TVX protein particles after a slow pH change to 0.01 M citrate, pH 5.0 at 4°C. Bar represents 100 nm.
The results show that the formation of small aggregates is unaffected by temperature and by pH in the range of pH 5.0 to 9.0. The precipitation that occurred at pH 4.0 probably reduced the effective protein concentration. The sedimentation of the remaining protein at 3S suggests that a concentration-dependent equilibrium might exist between the 3S (dimers) and 8S (hexamers and/or heptamers) species. Experiments at low protein concentrations have not yet been done to confirm this.

3.7.4 Discussion

The results show that the polymerization of TVX protein occurs only at pH 5.0 and is unaffected by temperature. The formation of small (7S) aggregates, which probably consist of pentamers and/or hexamers, is not affected by pH in the range of 5.0 to 9.0 or by temperature in the range of 4 to 20°C.

Particle formation at pH 5.0 indicates that TVX protein polymerization may involve carboxyl-carboxylate interactions like those involved in TMV assembly (Caspar, 1963; Stubbs et al., 1977).
4.1 Introduction

Hanging drops containing viral coat protein were prepared as described in Materials and Methods. Drops containing BCV protein were prepared at pH 6.0, 7.0 and 8.0, and incubated at 22°C. Those containing the proteins of FTV, VMV or PVX were prepared at pH 6.0 and 8.0. The drops containing FTV and VMV proteins were incubated at 22°C, while those containing PVX protein were incubated at 24°C and 4°C.

All samples were checked for birefringence using polarized light microscopy, before the wells were opened. Aliquants of the drops were then examined using electron microscopy.

All samples are referred to by the concentration of \((\text{NH}_4)_2\text{SO}_4\) in the well.

The results are summarized in Table 8.

4.2 BCV Protein

Crystal formation was observed at 10-25% saturated \((\text{NH}_4)_2\text{SO}_4\) at pH 6.0, at 10-20% saturated \((\text{NH}_4)_2\text{SO}_4\) at pH 7.0 and at 15-35% saturated \((\text{NH}_4)_2\text{SO}_4\) at pH 8.0 (Fig. 38).

Rod formation occurred over a wide range of \((\text{NH}_4)_2\text{SO}_4\) concentrations at pH 6.0, 7.0 and 8.0 (Table 8). An electron micrograph of
Table 8. Particle formation by the coat proteins of several potexviruses in hanging drops with \((\text{NH}_4)_2\text{SO}_4\)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Particle</th>
<th>pH</th>
<th>Temperature</th>
<th>% Saturated ((\text{NH}_4)_2\text{SO}_4) in well</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCV (^a)</td>
<td>Rods</td>
<td>6.0</td>
<td>22</td>
<td>0-40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.0</td>
<td>22</td>
<td>0-50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.0</td>
<td>22</td>
<td>0-40</td>
</tr>
<tr>
<td>PVX (^b)</td>
<td>Rods</td>
<td>6.0</td>
<td>24</td>
<td>15-50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.0</td>
<td>4</td>
<td>20-50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.0</td>
<td>24</td>
<td>15-40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.0</td>
<td>4</td>
<td>25</td>
</tr>
<tr>
<td>FTV (^c)</td>
<td>Rods, paracrystals, incomplete ropes</td>
<td>6.0</td>
<td>22</td>
<td>15-40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.0</td>
<td>22</td>
<td>20-30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.0</td>
<td>22</td>
<td>45-50</td>
</tr>
<tr>
<td>VMV (^b)</td>
<td>Rods</td>
<td>6.0</td>
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<td></td>
<td>Ropes</td>
<td>6.0</td>
<td>22</td>
<td>15</td>
</tr>
</tbody>
</table>

\(^a\) also formed rods in the sedimentation experiments (short ones at pH 4.0 and 5.0; long ones at pH 6.0-8.0)

\(^b\) no polymerization in the sedimentation experiments

\(^c\) formed rods and ropes in the sedimentation experiments at pH 5.0-9.0, and paracrystals at pH 4.0 and 5.0
Figure 38. Polarized light micrographs of hanging drops containing BCV coat protein at pH 8.0, 22°C.

(A) 15% saturated (NH$_4$)$_2$SO$_4$; (B) 20% saturated (NH$_4$)$_2$SO$_4$.

Magnification is about 200x.
BCV protein rods is shown in Figure 39.

The results show that, unlike the proteins of PVX, FTV and VMV, BCV protein formed the same type of structures in the presence of \((NH_4)_2SO_4\) as in its absence in the hanging drops and the sedimentation experiments.

4.3 PVX protein

Crystal formation occurred at 24°C at 5-40% saturated \((NH_4)_2SO_4\) pH 6.0, and at 35% and 40% saturated \((NH_4)_2SO_4\) pH 8.0. No crystals formed at 4°C.

Rod formation occurred at both temperatures. The results are summarized in Table 8. Electron micrographs of typical PVX protein rods are shown in Figure 40.

The results show that PVX protein polymerized in the presence of \((NH_4)_2SO_4\) at 24°C and 4°C, but requires higher concentrations of the salt than BCV protein.

4.4 FTV protein

Crystals formed at 20-50% saturated \((NH_4)_2SO_4\) at pH 6.0 and 8.0.

Rods and paracrystals of FTV protein formed at fairly high concentrations of \((NH_4)_2SO_4\), as listed in Table 8. A portion of a paracrystal is shown in Figure 41. Some incomplete or broken ropes appeared at 45% and 50% saturated \((NH_4)_2SO_4\) at pH 8.0.
Figure 39. Electron micrograph of BCV protein in a hanging drop at pH 8.0, 22°C, with 20% saturated (NH₄)₂SO₄ in the well. Bar represents 100 nm.
Figure 40. Electron microscopy of PVX protein particles in a hanging drop with 50% saturated (NH₄)₂SO₄ at pH 6.0, 4°C. (B) is a higher magnification than (A). Bar represents 100 nm.
Figure 41. Electron micrograph of a paracrystal of FTV protein formed in 25% saturated (NH₄)₂SO₄ at pH 8.0. Bar represents 100 nm.
The results show that FTV protein polymerized in two ways: ropes predominated in the absence of \((\text{NH}_4)_2\text{SO}_4\) whereas paracrystals predominated at relatively high concentrations of \((\text{NH}_4)_2\text{SO}_4\).

4.5 VMV protein

Crystals formed at 10-15% saturated \((\text{NH}_4)_2\text{SO}_4\) at pH 6.0 (Fig. 42) and at 5-10% saturated \((\text{NH}_4)_2\text{SO}_4\) at pH 8.0.

The results, summarized in Table 8, show that VMV protein polymerized in the presence of low concentrations (0-15% saturated) of \((\text{NH}_4)_2\text{SO}_4\). Rod formation (Fig. 43A, B), which occurred at both pH 6.0 and 8.0, must have a low rate constant, since it did not occur in the sedimentation experiments, but did occur in hanging drops in the absence of \((\text{NH}_4)_2\text{SO}_4\). Intertwined particles (ropes) (Fig. 43C) formed only at 15% saturated \((\text{NH}_4)_2\text{SO}_4\) at pH 6.0.

4.6 Discussion

McPherson (1976) described methods for the crystallization of proteins. The methods rely upon reducing the solubility of the protein thereby allowing protein molecules to come closer together.

One such method involves the use of \((\text{NH}_4)_2\text{SO}_4\) with vapour phase techniques. Its effect on protein solutions is discussed by Lewin (1974). In solution, the \(\text{NH}_4^+\) ion is only slightly hydrated whereas the \(\text{SO}_4^{2-}\) ion is highly hydrated. Additional water is involved in the formation of hydrated ion pairs. Therefore, the addition of \((\text{NH}_4)_2\text{SO}_4\) reduces the mole fraction of bulk water. Since the hydrated anion \((\text{SO}_4^{2-})\) acts as
Figure 42. Polarized light micrograph of a hanging drop containing VMV protein crystals at pH 6.0 in 10% saturated (NH₄)₂SO₄. Magnification is about 200x.
Figure 43. Electron micrographs of particles of VMV coat protein in hanging drops. Rods in (A) 0% saturated (NH₄)₂SO₄, pH 8.0 and (B) 10% saturated (NH₄)₂SO₄, pH 6.0. (C) Rope formed in 15% saturated (NH₄)₂SO₄, pH 6.0. Bar represents 100 nm.
an H... acceptor, it does not destabilize associated water structures 
\[ ([H_2O]_n)^{\text{}} \] in the bulk medium. This complements its effect in reducing the mole fraction of water because the mole fraction of associated water, \((H_2O)_n\), is lower than that of unassociated water, \((H_2O)_1\). Thus, the main effect of \((NH_4)_2SO_4\) on a protein solution is to reduce the amount of water available to solvate the protein (i.e. increases the effective concentration of protein), which allows the protein molecules to interact.

In addition, \((NH_4)_2SO_4\) displaces the equilibrium of the protein away from hydrated random coils and toward \(\alpha\)-helical and \(\beta\)-conformations. This makes the protein less soluble in water. Also, the reduced availability of water enhances interactions between hydrophilic groups because less water is available for shielding in the presence of \((NH_4)_2SO_4\).

Ammonium sulfate also increases surface tension and interfacial tension. Combined with its effect in reducing the mole fraction of water, this enhances intermolecular hydrophobic group associations.

In summary, \((NH_4)_2SO_4\) affects proteins in several complementary ways which reduce protein solubility and allow protein molecules to interact.

If the \((NH_4)_2SO_4\) concentration is sufficiently high, then the protein will precipitate. A series of concentrations were used in the hanging drops so that at some point below precipitating concentrations of \((NH_4)_2SO_4\), polymerization and crystallization might result.
Crystallization may also be induced by using a pH near the isoelectric pH (pI) of a protein, where the net charge is minimal. At the pI, electrostatic repulsion between molecules is at its lowest and the proteins are their least soluble and least hydrated (Lewin, 1974). These factors make the protein molecules more likely to interact at the pI than at other pH levels.

PVX protein polymerized only in hanging drops at fairly high concentrations of \((\text{NH}_4)_2\text{SO}_4\) (see Table 8), which would reduce the amount of available water and enhance intermolecular interactions between hydrophobic groups and also between hydrophilic groups.

The coat protein of VMV polymerized only in hanging drops at low concentrations of \((\text{NH}_4)_2\text{SO}_4\) (0-15% saturated in the mother liquor). No particles formed in the sedimentation experiments in which \((\text{NH}_4)_2\text{SO}_4\) was absent. Since particle formation occurred in hanging drops lacking the salt, the polymerization process must have a low specific rate constant. The effect of low concentrations of \((\text{NH}_4)_2\text{SO}_4\) in reducing water availability may make certain intermolecular interactions sufficiently favourable to increase the rate constant and the equilibrium constant of the polymerization reactions.

In hanging drops equilibrated with 15% saturated \((\text{NH}_4)_2\text{SO}_4\), VMV protein particles aggregated into "ropes" at pH 6.0 but not at pH 8.0. Charge may be a factor. Since the net negative charge would be greater at pH 8.0 than at pH 6.0, electrostatic repulsion between molecules and/or particles would be greater at the higher pH level. Charge density and therefore the degree of hydration would also be
greater at pH 8.0 than at pH 6.0. These two factors appear to be sufficient to prevent particles from getting close enough together to intertwine at pH 8.0.

