CHAPTER 1

INTRODUCTION

Tomato bushy stunt virus (TBSV) is a member of a fairly large class of plant viruses that are spherical particles of 30nm in diameter when seen in the electron microscope. They all contain single-stranded ribonucleic acid (RNA) enclosed by a shell of protein. They are not the smallest plant viruses known, there being a class of smaller RNA and protein viruses (including satellite tobacco necrosis virus, STNV) and a class of viroids containing nucleic acid only. They are nevertheless fairly simple in construction and, because they have a precise chemical structure, are accessible to study by the x-ray crystallographic method. They offer an ideal experimental system for the study of protein-nucleic acid and protein-protein interactions. A classification of plant viruses from the point of view of their physical, serological and infectious properties has been attempted by B. D. Harrison et. al. (1971). Two of the sixteen groups of viruses delineated contain members whose expansion/contraction (see below) properties have been investigated. Group 10 contains brome-grass mosaic virus (BMV) and cowpea chlorotic mottle virus (CCMV); group 11 contains TBSV. Though not classified, the related southern bean mosaic virus (SBMV) and turnip crinkle virus (TCV) are probably in group 11 (Ziegler, et. al.,

1974). Since no single virus has yet been selected for exclusive study, we will consider experimental results for all of the above, bearing in mind the known differences between them.

Each particle of TBSV has a molecular weight of about 9×10^6 (Williams and Backus, 1949) of which 1.5×10^6 is a single strand of RNA and the remainder is a protein shell surrounding this. The protein molecular weight is 40,000 (Ziegler, et. al., 1974) and there are 180 copies per virion. The protein capsid is relatively inert, being stable at a wide range of temperatures and pH values, as well as resistant to proteolytic enzymes and mild denaturants. This certainly serves the virus as a protective sheath during transmission from plant to plant. TBSV infects tomato plants and a wide range of other hosts. No specific transmission mechanism is known; it is possible to infect a plant by abrading the surface in the presence of the virus, but it is quite possible that the plant can take up virus by endocytosis, and this may be the normal entry route in vivo. Once the virus is inside the cell cytoplasm, some trigger mechanism releases the RNA that then acts as a message and positive replicative template to form new virus particles which self-assemble in solution (Bancroft, 1970; Casjens and King, 1975) and are released after the plant dies.

All of the above mentioned viruses have the pro-

perty of in vitro 'expansion', a structural phase transition from the transmissible 'compact' state to a new state in which the particles are increased in diameter by about 10%, as seen by a variety of techniques (Luftig, 1967; Incardona and Kaesberg, 1964). The transition occurs spontaneously under the right conditions of pH, ionic strength and divalent cation concentration and is at least partially reversible (see below). The physiological significance of the expansion is unknown, and is considered by some to be "an in vitro artefact" (Jacrot et. al., 1977). It is possible that the expanded state is an intermediate in the virus disassembly. Circumstantial evidence for this hypothesis is that the expanded state (unlike the compact one) is proteolytically sensitive (Pfeiffer and Hirth, 1975) and would therefore be rapidly degraded in the cytoplasm of the infected cell, thereby releasing its gene-containing RNA. Durham et. al. (1977) have proposed additionally that the difference in calcium concentration between the cytoplasm (10^{-6}M) and the intercellular fluid (10^{-3}M) is used by the virus to recognise the interior of the cell and trigger the disassembly. They speculate that the mechanism may be universal for simple viruses and that this may explain the widespread appearance of calcium-binding sites in virus structures. It is possible instead (or indeed, in addition) that the expanded state is an <u>assembly</u> intermediate. Several of the above viruses are known to self-assemble into empty capsids in the absence of RNA (Adolph and Butler,

1974). An expanded capsid might contain holes in its surface large enough for the RNA to enter so that it can coil up loosely inside, perhaps driven electrostatically by a positively charged capsid interior; the final calcium binding step would then compress the ball of RNA into the compact state.

The widespread observation of the expansion phenomenon among different viruses and the ubiquitous involvement of divalent cations suggest that these are fundamental in some way to the viral life cycle. Whether or not this is true, the expansion is an example of an allosteric conformation change at least as large as that of Haemoglobin (Perutz, 1970) and deserves detailed investigation in its own right. This is the purpose of the present work.

TBSV samples were partly provided by J. Witz and colleagues at Strasbourg, and partly prepared at Harvard by J. Hogle. Plants of Datura stramonium were infected with TBSV by abrasion with carborundum powder. Electron micrographs of leaf smears two weeks afterwards showed a high concentration of virus. The leaves were then harvested and homogenised in a minimum volume of water. The virus was isolated by differential centrifugation in yields of up to 5% of the dry weight of the plant.

1.1 Structure of TBSV.

It has long been observed that many simple viruses have one of two general morphologies: cylindrical rods and spheres. Such viruses have been termed 'regular' viruses. Crick and Watson (1956) proposed that this is a direct consequence of the employment of aggregates of small globular proteins into regular shapes with either helical (rods) or cubic point group symmetry (spheres). In this way, every protein molecule can have an exactly conserved contact with its neighbours in the superstructure. The theory, with certain modifications elaborated below, has been confirmed by careful electron microscope studies for a large number of helical and spherical viruses, as well as other macromolecular assemblies (reviewed by Horne and Wildy, 1961).