The protein of FTV polymerized in the sedimentation experiments, in which \((\text{NH}_4)_2\text{SO}_4\) was absent. The predominant aggregates were ropes, which formed at pH 5.0-8.0 (see section 3.5). However, paracrystals predominated at low pH, where electrostatic repulsion and charge-induced hydration are probably lowest. This could allow paracrystal formation, in which the particles are packed more closely than in the intertwined aggregates (ropes).

Paracrystals also predominated in the presence of fairly high concentrations of \((\text{NH}_4)_2\text{SO}_4\) (see Table 8) at pH 6.0 and 8.0. This is almost certainly due to the effect of the salt in reducing the mole fraction of bulk water and enhancing intermolecular interactions. It seems reasonable that paracrystals, rather than ropes, would predominate under such conditions since the former are more closely packed.

The results of the sedimentation experiments (section 3.2) showed that BCV protein polymerizes in the absence of \((\text{NH}_4)_2\text{SO}_4\) but, unlike FTV protein, did not form paracrystals at low pH. This is probably because the pH-mediated intersubunit interactions, which regulate particle formation by BCV protein and appear to involve histidine, (see section 3.3.2) are weakest at low pH.

The presence of \((\text{NH}_4)_2\text{SO}_4\) in the hanging drops did not induce the formation of BCV protein paracrystals. The effect of the salt
in raising the ionic strength and reducing the amount of available water may have simply stabilized the interactions required for particle formation, but did not appear to promote interactions between particles. It is possible that the latter may have been prevented by electrostatic repulsion, since the negative charge on the particles might be fairly high at neutral pH levels.
CHAPTER 5
STRUCTURAL ANALYSES

5.1 Structural analysis of BCV protein particles

Flexible rod-shaped particles of BCV protein formed in the absence and the presence of \((\text{NH}_4)_2\text{SO}_4\) (see Figs. 14, 16A, 39). Their structure was determined by optical diffraction from electron micrographs. The results are summarized in Table 9.

The particles which formed in the absence of \((\text{NH}_4)_2\text{SO}_4\) produced two major reflections (Fig. 44). An off-meridional reflection occurred at a reciprocal spacing of 35.5 Å \((n=10, \sigma=2.1 \text{ Å})\) and had a \(2\pi r_n\) value of 2.14 \((\sigma=0.39)\). This would originate from a \(J_1\) Bessel function and indicates that the particles were single helices with a pitch of 35.5 Å.

The second reflection occurred near the equator. The value of \(\zeta_l/\zeta_n\) was 3.7 \((\sigma=0.21)\), indicating that \(l=2\) and the true repeat was 7 turns. The value of \(2\pi r_n\) for the near-equatorial reflection was 9.87 \((\sigma=0.69)\). It probably originated from a \(J_9\) Bessel function.

The particles which formed in the presence of \((\text{NH}_4)_2\text{SO}_4\) produced similar diffraction patterns. An off-meridional reflection occurred at a reciprocal spacing of 34.1 Å \((n=7, \sigma=0.5 \text{ Å})\), with a \(2\pi r_n\) value of 2.14 \((\sigma=0.55)\). This indicates single helical geometry, with a pitch of 34.1 Å. A near-equatorial reflection produced a value of 3.56 \((\sigma=0.12)\) for \(\zeta_l/\zeta_n\) again indicating that \(l=2\) and the true repeat was 7 turns. The value of \(2\pi r_n\) for this reflection was 10.88 \((\sigma=1.38)\). It probably originated from a \(J_9\) Bessel function.

180
Table 9. Optical diffraction of particles of ECV protein

<table>
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<th>Reflection</th>
<th>Off-meridional</th>
<th>Near-equatorial</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\left(\text{NH}_4\right)_2\text{SO}_4^{a_j}$</td>
<td>$\text{Pitch} \pm \sigma$</td>
</tr>
<tr>
<td>Protein</td>
<td>+</td>
<td>34.1±0.5</td>
</tr>
<tr>
<td>Protein</td>
<td>-</td>
<td>35.5±0.5</td>
</tr>
<tr>
<td>Combined</td>
<td>±</td>
<td>35.0±1.8</td>
</tr>
<tr>
<td>Virus $^{b_j}$</td>
<td></td>
<td>35.0</td>
</tr>
</tbody>
</table>

$a_j$ = absent, $+ = present$

$b_j$ Richardson et al., 1981
Figure 44. Optical diffraction pattern of a BCV protein particle formed in 0.01 M citrate, pH 8.0.
The data indicate that particles of BCV protein, formed in the absence and the presence of (NH₄)₂SO₄, had the same structure. When the data for the two groups were combined, the off-meridional reflection showed that the pitch was 35.0 Å (n=17, σ=1.8 Å). The value of 2πR₀ was 2.14 (σ=0.45), indicating a J₁ Bessel function and a single helix. The near-equatorial reflection produced a value of 3.68 (σ=0.21) for c₁/cₙ, implying the k=2 and that the true repeat was 7 turns. The value of 10.29 (σ=1.11) for 2πR₀ indicated that this reflection originated from a J₉ Bessel function.

Optical diffraction of native BCV was described by Richardson et al (1981). The virus has a pitch of 35 Å. The near-equatorial reflection on the diffraction patterns gives a value of 3.7 (σ=0.1) for c₁/cₙ. This means that the reflection occurs on the second layer line and that the virus has a true repeat of 7 turns. The value of 2πR₀ for this reflection is 10.3 (σ=1.4), indicating that it originates from a J₉ Bessel function.

The data obtained from optical diffraction of BCV protein particles formed in the absence of the presence of (NH₄)₂SO₄ are consistent with the structure of native BCV: a single helix with a pitch of 35 Å, and 8 5/7 subunits/turn.

5.2 Structural analysis of FTV protein paracrystals

The coat protein of FTV formed paracrystals in hanging drops equilibrated in 15-40% saturated (NH₄)₂SO₄ at pH 6.0 and in 20-30% saturated (NH₄)₂SO₄ at pH 8.0. Electron microscopy showed that individual particles were rare and single-layer aggregates were absent in these
preparations. A few paracrystals formed in 0.01 M citrate, pH 5.0 in the sedimentation experiments (section 3.5) and single layers of aggregated particles with small paracrystalline regions (see Fig. 29B) were also present.

Electron micrographs of the paracrystals (Fig. 45) indicated that the rods were interpenetrated because the particles seemed wider at the ends of the paracrystals than in the middle. This was confirmed using optical diffraction. Unmasked paracrystals produced equatorial reflections at spacings of 100 Å.

The optical diffraction patterns provided some information about the structure of the particles when the mask was applied at the edges of the paracrystals (Fig. 46). A reflection occurred near the meridian at a reciprocal spacing of 34.2 Å (n=22; o=1.2) with a $2\pi R_n$ value of 2.28 (o=1.59). This means that the particles have helical geometry with a pitch of 34.2 Å.

A near-equatorial reflection occurred that was weak and rare on diffraction patterns from individual particles. However, it was strong on some diffraction patterns from paracrystals, when a very small area was masked. This reflection occurred at a reciprocal spacing of 172.4 Å (n=6, o=4.2 Å), giving a repeat of 5.03± 0.15 turns. It had a $2\pi R_n$ value of 10.24 (n=6; o=1.16), indicating that it originated from a $J_9$ Bessel function.

Native FTV has a pitch of 35 Å, a true repeat of 5 turns, and it produces a near-equatorial reflection with $2\pi R_n = 10.8± 1.9$ (Richardson
Figure 45. Electron microscopy of FTV protein paracrystals formed in 0.01 M citrate pH 5.0. The apparent diameter in the middle of the paracystal is less than the actual rod diameter, which can be seen at the end of the paracystal. Bar represents 100 nm.
Figure 46. Optical diffraction pattern of a FTV protein para-cystal, showing the off-meridional reflection (1/35 Å) and the near-equatorial reflection (1/175 Å).
et al., 1981).

The data obtained from optical diffraction of FTV protein particles and paracrystals are consistent with the structure of the native virus. The paracrystals are therefore composed of virus-like protein particles.

5.3 **Structural analysis of PVX protein particles**

Electron micrographs of PVX protein particles, which formed in hanging drops equilibrated with 15-50% saturated (NH₄)₂SO₄ at pH 6.0 and 8.0, at 24°C and 4°C, were used for optical diffraction (Figs. 40, 47A). All the diffraction patterns showed strong meridional reflections at a reciprocal distance from the equator of 34.3 Å (n=13, σ=0.81 Å, ε=2), indicating a stacked disk structure (Fig. 47). There was no evidence of pairing between adjacent disks, like that in the stacked disks of VMV protein.

A minor reflection occurred at a reciprocal distance from the equator of 68.1 Å (n=10, σ=3.2 Å) (Fig. 47C, 48) on ε=1. This means that the true repeat is 2 and that subunits on adjacent disks alternate in position. Using \( r=65 \) Å, \( 2\pi\sigma \), for this reflection was 9.18 (σ=2.45). This means that the order of the Bessel function falls in the range of \( J_5 - J_10 \), but is probably \( J_8 \) or \( J_9 \). Therefore, the number of subunits/disk is 8 or 9. Adjacent disks must be skewed with respect to each other, as shown in the radial projections (Fig. 49). For example, with 9 subunits/disk, adjacent disks would be rotated 20° and with 8 subunits/disk, they would be rotated 22.5°, with respect to each other.
Figure 47. Electron microscopy and optical diffraction of PVX protein particles formed in 20% saturated \((\text{NH}_4)_2\text{SO}_4\) at pH 8.0, 24°C. (A) Electron micrograph (B) Electron micrograph of the particle diffracted in (C). Bar represents 100 nm.
Figure 48. Optical diffraction pattern of a PVX protein particle formed in 15% saturated (NH₄)₂SO₄, pH 6.0 at 24°C.
Figure 49. Radial projections of stacked disk particles, with the disks 35 Å apart and a true repeat of 2.

(A) 8 subunits/disk. (B) 9 subunits/disk. r=65 Å

The (n,λ) coordinates are marked in the diagrams.
A. \( \frac{35A}{2} \) \\
B. \( \frac{35A}{2} \)
In summary, PVX protein particles were composed of stacked disks 34.3 Å apart, containing 8 or 9 subunits/disk. The true repeat was 2 and adjacent disks were rotated 22.5° or 20°, respectively, with respect to each other.

5.4 Structural analysis of VMV protein particles

Rod-shaped particles of VMV coat protein (Table 8; Fig. 43) formed in hanging drops at pH 6.0 and 8.0 in 0-15% saturated (NH₄)₂SO₄. Their structure was determined using optical diffraction and radial projections of structural models.

Particle diameters were measured from diffraction patterns obtained from the electron micrographs. A value of 126.5 Å (σ=6.9 Å) was found from groups composed of 2-3 particles side-by-side. Single particles had diameters of about 140 Å. This indicated that the protein particles had the same diameter as the virus and that packed diameters were slightly smaller than unpacked ones. A value of r=65 Å was used in calculating 2πr/σ from the diffraction patterns. The location of reflections from particles in arrays, in reference to the meridional and equatorial axes, did not differ significantly from those from single particles. Thus, the values of 2πr/σ did not segregate with respect to the number of particles that were diffracted.

Optical diffraction of the VMV protein particles (Fig. 50A) showed a strong reflection at 1/36 Å (a periodicity of 36 Å, σ=1.4 Å). Its displacement from the meridian suggested a helical structure with more than one helix and possibly, but not necessarily, more than one start. This point is illustrated in Figure 51. Optical filtering, which was
Figure 50. Optical diffraction patterns of particles of VMV coat protein. (A) One particle (B) Several parallel particles.
Figure 51. Radial projections of different particle types: single helix; 2-start helix; 2 parallel helices.

(A) a single helix with a pitch of 35 Å. (B) Two parallel helices with 35 Å between them. The pitch is 70 Å. At the bottom of the projection, the two helices are shown to be in register with respect to the horizontal axis of the rod. This makes it a 2-start helical particle. (C) The same two parallel helices shown out of register with respect to the horizontal axis. This is not 2 starts but merely 2 parallel helices. (A) will produce a J₁ reflection at 1/35 Å. (B) and (C) will both produce a J₂ reflection at 1/35 Å.
used to reconstruct particles using this reflection, also indicated multiple helices (Fig. 52).

The $2\pi r_n$ values were calculated for the position of the $1/36$ Å reflection. Measurements were taken from 20 particles or groups of particles. The groups of particles consisted of 2-3 particles in parallel, 5-6 particles in rafts of 1 or 2 layers and three-dimensional arrays of particles. Most of the three-dimensional groups and some of the rafts produced more than one reflection at a distance of $1/36$ Å from the equator (Fig. 50B). As a result, 30 $2\pi r_n$ values were obtained from 20 diffraction patterns. The mean $2\pi r_n$ was 8.37 ($\sigma=2.36$ Å). A histogram of the data is shown in Figure 53. The bimodal distribution of $2\pi r_n$ indicates that the samples contained 2 types of particles with different numbers of helices.

The $2\pi r_n$ values were listed in order of increasing magnitude and the cumulative variance ($s'$) was calculated (Table 10). A graph of $2\pi r_n$ plotted against the change in cumulative variance ($\Delta s'$) is shown in Figure 54. A sharp increase in $\Delta s'$ indicates the beginning of a new class. On this basis, $2\pi r_n$ values up to 7.06 were placed in the first class and all values above 7.06 were placed in the second class. This meant that there were 2 types of rods with $2\pi r_n$ values of 5.98 ($\sigma=0.64$, $n=13$) and 10.20 ($\sigma=1.27$, $n=17$) at $1/36$ Å, which indicated 5 and 9 helices ($J_5$ and $J_9$), respectively. The terminology for describing multi-helical particles is illustrated in Figure 55.

Another strong reflection occurred in the diffraction patterns (Fig. 50B). It reflected a periodicity of 143.3 Å ($\sigma=4.9$ Å) or 144 Å.
Figure 52. Optical reconstruction of a VMV protein particle. Its diffraction pattern is shown in Figure 50B. (A) unfiltered (B) using only the 1/36 Å reflection.
Figure 53. Histogram of $2\pi r_R$ values for the 1/36 Å reflection from VMV protein particles. $n=30$, from 20 diffraction patterns.
Table 10. Values of $2\pi r R_n$, from the 1/36 Å reflection, in increasing order of magnitude, with cumulative variance ($s'$) and the change in cumulative variance ($\Delta s'$).

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<th>$2\pi r R_n$</th>
<th>$s'$</th>
<th>$\Delta s'$</th>
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<tr>
<td>6.13</td>
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Figure 54. The $2\pi R_n$ values, for the 1/36 Å reflection from VMV protein particles against the change in cumulative variance ($\Delta s'$) from $2\pi R_n$ values listed in increasing order of magnitude.
Figure 55. Terminology for describing a multi-helical particle. This diagram shows a radial projection of 5 parallel helices. The distance between adjacent helices is 35 Å. The pitch equals this distance x the number of helices (5x35 Å or 175 Å, in this case). The crystallographic repeat or vertical repeat is the smallest asymmetric unit and in this case is smaller than either the true repeat or the pitch. Bar represents 50 Å.
DISTANCE BETWEEN ADJACENT HELICES 35 Å

VERTICAL OR CRYSTALLOGRAPHIC REPEAT 140 Å

PITCH 175 Å

TRUE REPEAT OF ONE HELIX 875 Å

5 PARALLEL HELICES

50 Å
(4x 36 Å) and had a mean value of $2\pi r \tau_1$ of 11.71 ($\sigma=1.36$). This indicated that the reflection originated from a $J_{10}$ Bessel function.

The $2\pi r \tau_1$ values and the order of the Bessel functions for the two groups of rods are listed in Table 11. The diffraction patterns indicate that there are two types of rod with 5 and 9 helices, 36 Å between adjacent helices and a vertical repeat of 144 Å. The pitch of the helices would be 180 Å and 324 Å respectively. Radial projections were drawn of models that fit these geometric criteria. They were compared to the data and assessed on the basis of the ($\kappa, \ell$) coordinates, determined according to the method of Klug et al. (1958), and the number of subunits/unit volume, as compared to the virus.

The main characteristics of the "5-start" models are listed in Table 12. The most suitable model is shown in Figure 56. It has 5 starts with 36 Å between adjacent helices, a pitch of 180 Å, 8 3/4 subunits/turn and a vertical repeat of 144 Å. The true repeat is 720 Å. The ($\kappa, \ell$) coordinates are shown in the figure. The transform (Fig. 57A) has a $J_{10}$ reflection on the first layer line and a $J_{-5}$ on the fourth layer line, which are consistent with the data. It also has a $J_{15}$ reflection on the second layer line and a meridional ($J_0$) reflection on the seventh layer line. These would be too far from the origin to appear on the diffraction patterns. This structure would also produce a $J_5$ reflection on the third layer line. This may be present on a few on the diffraction patterns but it is neither strong nor common. The number of subunits/unit volume is 1.03x the number in the virus. The ratio of $R_1:R_4$, where $R_1$ and $R_4$ are the distances between the maxima and the meridian on the first and fourth layer lines, respectively, is 1.96 for the
The $2\pi R_n$ values for the principal reflections and the order of the Bessel functions implied by them, for the 2 types of VMV protein particles.

<table>
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<th>$\ell$</th>
<th>Periodicity ($\text{Å}^{-1}$)</th>
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<th>Type 2</th>
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<td>4</td>
<td>36</td>
<td>5.98±0.64$^a$</td>
<td>5</td>
</tr>
<tr>
<td>1</td>
<td>144</td>
<td>11.71±1.36$^c$</td>
<td>9-11</td>
</tr>
</tbody>
</table>

$^a$ $J_5$ circular = 6.42

$^b$ $J_9$ circular = 10.71

$^c$ $J_{10}$ circular = 11.77
Table 12. Models for Type I particles with 5 parallel helices, 36 Å between adjacent helices and a vertical repeat of 144 Å were constructed. The number of real starts, the absolute value of the \( n \) coordinate at \( \varepsilon = 1 \) and \( \varepsilon = 4 \), and the number of subunits/unit volume as compared to the virus are listed.

| # starts | # subunits/turn | \[|n|\] \( \varepsilon = 1 \) | \( \varepsilon = 4 \) | Ratio of subunit density in protein particle to that in the virus |
|----------|-----------------|---------------------|---------------------|---------------------------------|
| 1        | 7 3/4           | 9                   | 5                   | 0.89                             |
| 5        | 8 3/4           | 10                  | 5                   | 1.03                             |
| 1        | 9 3/4           | 10                  | 5                   | 1.11                             |
Figure 56. Radial projection of the most suitable model for Type 1 VMV protein particles. There are 5 starts, a pitch of 180 Å, 36 Å between adjacent helices, a vertical repeat of 144 Å and 8 3/4 subunits/turn. The true repeat is 720 Å. The \((n, \ell)\) coordinates are marked on the diagram. Bar represents 50 Å.
Figure 57. Transforms from radial projections of models for the structure of VMV protein particles. (A) 5-start particle with 8 3/4 subunits/turn. (B) 9 parallel helices with 8 3/4 subunits/turn. (C) 9 parallel helices with 7 3/4 subunits/turn.
particles and 1.83 for the model.

If the 5-start model were constructed with 7 3/4 subunits/turn, there would be 5 parallel helices but not 5 starts. A J-9 reflection would occur on the first layer line and the number of subunits/unit volume would be 0.89 times the number in the virus. This model is less suitable than the previous one.

With 9 3/4 subunits/turn, instead of 8 3/4, there would be 5 parallel helices but not 5 starts. A J-10 reflection would appear on the first layer line. The number of subunits/unit volume would be 1.11 times the number in the virus. Therefore, 9 3/4 subunits/turn is less suitable than 8 3/4 subunits/turn.

The main characteristics of the "9-start" models are listed in Table 13. The most suitable model is shown in Figure 58. It has 36 Å between adjacent helices, a pitch of 324 Å, 8 3/4 subunits/turn and a vertical repeat of 144 Å. The true repeat is 129 Å. There are 9 parallel helices but not 9 starts. The (n,λ) coordinates are marked on the diagram and the transform is shown in Figure 57B. A J-11 reflection appears on the first layer line (1/144 Å). This is consistent with the data which shows 2πrR = 11.71 (c=1.36). There is a J-9 reflection on the fourth layer line (1/36 Å), which is also consistent with the data. The model also produces a J-12 reflection on the second layer line and a J-7 reflection on the seventh layer line. These would be at the limits of resolution of diffraction and were not observed on the patterns. This model would also produce a J-2 reflection on the third layer line. This unfortunately was not seen on the diffraction
| # starts | # subunits/turn | $|n|$ | $\varepsilon=1$ | $\varepsilon=4$ | # subunits/unit volume: # in virus |
|----------|----------------|-------|--------------|--------------|----------------------------------|
| 1        | 7 3/4          | 10    | 9            |              | 0.89                             |
| 3        | 8 1/4          | 6     | 9            |              | 0.94                             |
| 1        | 8 3/4          | 11    | 9            |              | 1.00                             |
| 3        | 9 3/4          | 12    | 9            |              | 1.11                             |
| 1        | 10 3/4         | 13    | 9            |              | 1.23                             |
| 9        | 11 1/4         | 9     | 9            |              | 1.29                             |
| 1        | 12 1/4         | 10    | 9            |              | 1.37                             |
Figure 58. Radial projection of the most suitable model for Type 2 VMV protein particles. There are 9 parallel helices, a pitch of 324 Å, 36 Å between adjacent helices, a vertical repeat of 144 Å and 8 3/4 subunits/turn. The true repeat is 1296 Å. The (n,ε) coordinates are marked on the diagram. Bar represents 50 Å.
patterns. The number of subunits/unit volume is 1.00x the number in the virus. The ratio of \( R_1 : R_4 \) is 1.15 for the particles. It is 1.20 for the model.