1.1.1 General Architecture.

Caspar and Klug (1962) have carefully considered the ways that identical protein molecules could aggregate to form spherical viruses. When it is considered that there must be a linear relationship between the length of the RNA strand to be encapsulated and the length of the coat protein, it is a fundamental design condition of a spherical virus that the volume of the cavity of the capsid be maximised with respect to the length of the protein. For this reason, it is clear that the virus must exploit the ways of packing the greatest number of protein subunits into a

shell, i.e. they must exploit the point groups of the highest order (see Falicov, 1967). All point groups containing mirror symmetry are excluded for structures made of L-amino acids, which are necessarily chiral. Strictly then, the largest allowed point group is the Abelian group n with $n > 60$, but this is clearly unsuitable for the construction of closed shells because the subunits would have to be extremely long and thin; moreover, there would have to be axial holes in the shell of circumference greater than 60 times the width of an atom, which would defeat its purpose of protection of the RNA. The next largest order point group without mirrors is 532 with 60 elements. It is the point group of the icosahedron, the largest platonic polyhedron. The organisation of a shell with this symmetry would have a subunit of protein in every 'icosahedral asymmetric unit', so that each one would be exactly equivalent to the other 59. The icosahedral asymmetric unit is a spherical sector with a cross section that is roughly an equilateral triangle as shown in figure 1.1; this is an ideal shape for a globular protein to adopt for the construction of shells. The icosahedral architecture appears to be universally used by the regular spherical viruses, which prompted Caspar and Klug (1962) to refer to them as 'icosahedral' viruses.

There is an additional packing freedom that the subunits can exploit to increase greatly the enclosed volume/protein length ratio. This was first recognised by

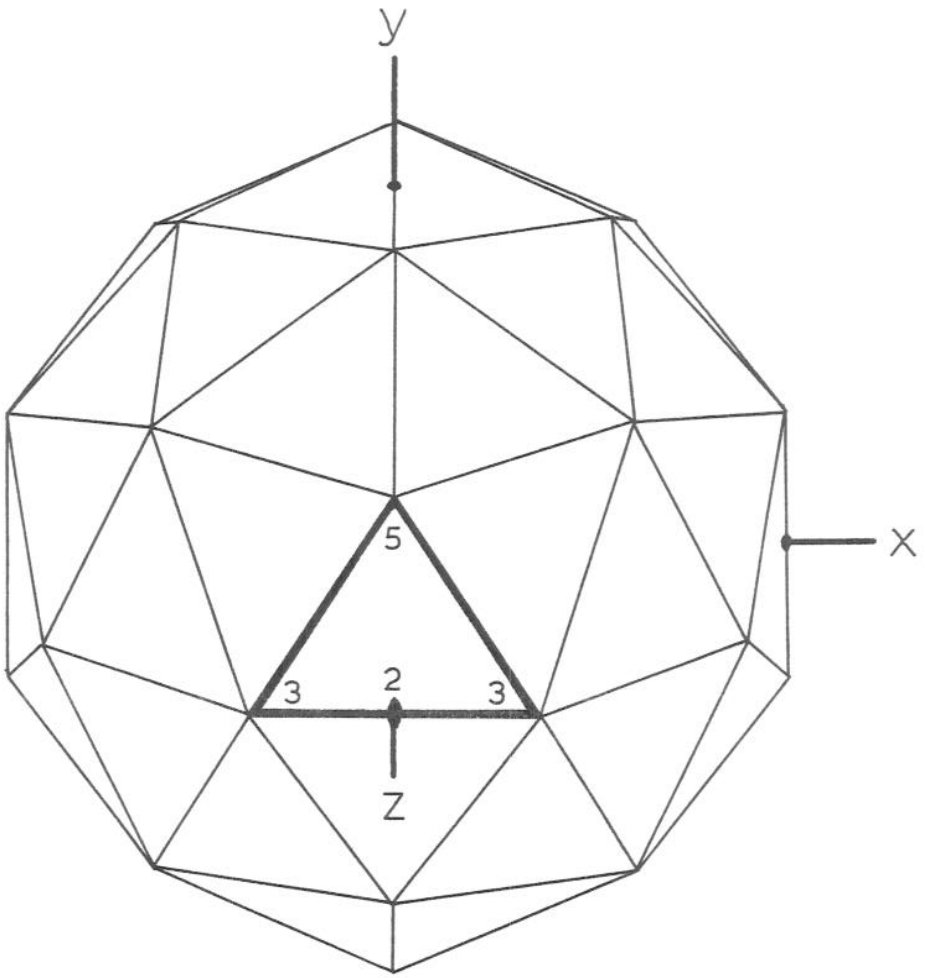

Figure 1.1. Definition of the icosahedral asymmetric unit. The vertices of this polyhedron are the intersection of the symmetry axes of the 532 point group with a sphere. The relation to the primitive icosahedron (5's only) and to its dual, the dodecahedron, (3's only) is clearly visible. The icosahedral asymmetric unit is the triangular pyramid with its apex at the origin that intersects one of the faces (highlighted). The 60 equivalent, point group related volumes of space correspond to the 60 faces of this polyhedron. The 5-, 3- and 2-fold axes are marked and the conventional coordinate frame of the virus is denoted.