Other models were constructed with 8 1/4, 9 3/4, 10 3/4, 11 1/4 and 12 1/4 subunits/turn. Some of their characteristics are listed in Table 13. They were all unsuitable either because of the number of subunits/unit volume in comparison to the virus or because the order of the Bessel function for the maximum on the first layer line was incompatible with the data, or both.

The radial projection of a rod with 9 parallel helices, a 36 Å between adjacent helices, a pitch of 324 Å, 7 3/4 subunits/turn, a vertical repeat of 144 Å and a true repeat of 1296 Å is shown in Figure 59. Its transform is shown in Figure 57C. It produces a \( J_{-10} \) reflection on the first layer line and a \( J_{-9} \) reflection on the fourth layer line, which is compatible with the data. The other \( (n, \ell) \) coordinates, \((11,2)\), \((1,3)\) and \((12,5)\) did not correspond to any reflections observed on the diffraction patterns. The ratio of \( R_1 : R_4 \) is 1.10 for the model, compared to 1.15 for data. The number of subunits/volume is 0.89x the number in the virus. This implies a 10% increase in the volume of each subunit as compared to those in the viral capsid. Therefore, this model is less suitable than that with 8 3/4 subunits/turn.

As previously mentioned, the optical diffraction patterns of most of the three-dimensional arrays had two reflections on the fourth layer line, corresponding to \( J_5 \) and \( J_9 \). In some cases there were numerous small reflections which spanned the range from \( J_5 \) to \( J_9 \). These results indicate that most of the three-dimensional arrays are composed of two
Figure 59. Radial projection of a model for Type 2 VMV protein particles. There are 9 parallel helices, a pitch of 324 Å, 36 Å between adjacent helices, a vertical repeat of 144 Å and 7 3/4 subunits/turn. The true repeat is 1296 Å. The \((n, \xi)\) coordinates are marked on the diagram. Bar represents 50 Å.
types of rods.

In summary, there are two types of VMV protein rods that formed in hanging drops at pH 6.0 and 8.0 in 0-15% saturated (NH₄)₂SO₄. They are both multiple helices with 36 Å between adjacent helices, a vertical repeat of 144 Å and 8 3/4 subunits/turn. One type of rod is a 5-start helix with a pitch of 180 Å and a true repeat of 720 Å. The other consists of 9 parallel helices, but not 9 starts, with a pitch of 324 Å and a true repeat of 1296 Å. Most of the three-dimensional arrays of particles contained both structures.

5.5 Structural analysis of TVX protein particles formed at pH 5.0

The coat protein of TVX was dialyzed against 0.01 M citrate pH 5.0 at 4°C for three days. Electron microscopy showed that the protein had formed particles (Fig. 37). Their structure was determined using optical diffraction.

The diameter of single particles, measured directly from the electron micrographs, was 140 Å (σ=9 Å). Using optical diffraction, the diameter of individual particles was 141 Å (σ=5 Å) and the packed diameter was 133 Å. These values indicate that some interpenetration occurred in packed particles of TVX protein at pH 5.0.

The diameter of the TVX protein particles is consistent with that of other viruses in the PVX group (Richardson et al., 1981; Tollin et al., 1979, 1981). Allowing for stain accumulation at 5-10 Å from the surface of the particles (Richardson et al., 1981), a radius of 65 Å was used in calculating 2πR values from the optical diffraction patterns of TVX protein particles at pH 5.0.
Optical diffraction patterns of TVX protein particles are shown in Figure 60. There appeared to be two major reflections at 1/36 Å and 1/72 Å. The values of $2\pi R_n$ were $3.54 \pm 0.20$ and $8.06 \pm 0.62$, respectively. These values suggested a J_{2-3} reflection at 1/36 Å and a J_{6-7} reflection at 1/72 Å (Table 14). This implied 2 or 3 parallel helices with 36 Å between adjacent helices. Reconstructions, using optical filtering, were consistent with this conclusion (Fig. 61). Radial projections of potential structures were drawn with 2 or 3 helices, 36 Å between adjacent helices and a vertical repeat of 72 Å.

The particles would have to be 2 or 3 parallel helices repeating in 1 or 2 turns, respectively in order to produce the 72 Å vertical repeat. Transforms were constructed from radial projections. The models were assessed on the basis of: 1) the number of subunits/unit volume or, since the rods were the same diameter as the virus, the number of subunits/unit length; 2) the position of the maxima on the first and second layer lines (1/72 Å and 1/36 Å, respectively); and 3) the ratio of $R_1 : R_2$, where $R_1$ and $R_2$ represent the distance between the reflection and the meridian on the first and second layer lines, respectively.

The transforms constructed from the radial projections of 2-start helices are shown in Figure 62. The parameters of the models are summarized in Table 15. With 6.0 subunits/turn and 2 apparent starts or parallel helices (Fig. 62A), the $(n, \lambda)$ coordinates may be consistent with the data but there are only 24 subunits/140 Å length, compared to 35 subunits in the same length of the virus. This makes it an unacceptable model.
Figure 60. Optical diffraction pattern of a TVX protein rod formed in 0.01 M citrate pH 5.0 at 4°C.
Table 14. The $2\pi R_n$ values for the principal reflections and the order of the Bessel function implied by them, for TVX protein particles at pH 5.0.

<table>
<thead>
<tr>
<th>$\ell$</th>
<th>Periodicity ($\text{Å}^{-1}$)</th>
<th>$2\pi R_n \pm \sigma$</th>
<th>$J_n$</th>
<th>$R_1:R_2 \pm \sigma$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>36</td>
<td>$3.54 \pm 0.20^{a_j}$</td>
<td>2-3</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>72</td>
<td>$8.06 \pm 0.62^{b_j}$</td>
<td>6-7</td>
<td>$2.33 \pm 0.28$</td>
</tr>
</tbody>
</table>

$^{a_j}$ $J_2$ circular = 3.06
$J_3$ circular = 4.32
$^{b_j}$ $J_6$ circular = 7.50
$J_7$ circular = 8.58
Figure 61. Optical reconstructions of a TVX protein particle formed in 0.01 M citrate pH 5.0 at 4°C. (A) Unfiltered (B) Using only the 1/36 Å reflection.
Figure 62. Transforms from radial projections of models for the structure of TVX protein particles at pH 5.0. There are 2 parallel helices with r=65 Å, pitch=72 Å and a repeat of 72 Å. (A) 2 apparent starts with 6.0 subunits/turn. (B) 2 real starts with 7.0 subunits/turn. (C) 2 real starts with 8.0 subunits/turn. (D) 2 apparent starts with 8.0 subunits/turn. (E) 2 real starts with 9.0 subunits/turn.
\[ l \]  
\[ (2,2) \circ (8,1) \circ (7,1) \circ (5,3) \]

\[ D \]

\[ l \]
\[ (2,2) \circ (10,1) \circ (8,1) \]

\[ E \]

\[ \]

\[ l \]
\[ (2,2) \circ (7,1) \circ (5,1) \circ (6,2) \circ (13,2) \]

\[ B \]

\[ l \]
\[ (2,2) \circ (5,2) \circ (2,2) \]

\[ C \]
Table 15. Models of TVX protein particles with 2 parallel helices, 36 Å apart with a pitch and vertical repeat of 72 Å.

<table>
<thead>
<tr>
<th># starts</th>
<th>1</th>
<th>2</th>
<th>2</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td># subunits/turn</td>
<td>6.0</td>
<td>7.0</td>
<td>8.0</td>
<td>8.0</td>
<td>9.0</td>
</tr>
<tr>
<td></td>
<td>6,8</td>
<td>5,7</td>
<td>8,0</td>
<td>7,9</td>
<td>8,10</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2</td>
<td>13</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>#1</td>
<td>6,8,2</td>
<td>9,2,6</td>
<td>7,9</td>
<td>8,10</td>
<td></td>
</tr>
<tr>
<td>#2</td>
<td>0.68</td>
<td>0.80</td>
<td>0.91</td>
<td>1.03</td>
<td></td>
</tr>
<tr>
<td>R1:R2</td>
<td>2.46, 3.16</td>
<td>2.10, 2.81</td>
<td>2.81, 3.51</td>
<td>3.16, 3.88</td>
<td></td>
</tr>
</tbody>
</table>
With 7.0 subunits/turn and 2 real starts (Fig. 62B), the \((n, \xi)\) coordinates are again consistent with the data but there are only 28 sub-units/140 Å length, compared to 35 in the same length of the virus. This is not a reasonable model.

With 8.0 subunits/turn and 2 real starts (Fig. 62C), a repeat occurs every 36 Å and all the reflections occur on the same layer line \((1/36 \, \text{Å})\). This is clearly inconsistent with the data.

With 8.0 subunits/turn and 2 apparent starts (parallel helices) (Fig. 62D), the positions of the maxima are consistent with the data. There are 32 subunits/140 Å length or 0.91x the number in the virus, which seems reasonable.

With 9.0 subunits/turn and 2 real starts (Fig. 62E), the values of \(n\) for the reflection on the first layer line are too high to be compatible with the data.

For 2 parallel helices, the most reasonable model has 8.0 subunits/turn and 2 apparent starts (Fig. 62D). There are 36 Å between adjacent helices with a pitch of 72 Å. The radial projection is shown in Figure 63.

With 3 parallel helices, the helices must repeat in 2 turns to produce a 72 Å periodicity. The characteristics of the models of these particles are summarized in Table 16.

With 7 1/2 subunits/turn and 3 real starts, the \((n, \xi)\) coordinates are compatible with the data. The transform of the radial projection for this model is shown in Figure 64A. The reflections with the coordinates \((0, 5)\) and \((3, 3)\) would be very faint because of the distance between the subunits. However, this structure has only 31 subunits/140 Å length or 0.89x the number of subunits in the same length of the virus. This makes it an unreasonable model.
Figure 63. Radial projection of a model for the structure of TVX protein rods at pH 5.0, with 2 apparent starts. The model has 8.0 subunits/turn, 2 parallel helices with a pitch of 72 Å and a vertical distance of 36 Å between them r=65 Å. The basic rectangle is 72 Å long. The (\(\pi, \epsilon\)) coordinates are marked. Bar represents 50 Å.
Table 16. Models for TVX protein rods with 3 parallel helices, 36 Å between adjacent helices, a pitch of 108 Å and a vertical repeat of 72 Å.

<table>
<thead>
<tr>
<th># starts</th>
<th># subunits/turn</th>
<th>( n )</th>
<th>Ratio subunit density in protein particles to that in virus</th>
<th>( R_1 : R_2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>7 1/2</td>
<td>6, 9</td>
<td>3</td>
<td>0.89</td>
</tr>
<tr>
<td>1</td>
<td>8 1/2</td>
<td>7, 10</td>
<td>3</td>
<td>0.97</td>
</tr>
<tr>
<td>1</td>
<td>9 1/2</td>
<td>8, 11</td>
<td>3</td>
<td>1.12</td>
</tr>
</tbody>
</table>
Figure 64. Transforms from radial projections of models for the structure of TVX protein particles at pH 5.0. There are 3 parallel helices with \( r=65 \, \text{Å} \), pitch=108 \( \text{Å} \) and a repeat of 72 \( \text{Å} \).

(A) 3 real starts with 7 1/2 subunits/turn. (B) 3 apparent starts with 8 1/2 subunits/turn.
With 8 1/2 subunits/turn and 3 apparent starts, the positions of the maxima are compatible with the data. The transform of the radial projection for this model is shown in Figure 64B. The J₄ reflection on the third layer line would be very faint. There are 34 subunits/140 Å length or 0.97x the number in the same length of the virus.

With 9 1/2 subunits/turn, Jₙ for the reflection on the first layer line and the number of subunits/140 Å length would be too high to make it a feasible model.

For 3 parallel helices, the most likely structure consists of 8 1/2 subunits/turn and 3 apparent starts. Each helix has a pitch of 108 Å, with 36 Å between adjacent helices. The radial projection is shown in Figure 65.

In order to determine which of the two models is most compatible with the data, the ratio of $R_1:R_2$ was calculated from the optical diffraction patterns, where $R_1$ and $R_2$ are the distances between the maxima and the meridian on the first and second layer lines, respectively. This ratio had a mean value of 2.33 ($\sigma=0.28$). Patterns from 11 single particles were used. In 4 out of 5 one-sided images, the reflections on the first and second layer lines were on opposite sides of the meridian. This means that in the two prospective models, where there are 2 reflections on the first layer line, the one that occurs has the lower Bessel function of positive value. The ratio of $R_1:R_2$ was calculated on that maximum. For the 3-start model (Figs. 64B, 65) $R_1:R_2=2.04$. For the 2-start model (Figs. 62D, 63) $R_1:R_2=2.81$. There-
Figure 65. Radial projection of the apparent 3-start model for the structure of TVX protein particles at pH 5.0. The model has 8 1/2 subunits/turn, 3 parallel helices with a pitch of 108 Å and a vertical distance of 36 Å between them. There is a vertical repeat of 72 Å and r=65 Å. The basic rectangle is 72 Å long. The (π,ξ) coordinates are marked. Bar represents 50 Å.
fore, the 3-start model (Fig. 65) is more compatible with the data than
the 2-start model. The differences between the model and the actual $R_1:R_2$
ratios imply that the particles were flattened.

A minor reflection sometimes occurred near the equator at 1/144 Å.
The value of $2\pi r_n$ for this reflection was 10.26±1.08, which corresponds
to a $J_{8-9}$ reflection. Radial projections were drawn of 2- and 3-start
helical models with 36 Å between adjacent helices and vertical repeats
of 72 Å and 144 Å. The transforms are shown in Figure 66. Characteristics
of the models are summarized in Table 17. In all cases, the
order of the Bessel function for the 1/72 Å reflection ($2=2$) was too
high in comparison to the data. Since the reflection at 1/72 Å was
much stronger and more common than the apparent reflection at 1/144 Å,
the latter was not considered to be a real reflection.

In summary, TVX protein in 0.01 M citrate, pH 5.0 at 4°C for
3 days forms rods of the same diameter as the virus. They consist of
3 parallel helices (not 3 starts) with a pitch of 108 Å, a vertical
distance of 36 Å between adjacent helices, a vertical repeat of 72 Å
and 8 1/2 subunits/turn.

5.6 Structural analysis of intertwined particles of VMV protein

Electron micrographs of samples from hanging drops equilibrated
with 15% saturated (NH$_4$)$_2$SO$_4$ at pH 6.0, 22°C showed intertwined particles
of VMV coat protein (see section 4.5). The structure of the aggregates,
referred to as "ropes", and the particles that compose them was determined
using optical diffraction.
Figure 66. Transforms from radial projections of models for the structure of TVX protein particles at pH 5.0. In (A)-(D) there are 2 parallel helices with $r=65\,\AA$, pitch$=72\,\AA$ and a repeat of $144\,\AA$. (A) 2 apparent starts with 8 1/2 subunits/turn. (B) 2 real starts with 8 1/2 subunits/turn. (C) 2 real starts with 9 1/2 subunits/turn. (D) 2 real starts with 7 1/2 subunits/turn. (E) 3 real starts with pitch$=108\,\AA$ and a repeat of $144\,\AA$ and 8 3/4 subunits/turn.
Table 17. Models incorporating a minor reflection at 1/144 Å
($\varepsilon=1$), which had $2\pi r R_n=10.26+1.08$. 1/72 Å becomes
$\varepsilon=2$, and 1/36 Å becomes $\varepsilon=4$. There is a distance of
36 Å between adjacent helices and the pitch= # helices
x 36 Å. The vertical repeat is 144 Å.

<table>
<thead>
<tr>
<th># helices</th>
<th># starts</th>
<th># subunits/turn</th>
<th>$\varepsilon=1$</th>
<th>$\varepsilon=2$</th>
<th>$\varepsilon=4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1</td>
<td>8.1/2</td>
<td>9</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>8 1/2</td>
<td>8</td>
<td>17</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>9 1/2</td>
<td>10</td>
<td>18</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>7 1/2</td>
<td>8</td>
<td>14</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>8 3/4</td>
<td>8</td>
<td>19</td>
<td>3</td>
</tr>
</tbody>
</table>
Figure 67 shows electron micrographs of the ropes. They have a diameter of 300-400 Å and consist of several intertwined particles. Some of the optical diffraction patterns (Fig. 68) show a near-equatorial cross which indicates a large pitch consistent with a helical structure. There were also two meridional or near-meridional reflections at 31 Å and 62 Å indicating that the particles are composed of stacked disks with two periodicities, rather than helices.

Optical reconstructions showed horizontal bands of uniform intensity if only the 31 Å reflection was used (Fig. 69A). When the 62 Å reflection was also included (Fig. 69B), the bands showed alternating high and low intensities. This means that the 31 Å reflection arose from stacked disks and the 62 Å reflection resulted from pairing between adjacent disks in the protein particle.

The 31 Å reflection was not always on the meridian. Stacked disks produce a meridional reflection and the cases of an off-meridional reflection indicate that there were helical grooves at a 15° angle to the horizontal axis of the particle. This was probably due to twisting of the particles in the rope so that the longitudinal axis of the particle was at a 75° angle to the horizontal axis of the rope. Twisting inevitably results when individual particles are intertwined.

Intertwined particles should produce a near-equatorial cross on the diffraction pattern, from which the pitch of the entire structure can be deduced. Although the near-equatorial cross was not always clear, values of 1400±200 Å could be obtained for the interparticle pitch. This value is consistent with that calculated from the informa-
Figure 67. Electron micrographs of ropes of VMV protein formed in 15% (NH$_4$)$_2$SO$_4$ at pH 6.0. Arrows indicate ends where the number of particles/rope can be counted. Bar represents 300 nm.
Figure 68. Optical diffraction pattern of VMV protein rope formed in 15% saturated (NH₄)₂SO₄ at pH 6.0. The main reflections are two sets of meridional reflections and the equatorial cross.
Figure 69. Optical reconstructions of VMV protein ropes.

(A) Only one pair of meridional reflections (1/31 Å) was used.

(B) Both sets of meridional reflections (1/31 Å and 1/62 Å) were used.
tion available on the geometry of the ropes. Examination of the
electron micrographs indicated that there are 4 protein particles
per rope. (The particles can be counted at the end of the rope (Fig. 67).)
Assuming that the particles have the same diameter as the virus (140 Å)
and interpenetrate to a packed diameter of about 135 Å, four particles
arranged around the centre of the rope would produce a diameter of
326 Å (Fig. 70A) which agrees with the measured diameter of 300–400 Å.

A right-angle triangle can be constructed in the longitudinal plane
of the rope (Fig. 70B,C) such that one side, along the longitudinal axis
of the rope, equals \( P/4 \) where \( P \) is the pitch of the rope helix. The
side along the horizontal axis of the rope corresponds to the 95.4 Å
between the centre of the rope and the centre of each particle. The
hypotenuse corresponds to the longitudinal axis of the particle. As
mentioned, there was a 15° angle between the longitudinal axes of the
particle and the rope, corresponding to the angle between the hypotenuse
and \( P/4 \) in the triangle. From this information, the pitch of the rope
is calculated to be approximately 1400 Å, which agrees with the measure-
ments made from the diffraction patterns.

In summary, the ropes of VMV coat protein are composed of four in-
dividual protein particles arranged in a helix so that the pitch is
approximately 1400 Å. Rope diameter is about 326 Å. There is a 15°
angle between the longitudinal axes of the particles and the rope, due
to twisting of the particles. Each particle is made of stacked disks
with a spacing of 31 Å. Adjacent disks are paired to give spacings of
62 Å.
Figure 70. Diagrams showing the structure of VMV protein ropes. (A) Cross-section. The particles are 140 Å in diameter but will interdigitate. The circles represent 135 Å, the packed diameter of the particles. (B) Partial long-section. (C) Long-section. 
P=pitch of rope, r=radius of rope. — = front side of rope. --- = back side of rope. Each line represents the center of a particle.
5.7 Structural analysis of intertwined particles of FTV protein

Structures made from intertwined particles of FTV coat protein ("ropes") were observed by electron microscopy (Fig. 71) of samples after fast and slow pH changes. The ropes usually formed at pH 4.0-9.0 at 20°C, but not at 4°C. They did not form in the presence of (NH₄)₂SO₄, whereas paracrystals of FTV protein did. In this respect, the coat proteins of FTV and VMV differ because ropes of VMV protein formed only in the presence of 15% saturated (NH₄)₂SO₄ at pH 6.0 (see section 4.5).

Optical diffraction of electron micrographs was used to determine the structure of the FTV protein ropes (Fig. 72). Near meridional reflections on the diffraction patterns showed a periodicity of 33±1 Å (n=12) which is probably the pitch of the individual intertwined particles. Near-equatorial reflections often produced a clear cross on the diffraction patterns. These reflections originate from the long helix of the rope. Measurement of these reflections indicates that there are 2 classes of long pitch: 680±79 Å (n=9) and 108±125 Å (n=8). Both classes, which did not differ in diameter, were observed under the same conditions.

The FTV protein ropes are approximately 250 Å in diameter. Examination of the ends of the ropes indicates that they consist of 3 individual particles (Fig. 71). Assuming that the particles have the same diameter as the virus and are interdigitated to a diameter of about 135 Å, the rope diameter can be calculated for ropes containing various numbers of particles. A rope consisting of 3 particles would have a diameter of 252 Å. Two particles twisted into a rope would have dimensions of 135 Å x 270 Å. Diameter measurements might not distinguish between the two
Figure 71. Electron micrographs of FTV protein ropes.

(A) after a slow pH change to 0.01 M MES, pH 6.0 at 24°C. Ropes composed of 2 rods (arrows) run vertically, while the rope running horizontally is composed of more than 2 rods.

(B) and inset: after a slow pH change to 0.01 M MES, pH 7.0 at 24°C. Inset and arrows show that the ends of the ropes consist of 3 rods. Bar represents 100 nm.
Figure 72. Optical diffraction of FTV protein ropes formed in 0.01 M glycine pH 9.0. The main features are the equatorial cross and the reflections near the meridian.
structures but the electron micrographs clearly show that, with the rare exception, there are more than 2 particles per rope. Figure 71A shows the difference: two ropes which obviously consist of 2 particles are marked with arrows; the main rope running more or less perpendicular to them definitely contains more than two particles and is the most common type. The ends of the ropes (Fig. 71B) appear to contain 3 particles. A rope containing four particles would have a diameter of 326 Å, which is inconsistent with the observations. Therefore, the FTV protein ropes must consist of three intertwined helical particles, each with a pitch of 33 ± 1 Å and a long pitch of 680 and 1050 Å.

5.8 Discussion

The optical diffraction patterns of flexuous helical plant viruses and the tubular particles composed of their proteins usually contain only two reflections. Their positions and the ratios deriving from them have necessarily formed the principal basis for solving structures. The assumption that subunit volume remains about the same in all structures composed of a given protein has been made to test the models.

The structure of the protein particles has not been described in terms of the helix with the smallest pitch but rather in terms of the helix producing the strongest reflection, which generally occurs at a spacing of 1/35 Å.

All of the particle types examined produced this reflection, indicating a common repeating unit, whether it be between adjacent helices of multi-helical or multi-start particles, between adjacent disks or, in
the case of the viruses and virus-like particles, between adjacent 
turns of a single helix. This indicates some similarity in bonding 
in all structures, as does the fact that the number of subunits/turn 
or disk varies only slightly from the number of subunits/turn in the 
viruses. However, there is some difference in the bonding in different 
particle types. For example, the intersubunit distance along the main 
helix in Type 2 VMV protein particles is 1.27 times that in VMV (although 
the subunit volumes are the same), while that between the subunits in 
stacked disks is 0.98 times that in VMV. Such differences may result 
from variations in bond angle, which can vary by approximately 5° in 
any direction (Caspar and Klug, 1962), or from differences in subunit 
orientation within different structures.

The length of the crystallographic and true repeats and the type 
and extent of interparticle aggregation varied among the protein products.

The proteins of BCV and FTV produced virus-like particles and did 
not appear to produce any other structures. This indicates that protein-
protein interactions must be very important in directing viral capsid 
assembly. There was no extensive aggregation between particles of BCV 
protein. The coat protein of FTV formed paracrystals, probably as a 
result of water extrusion at low pH and in the presence of \((\text{NH}_4)\text{SO}_4\) 
(see section 4.6). The interparticle distance was about 100 Å, indicat-
ing deep interpenetration, which is not uncommon among the potexviruses, 
especially under conditions of low humidity (Tollin et al, 1979, 1981; 
Radwan et al, 1981). Therefore, the structure and conformation of the 
FTV protein particles composing the paracrystals and the BCV protein 
particles are probably the same as in the viral capsids.
However, at non-acidic pH levels in the absence of (NH₄)₂SO₄, the FTV protein particles, which appear to be virus-like, intertwined into ropes. This suggests that the outer surfaces of the subunits differ from those in the virus, which does not intertwine. The conformation of the outer surfaces of the FTV protein subunits may change when the inner surface interacts with the RNA, preventing the virus particles from intertwining. Alternatively, the movement of the subunits may be less restricted in the absence of the RNA, enabling the particles to intertwine by interacting at points that are not available in the presence of (NH₄)₂SO₄. Tremaine and Agrawal (1972) found that intact PVX did not dissociate when treated with trypsin, which removed 19 amino acids from each subunit. However, the trypsin-treated virus intertwined into rope-like aggregates composed of either 2 or 3 particles.

The proteins of PVX, VMV and, so far, TVX do not form virus-like particles. This indicates that the role of protein-RNA interactions in directing capsid assembly is more important for these viruses than for BCV or FTV.

The coat protein of PVX formed particles composed of disks or rings that were 34 A apart. The rings were composed of 8 or 9 subunits. They were not paired, which suggests that the particles were polar (i.e., disks all face in the same direction along the particle) and may be variants of the viral capsid (Finch and Klug, 1971). However, there is not necessarily any relationship between the assembly of the stacked disks and of the virus, since the sub-assembly product of the stacked disks may not be able to form in the absence of (NH₄)₂SO₄.
The protein of VMV formed three types of particle: stacked disks which intertwined into ropes, 5-start helices and 9 parallel helices. Both types of multi-helical particle formed in hanging drops equilibrated with 0-15% saturated (NH₄)₂SO₄ at pH 6.0 and 8.0, in what appears to be a slow reaction. With 15% saturated (NH₄)₂SO₄ at pH 6.0, ropes also formed. There are two ways that two or three particle types could form simultaneously: 1) by separate pathways from a mixture of different sub-assembly products; 2) via different pathways from a common sub-assembly product.

Optical reconstructions showed that the stacked disk particles composing the ropes are paired in a way that suggests polarity (disks facing in the same direction). They might be variants of virus structure, although the spacing of the disks is smaller than the pitch of the capsid helix and the diffraction patterns provide no information on the number of subunits/disk (it is probably masked by the equatorial cross). There is no evidence to indicate whether these particles are assembled from single- or double-layered disks. As with the stacked rings of PVX protein, the VMV protein stacked disks may form from a sub-assembly product that occurs only in the presence of (NH₄)₂SO₄.

Preliminary reconstitution experiments indicate that the optimal pH for VMV assembly is pH 6, where both types of multi-helical particle can slowly form. Interaction of the sub-assembly product with the RNA may make intermediates for multi-helix formation energetically unfavourable. The rate of capsid assembly in comparison to that of multi-helix assembly and the stability of virus particles may also contribute to the exclusive formation of single-helical-capsids in the virus.
Multi-start and multi-helical particles may be variants of capsid structure. The vertical distance between adjacent helices or turns is constant and the number of subunits/turn in the multi-helical particles varies only slightly from that in VMV. Stacked disk particles of TMV protein have been proposed as variants of virus structure (Finch and Klug, 1971) in view of their polarity and the perturbations of the protein subunits in the Dahlemense strain of TMV. The protein of PVY forms stacked disks that are, with time, converted into capsid helices in the presence of the RNA (McDonald and Bancroft, 1977). Both types of structure have been observed in the same particle, which suggests a structural relationship, although it is not known whether or not an intermediate dissociation step took place. The protein of NMV, a potexvirus, forms stacked disks which are converted into 2-start helices (Bancroft et al., 1980). Again, it is not known whether successive dissociation of the disks occurs as the conversion proceeds.

Although this type of structural variation in the polymerized proteins of helical plant viruses is well documented, the degree of relationship between the capsid helix, stacked disks and multiple helices and starts is not understood. What is clear from the results is that VMV protein is able to assemble in more than one way when not restricted by the effects of protein-RNA interactions.

The coat protein of TVX forms particles in the absence of \((\text{NH}_4)_2\text{SO}_4\) at pH 5.0. Although rapidly-sedimenting material appears at pH 6.0 and 7.0, no particles have been observed using electron microscopy. At pH 5.0, TVX protein forms particles composed of 3 parallel helices. Although these particles may be variants of virus structure, it is not known whether
they form from a sub-assembly product that is required for virus assembly. The results do indicate, however, that protein-RNA interactions are important for capsid formation during the assembly of TVX.
CHAPTER 6

GENERAL DISCUSSION

The sub-assembly processes, protein-protein interactions and protein-RNA interactions involved in the assembly of the potexviruses, which are structurally similar (see section 1.6), will be discussed in this chapter. This discussion will show that an assembly pathway cannot necessarily be deduced from the symmetry of the final products, nor do related viruses necessarily share the same assembly pathway.

6.1 Sub-assembly products

6.1.1 Size approximation

Amino acid determinations on the coat proteins of several potexviruses (Table 18), performed by M. Short (unpublished) indicate that the proteins of BCV, VMV, FTV and PVX have a molecular weight of approximately 22,000 d, as does PMV protein (Erickson et al, 1983), although higher molecular weight values have been reported for PVX protein (Miki and Knight, 1968; Tremaine and Agrawal, 1972; Goodman, 1975; Koenig and Lesemann, 1978). It seems likely that the proteins of these potexviruses are as highly hydrated as that of PMV (Erickson et al, 1983) in view of their similarities in composition, molecular weight, virus particle structure (Richardson et al, 1981) and the degree of particle interpenetration of the viruses, especially in environments of low humidity (Tollin et al, 1967, 1979; Radwan et al, 1981; Richardson et al, 1981). It is therefore reasonable to approximate the size of the small aggregates of the potexvirus proteins examined here in the same way as was done for PMV protein by Erickson et al (1983).
Table 18. Amino acid composition of 4 potexvirus proteins $^a_j$

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>VMV</th>
<th>FTV</th>
<th>PVX</th>
<th>BCV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys</td>
<td>5</td>
<td>13</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td>His</td>
<td>1</td>
<td>1</td>
<td>2</td>
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<tr>
<td>Arg</td>
<td>9</td>
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<td>Asp</td>
<td>14</td>
<td>25</td>
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<td>18</td>
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<tr>
<td>Thr</td>
<td>25</td>
<td>16</td>
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<td>17</td>
</tr>
<tr>
<td>Ser</td>
<td>17</td>
<td>9</td>
<td>14</td>
<td>25</td>
</tr>
<tr>
<td>Glu</td>
<td>18</td>
<td>20</td>
<td>16</td>
<td>24</td>
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<tr>
<td>Pro</td>
<td>17</td>
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<tr>
<td>Gly</td>
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<td>7</td>
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<tr>
<td>Ala</td>
<td>27</td>
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<td>38</td>
<td>22</td>
</tr>
<tr>
<td>Cys</td>
<td>2</td>
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<td>3</td>
<td>2</td>
</tr>
<tr>
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<td>8</td>
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<td>11</td>
<td>12</td>
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<td>6</td>
<td>1</td>
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<td>11</td>
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<td>24</td>
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<tr>
<td>Tyr</td>
<td>3</td>
<td>7</td>
<td>2</td>
<td>5</td>
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<td>8</td>
<td>10</td>
<td>11</td>
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<td>3</td>
</tr>
<tr>
<td></td>
<td>204</td>
<td>192</td>
<td>207</td>
<td>215</td>
</tr>
</tbody>
</table>

Molecular Weight

- 21,473
- 21,171
- 22,160
- 23,316

$a_j$ M. Short, unpublished
The results have shown, in some detail, the behaviour of several potexvirus proteins under a variety of conditions. There is no indication that 14S disk aggregates are formed by the proteins of PVX, BCV, ETV, VMV and TVX, whereas those of PMV (Erickson and Bancroft, 1978; Erickson et al., 1983) and CYMV (Bancroft et al., 1979) (both potexvirus) form double-layered disk aggregates which may be involved in virus assembly. Disks have also been observed with the proteins of several unrelated viruses, such as TMV (Butler and Klug, 1971; Durham, 1972; Klug, 1972; Okada and Ohno, 1972; Richards and Williams, 1972), TRV (Semancik and Reynolds, 1969; AbouHaidar et al., 1973; Fritsch et al., 1973; Morris and Semancik, 1973) and BSMV (Atabekov et al., 1968, 1970) (rigid helical viruses).

Table 19 summarizes the results from studying small aggregates formed by the potexvirus proteins, including results that have been presented here.

6.1.2 The sub-assembly of PVX

The initial work published on the behaviour of PVX protein, which was not very detailed, indicated that this protein might form disk or ring aggregates. Kaftanova et al. (1975) and Goodman et al. (1975) reported the formation of a 10-15S species, which Goodman et al. (1976) intuitively suggested was a single ring composed of 9 subunits. Assuming that PVX protein is as highly hydrated as PMV protein and sediments accordingly (Erickson et al., 1983), this species might be a mixture of single rings and two-layer disks. Kaftanova et al. (1975) observed disks in the electron microscope, and stacked disks upon aging of the
Table 19. The formation of small aggregates of potexvirus proteins
(concentration = 3 mg/ml; 20°C)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sedimentation Coefficient (S)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 5±1</td>
</tr>
<tr>
<td>PMV</td>
<td>14</td>
</tr>
<tr>
<td>CYMV</td>
<td></td>
</tr>
<tr>
<td>BCV</td>
<td>7</td>
</tr>
<tr>
<td>FTV</td>
<td>4</td>
</tr>
<tr>
<td>PVX</td>
<td>4 or 10</td>
</tr>
<tr>
<td>VMV</td>
<td>7</td>
</tr>
<tr>
<td>TVX</td>
<td>8</td>
</tr>
</tbody>
</table>

a) Erickson and Bancroft, 1978

b) Bancroft et al, 1979
protein. It is possible that these stacks were the result of proteolytic cleavage as were the stacked disks of TMV protein observed by Durham (1972a). PVX protein is apparently cleaved by plant proteases under certain conditions if the purification process is done slowly (Koenig et al., 1970; Tremaine and Agrawal, 1972). We did not observe disks or stacked disks except in the hanging drops in the presence of high concentrations of \((NH_4)_2SO_4\) (sections 4.3, 5.3). The 10S species was observed during analytical ultracentrifugation (Table 3) at pH 5.0 after a fast pH change at 4°C, where it was the only species, and as a minor species at pH 8.0 and 9.0 after a slow pH change at 20°C.

Reconstitution of PVX proceeds best at or slightly above pH 6.0 at low ionic strength and is inhibited by low temperature (Kaftanova et al., 1975; Goodman et al., 1976). Under these conditions, the 10S species did not form (Table 3). Disks were observed by Kaftanova et al. (1975) and Goodman et al. (1976) only under conditions that were unsuitable for reconstitution, either the pH or the ionic strength being too high. Goodman et al. (1976) pointed out that this does not rule out the possibility of disk involvement in assembly, in view of the small quantities that would be required for such an aggregate to participate in initiation.

In summary, the results presented here show no disk formation by PVX protein in the absence of \((NH_4)_2SO_4\), even in 0.2 M NaCl at pH 6.0. Even when 10S material was present, no disks were observed in the electron microscope, although even this does not completely rule out the possibility of their presence (Erickson et al., 1983). In view of the predominance of the disk-type aggregates of TMV (Fraenkel-Conrat
and Singer, 1959; Klug, 1972), TRV (AbouHaidar et al., 1973; Morris and Semancik, 1973), BSMV (Atabekov et al., 1968, 1970; Gumpf and Hamilton, 1968), PMV (Erickson and Bancroft, 1978) and CYMV (Bancroft et al., 1979) under optimal reconstitution conditions, it seems unlikely that such an aggregate is an important sub-assembly product for PVX. This leaves two possibilities: the 4S material, which probably consists of trimers and was the most common PVX protein aggregate (Table 3) and/or the 8S material, which probably consists of heptamers and/or octamers and formed after a slow pH change at pH 6.0, 20°C (suitable conditions for reconstitution). The sub-assembly aggregate for PVX is therefore much smaller than the disks of TMV, TRV, BSMV, PMV and CYMV proteins.

6.1.3 The sub-assembly of BCV

The behaviour of BCV protein (sections 3.2 and 3.3) did not indicate that a disk structure is involved in the assembly of this virus. The protein polymerizes extensively into virus-like particles which are shortest at acid pH levels. At low pH and temperature, slowly-sedimenting species (24S at pH 5.0, 36S at pH 4.0, see Table 1) occurred which could represent double or triple disks, respectively (i.e. 4 or 6 stacked rings) but are more likely to represent 4 or 6 helical turns (i.e. limited helical growth rather than a sub-assembly aggregate for helix formation). If the 24S species, which occurred at pH 5.0, 4°C, represents the formation of a sub-assembly product under doubly unfavourable conditions of pH and temperature, then its addition to a reconstitution mixture might be expected to enhance the reconstitution, either quantitatively or kinetically. If this species simply represents limited
helical growth, rather than being a sub-assembly product, then its addition to a reconstitution mixture would be expected to inhibit or else not affect the outcome of the reaction. At this point, it is not possible to tell whether the 24S species is a 4-ring sub-assembly product. However, this seems unlikely in view of its apparent absence or low concentration under conditions which are suitable for the re-assembly of the virus (pH 7.0, 25°C).

Preliminary reconstitution experiments (data not shown) with the protein and RNA of BCV in a 20:1 ratio at 25°C indicate that the optimal pH for the assembly of this virus is pH 7.0. This is also the optimal pH for the assembly of the protein. Apart from long helical particles, the only other species of BCV protein present at pH 7.0 sedimented at 7S. It probably consists of hexamers. This means that if a larger sub-assembly product exists (e.g. an 18-subunit disk), it would have to be present in undetectable quantities and therefore would probably participate in nucleation only (not in elongation) and have elongation proceed very rapidly upon it. Low temperature inhibits BCV protein polymerization and therefore might be expected to reveal a sub-assembly aggregate. However, it increased the proportion of small aggregates (7S) which are probably hexamers, but did not reveal a sub-assembly product intermediate between the hexamers and the long helical particles. Therefore, the sub-assembly product for BCV assembly is probably a hexamer (the 7S species).

6.1.4 The sub-assembly of FTV

The coat protein of FTV polymerizes into particles that appear to
be virus-like (see sections 3.5, 5.2, 5.7) at pH 4.0 to 9.0. Small aggregates which sediment at about 6 or 7S at pH 6.0 to 8.0 probably consist of hexamers. These may form from the 4S species which occurs at low pH (pH 4.0 and 5.0) after a fast pH change (probably before equilibrium is established), and after a slow pH change at pH 7.0 in the presence of 0.1 M KCl or at low protein concentration.

There was no FTV protein species that could represent a single (10S) or double (14S) ring of subunits, such as those formed by PMV and CYMV proteins (see Table 4). The 31S species which formed at pH 9.0, 4°C after a fast pH change is unlikely to be a sub-assembly aggregate, for the formation of a helix. It would have to contain six 9-subunit disks and would probably be too large to make the transition from cylindrical to helical geometry. It is more likely to represent limited (6-turn) helix formation under somewhat unfavourable conditions of pH and temperature. Helix formation by FTV protein appears to resemble that of BCV protein in two respects: both form virus-like particles over a wide range of pH and appear to have a hexamer as a sub-assembly product.

6.1.5 The sub-assembly of VMV

The coat protein of VMV did not form virus-like particles under any of the conditions examined. Tubular polymers formed only in the hanging drops indicating that the specific rate constant for their formation was very low. As was the case with PVX protein, the occurrence of stacked disks in the presence of \((\text{NH}_4)_2\text{SO}_4\) (15% saturated) does not necessarily indicate that their sub-assembly product, which is presumably a disk of some type, is also involved in helix formation.
The small aggregates of VMV protein sedimented at approximately 7S regardless of the pH and temperature, within the usual range. This species probably represents a hexamer. Sometimes it sediments more rapidly (8 or 9S) and may also contain some heptamers and/or octamers.

Preliminary reconstitution experiments (data not shown) indicate that the optimal pH for the assembly of VMV is pH 6.0. At this pH, the coat protein alone slowly forms two types of helical particle with 9 parallel helices or 5 starts. The sedimentation experiments (see Table 6) indicate that prior to the formation of these tubular particles, the protein exists as a 9S aggregate. This is probably not a disk and is a smaller aggregate than the sub-assembly products for PMV and CYMV assembly.

The results indicate that the sub-assembly steps in the assembly of VMV involve a 3-5S species, which probably consists mainly of trimers, and a species which sediments at approximately 7S, which probably consists mainly of hexamers and possibly heptamers and/or octamers.

6.1.6 The sub-assembly of TVX

The coat protein of TVX forms particles composed of 3 parallel helices after a slow pH change to pH 5.0. Rapidly-sedimenting material was present at pH 6.0 and 7.0 but appears to represent denatured or precipitated protein, since no large ordered polymers were observed in the electron microscope. Over the range of pH 4.0 to 9.0, the small aggregates sediment at 8S. This species probably consists mainly
of heptamers.

In summary, hexamers or heptamers appear to be the main sub-assembly product for BCV, FTV, VMV PVX and TVX proteins. These aggregates are in equilibrium with trimers, perhaps with the exception of TVX protein which has not been examined at low concentration. None of the above proteins form disks or ring aggregates in detectable quantities.

A sub-assembly process may consist of more than one step. It is possible that the formation of 14S disks of PMV and CYMV proteins is preceded by the formation of smaller aggregates. Indeed, the proteins of PMV and CYMV form aggregates which are smaller than the 14S disks (see Table 19; Erickson and Bancroft, 1978; Bancroft et al, 1979). Two species of PMV protein occur at pH 10.0, which sediment at 3 and 8S and probably consist of dimers and heptamers. CYMV protein forms a 4S species (probably trimers) which coexists with the 14S disks. It also forms a 7S (probably hexamers) at low temperature, pH or protein concentration.

It appears that the formation of a hexamer or heptamer of protein is a step common to the sub-assembly of all potexviruses and that this may proceed from a trimer in all cases, the trimer forming from monomers and perhaps dimers. The polymerization of the small aggregates into 14S disks, however this may occur, is not common to all potexviruses, and may not be a requirement for reconstitution.

6.2 Protein–protein interactions

6.2.1 Nature of the interactions

The aggregation of TMV protein into disks or helical particles is
controlled by co-operative hydrogen bonding in carboxyl-carboxylate pairs (Caspar, 1963; Butler et al., 1972). Carboxylate clusters may also be involved in the regulation of helix formation by PMV and CYMV proteins (AbouHaidar and Erickson, 1983; Bancroft et al., 1979) as well as in the assembly of CCMV (Bancroft et al., 1967).

Of the five potexvirus proteins examined here, only the proteins of BCV and FTV formed virus-like particles. These helices formed over the range of pH 4.0 to 9.0 for both proteins. Carboxyl-carboxylate pairs could not be maintained up to pH 9.0 and therefore cannot alone control helix formation by BCV and FTV proteins. Thus, the role of carboxyl-carboxylate interactions is not universal in controlling the assembly of helical plant virus capsids, in the way that they do for TMV.

The results of experiments described in Chapter 3 show that the polymerization of BCV protein probably involves an interaction between the imidazole group of histidine and a basic amino acid residue. A similar interaction appears to be involved in the formation of stacked disks of PVY protein at pH 6.0 to 9.0 (McDonald et al., 1976), which can be converted into helices upon the addition of RNA (McDonald and Bancroft, 1977). No large tubular polymers of PVY protein formed at acidic pH levels, indicating that carboxyl groups are not involved in the polymerization of this protein (McDonald et al., 1976).

A second interaction, involving a carboxyl side chain and a more basic residue, may produce the limited equilibrium polymerization of BCV protein at acidic pH and complement the first interaction at neutral
pH (see section 3.3.2).

The coat protein of FTV also polymerizes over a wide pH range (pH 4.0 or 5.0 to 9.0), indicating that this process is not controlled by carboxyl-carboxylate pairs, at least not at the higher pH levels. However, its polymerization at low pH is quite extensive, unlike that of BCV protein, and is probably regulated by the interaction of a carboxyl side chain and a basic amino acid, such as lysine, as discussed in section 3.5.7.

The results imply that the nature of the interactions between amino acids that control capsid assembly is not the same for all helical plant viruses or even for all potexviruses.

6.2.2 The importance of protein-protein interactions

The assembly of a virus involves two fundamental types of interaction: protein-protein interactions and protein-RNA interactions. The importance of each can be inferred from the behaviour of the protein in the absence of the nucleic acid. For example, the ability of TMV protein to form virus-like helices, in the absence of the RNA, at acidic pH levels indicates that protein-protein interactions are important in directing capsid formation during TMV assembly. However, protein-RNA interactions are obviously important in allowing the process to occur in vivo and to be specific (i.e. for the protein to encapsidate the viral genome in a cellular environment).

The formation of tubular particles by the potexvirus proteins is
summarized in Table 20.

The coat protein of PMV forms virus-like particles at pH 4, which is too low for reconstitution to occur (Erickson et al., 1976; Erickson and Bancroft, 1978), indicating that the respective role of the two types of interaction in PMV assembly is probably quite similar to TMV.

CYMV protein forms virus-like helices at pH 5.0-5.5 but under these conditions wide helical particles containing about 12 or 13 subunits/turn also occur (Bancroft et al., 1979). At pH 8.0, stacked disks or rings occurred. These results indicate that some of the information necessary for CYMV capsid formation is contained within the protein subunits but that protein-RNA interactions must be more important than they are to PMV in directing the assembly of the structurally correct capsid.

As shown in Table 20, NMV protein does not form virus-like particles in the absence of the RNA. It forms short stacked disks that, with time, are converted into two types of two-start helices (Bancroft et al., 1980). Although this protein can polymerize at pH 5, protein-RNA interactions appear to be quite important in directing the assembly of a single-helical virus capsid.

The coat proteins of BCV and FTV both assemble into virus-like particles over a wide range of pH as well as in the presence of (NH$_4$)$_2$SO$_4$. Neither of these proteins formed any other type of structure. Clearly, protein-protein interactions have a very important role in the assembly
Table 20. The formation of tubular polymers of viral proteins from the potexviruses

<table>
<thead>
<tr>
<th>Protein Source</th>
<th>Conditions</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMV a)</td>
<td>pH 4</td>
<td>virus-like single helix, 9 1/3 subunits/turn, morphological groups of 7</td>
</tr>
<tr>
<td></td>
<td>40% 2-methyl-2,4-pentanediol, pH 6</td>
<td></td>
</tr>
<tr>
<td>CYMV b)</td>
<td>pH 8</td>
<td>Paracrystals of stacked disks</td>
</tr>
<tr>
<td></td>
<td>pH 5.0-5.5</td>
<td>Helical 130 Å wide, 8 4/5 subunits/turn, virus-like</td>
</tr>
<tr>
<td></td>
<td>pH 5.0-5.5</td>
<td>Helical, 1.25-1.5x wider, 12-13 subunits/turn</td>
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<tr>
<td>NMV c)</td>
<td>pH 5, 1 day</td>
<td>Short stacked disks</td>
</tr>
<tr>
<td></td>
<td>pH 5, 3-4 days</td>
<td>Long, 2-start helical rods, repeating in 1 and 3 turns</td>
</tr>
<tr>
<td>BCV</td>
<td>pH 4,5</td>
<td>Short rods</td>
</tr>
<tr>
<td></td>
<td>pH 6-9</td>
<td>Long, virus-like rods</td>
</tr>
<tr>
<td>PVX</td>
<td>pH 6,8, 15-40% saturated (NH₄)₂SO₄</td>
<td>Stacked disks</td>
</tr>
<tr>
<td>VMV</td>
<td>pH 6,8 0-15% saturated (NH₄)₂SO₄</td>
<td>Multi-helical rods: 5-start; 9 parallel helices</td>
</tr>
<tr>
<td></td>
<td>pH 8, saturated (NH₄)₂SO₄</td>
<td>Pair stacked disk rods, intertwined in groups of 4</td>
</tr>
<tr>
<td>FTV</td>
<td>pH 5-9</td>
<td>Virus-like rods, intertwined in groups of 3</td>
</tr>
<tr>
<td></td>
<td>pH 5</td>
<td>Virus-like rods in paracrystals</td>
</tr>
<tr>
<td></td>
<td>pH 6, 15-40% saturated (NH₄)₂SO₄</td>
<td>Virus-like rods in paracrystals</td>
</tr>
<tr>
<td></td>
<td>pH 8, 20-30% saturated (NH₄)₂SO₄</td>
<td>Virus-like rods in paracrystals</td>
</tr>
<tr>
<td>TVX</td>
<td>pH 5</td>
<td>3 parallel helices, repeating in 2 turns</td>
</tr>
</tbody>
</table>

a) Erickson et al., 1976
b) Bancroft et al., 1979; Tollin et al., 1981
c) Bancroft et al., 1980
of BCV and FTV.

The proteins of PVX, VMV and TVX have not formed virus-like particles under any of the conditions yet investigated. PVX protein formed unpaired stacked disks in the presence of relatively high concentrations of (NH₄)₂SO₄, VMV protein formed paired stacked disks, 5-start helices and particles composed of 9 parallel helices, and TVX protein formed particles composed of 3 parallel helices. Protein-RNA interactions must be much more important in the assembly of PVX, VMV and TVX than in the assembly of BCV, FTV, PMV and CYMV.

6.2.3 Protein-protein interactions and specificity of assembly

The proteins of helical plant viruses previously studied were unable to assemble under conditions suitable for reconstitution.

For example, PMV protein forms virus-like particles at pH 4.0 (Erickson et al., 1976) while the optimal pH for PMV assembly is pH 8.0 (Erickson and Bancroft, 1978). CYMV protein forms virus-like particles and wide particles at pH 5.0-5.5 but reconstitution occurs best near neutrality (Bancroft et al., 1980; Tollin et al., 1981). TMV protein assembles into virus-like particles at lower pH than that optimal for reconstitution (Fraenkel-Conrat and Williams, 1955; Lauffer and Stevens, 1968). TRV protein assembles best at a higher temperature than that optimal for assembly of the virus (Morris and Semancik, 1973; Semancik and Reynolds, 1969). With the "C" strain, both processes occur slightly above neutrality but the CAM strain has different pH requirements for the two processes (AbouHaidar et al., 1973). BSMV protein may assemble at a pH suitable for reconstitution, but its temperature and cation require-
ments are different (Atabekov et al., 1968, 1970). However, the protein of BCV forms virus-like particles at the optimum pH for reconstitution. FTV protein forms virus-like particles in the range of pH 5.0-9.0 which probably encompasses the optimal pH for reconstitution, although this has not yet been examined.

Some control must be exerted in vivo to prevent the formation of empty capsids. This presumably occurs in the sequence of production of viral RNA and protein. Protein synthesis probably begins at the stage when a pool of RNA is present so that large quantities of protein capable of self-assembling would not accumulate before the appearance of the RNA that it should encapsidate.

6.3 Protein-RNA interactions

The most efficient way to start helical growth, in mechanical and energetic terms, is with a closed structure such as a ring or disk of protein. This idea has been supported by the predominance of disk aggregates of the proteins of TMV, TRV, BSMV, PMV and CYMV, which were discussed previously. Disks are required to initiate helical growth in the assembly of TMV (Butler, 1971, 1972; Butler and Klug, 1971; Okada and Ohno, 1972; Richards and Williams, 1972) and TRV (AbouHaidar et al., 1973; AbouHaidar, 1976), and are thought to be involved in the nucleation of PMV and CYMV assembly.

The initiation region on TMV-RNA is about 150 nucleotides long (Zimmern and Butler, 1977) and is thought to contain a core nucleation sequence of about 50 nucleotides (Zimmern, 1977), which is about the
right length to bind to a disk of protein containing 17 subunits with 3 nucleotides per subunit.

The initiation of PMV assembly occurs within the first 200 nucleotides on the 5' end of the RNA (AbouHaidar and Bancroft, 1978) but the nucleation sequence is believed to be shorter than this, since elongation occurs very rapidly. The sequence of the first 139 nucleotides of PMV-RNA was determined by AbouHaidar (AbouHaidar and Erickson, 1983). It contains 8 consecutive repeats of the sequence N(C or A)AAA, from the first 40 nucleotides after the 5' CAP. With 5 nucleotides/protein subunit, a sequence of 40 nucleotides would be about the right length to specifically bind to a disk of 9 subunits (i.e. one turn).

The results that have been presented imply that a hexamer or heptamer acts as the largest possible sub-assembly product for BCV, FTV, VMV, PVX and TVX. If, in fact, disks are not formed by the proteins of these viruses, then a recognition sequence of much less than 40 nucleotides would have to be involved in nucleating assembly. For example, a sequence that recognizes a linear hexamer, with 5 nucleotides/subunit, would consist of about 30 nucleotides. However, a bilayer hexamer would be more likely to occur since the number of intersubunit bonds would be greater and the bound RNA segment would be protected on both sides. In this case, the recognition sequence would be only 15 nucleotides in length.

Once the first hexamer (or heptamer) had bound to the recognition sequence on the RNA, other protein aggregates of the same or smaller
size might bind to adjacent nucleotides, producing the first turn of the helix. Alternatively, the binding of the next aggregates might result in the formation of a disk directly upon the RNA. This initiation complex would then have to make a transition from cylindrical to helical geometry before elongation could proceed.

Even if disk formation does occur on the RNA of the potexviruses examined here, there may still be a recognition sequence small enough to specify the binding site of a small protein aggregate during the first step of initiation of assembly.

Some of the icosahedral plant viruses, such as CCMV, appear to have dimers or trimers as sub-assembly products in shell formation. This implies that the region of specific interaction with their nucleic acid must be no greater than about a dozen nucleotides (see Makowski, 1980). Perhaps the interactions between proteins and such small RNA sequences are more specific and more common than was previously believed, if in fact such an interaction is involved in the specificity of virus assembly.

Protein-RNA interactions must have the most important role in specifying the structure of PVX, VMV and TVX, the proteins of which cannot form virus-like particles, as opposed to PMV, CYMV, FTV and BCV. The interaction between the protein and the nucleic acid may expose protein surface groups that can participate in protein-protein interactions to stabilize the viral capsid. This might occur as a result of the release of water that was directly or indirectly bound to those regions of the subunit, or a conformational change in the subunit surface.
REFERENCES