Caspar and Klug (1962). They demonstrated how a two-dimensional hexagonal array of subunits can be turned into a closed shell by reducing the number of subunits meeting at twelve of the sixfold axes from six to five. The equilateral triangle formed by any three adjacent fivefolds becomes a face of the icosahedron that is formed. The allowed number of subunits in the icosahedral asymmetric unit is limited to the values of the 'triangulation number', $T = 1, 3, 4, 7, 9, \ldots h^2+k^2+hk$ with integral h and k, which leads to the prediction that the total number of subunits in the whole virus is $60T = 60, 180, 240, 420, \ldots$ The observed pattern in the known sizes of spherical viruses strongly suggests that this method of packing is indeed employed.

The transformation from plane to shell is exact for a 2-dimensional net, but for protein subunits of finite thickness it involves a certain amount of approximation in the angular orientation of the two subunits participating across a contact. This could be accommodated by some flexibility of relevant amino acid side chains or by other design, so that more than one subunit can pack into each icosahedral asymmetric unit. The adjective phrase 'quasi equivalent' is used to refer to subunits inside the same icosahedral asymmetric unit that are related by this approximate local symmetry. The suggestion of quasi equivalent packing was completely novel when it was proposed by Caspar and Klug (1962), but there is now a large amount of evidence

in its favour and it is now accepted as a general principle. The achievement of quasi equivalence at the molecular level is one of the most interesting and ingenious features of virus structure.

1.1.2 Electron Microscopy.

Strong support for the theory of quasi equivalence came from a detailed analysis of negatively stained electron microscope images of TBSV and TCV (Finch et. al., 1970). The images of both viruses are stained spheres with 90 towers projecting above the stain. The interpretation is that these viruses have T = 3 surface lattices with dimer clustering of the 180 subunits along the 30 point group 2-fold axes and 60 quasi 2-fold axes that arise because of the existence of the three quasi equivalent subunits in the icosahedral asymmetric unit. To confirm this result, a 'gallery' of calculated views of the virus from many different directions was produced and these were compared with the individual images. Closer examination of the images revealed an additional unstained feature on the particle 5-fold axes.

1.1.3 Solution Scattering.

An homogeneous preparation of spherical particles has a distinct x-ray or neutron scattering pattern at small angle (Guinier, 1963) consisting of concentric rings of

varying intensity. Analysis of the relative intensities of the these rings can permit a calculation to be made of the spherically averaged electron density as a function of radius. Contrast matching methods with sucrose or salt (Harrison, 1969) or, more effectively, D_2O (Jacrot et. al., 1977) allows the protein and RNA containing fractions of this density to be distinguished.

A number of spherical plant viruses have been studied by this method, including TBSV (Harrison, 1969; Chauvin, 1978). The conclusion for TBSV is that, in the spherically averaged structure, there are shells of protein between 50 and 80Å and also between 110 and 158Å, plus a shell of RNA between 80 and 110Å. The method has been used to study the virus expansion properties and these results are discussed below.

1.1.4 Crystallographic Study.

The greatest amount of information about the structure of TBSV comes from the high resolution analysis of crystals of the virus (Caspar, 1956; Jack et. al., 1975; Harrison and Jack, 1975; Schutt, 1976; Winkler et. al., 1977; Harrison et. al., 1978).

The crystals studied were first obtained by Bernal et. al. (1938) and their diffraction pattern was shown to belong to a cubic lattice by Hodgkin (1950). The crystal space group is I23 with body centred cubic packing. The two

virus particles per unit cell must therefore have at least 23 symmetry and the presence of strong radial 'spikes' in the diffraction pattern along the directions of the symmetry axes indicated that the particle symmetry was 532 (Caspar, 1956).

The high resolution structure was calculated using this icosahedral symmetry for the refinement of phases (see chapter 5) and so shows only averaged detail of components with less symmetry than this. Apart from a few unconnected features visible in 8 Ångstrom resolution maps, the region of the virus inside a radius of 110Å is completely devoid of density, indicating that the RNA and inner protein shell are either disordered or have a symmetry less than 532. This statement is supported by the observation that only the C-terminal 70% of the polypeptide chain is accounted for in a chain trace of the ordered part of the structure.

Figure 1.1 shows the icosahedral geometry of the virus with the icosahedral asymmetric unit highlighted. An enlarged view of the icosahedral asymmetric unit is shown in figure 1.2 with the subunit boundaries of the coat protein to demonstrate the packing of these. The standard nomenclature of the three quasi equivalent positions and the various contacts is specified. Each subunit has two large domains occupying the C-terminal 70% of the sequence (figure 1.3) and separated by a short hinge region, denoted 'h'. The surface of the virus is closed by the 'S' (surface) domains

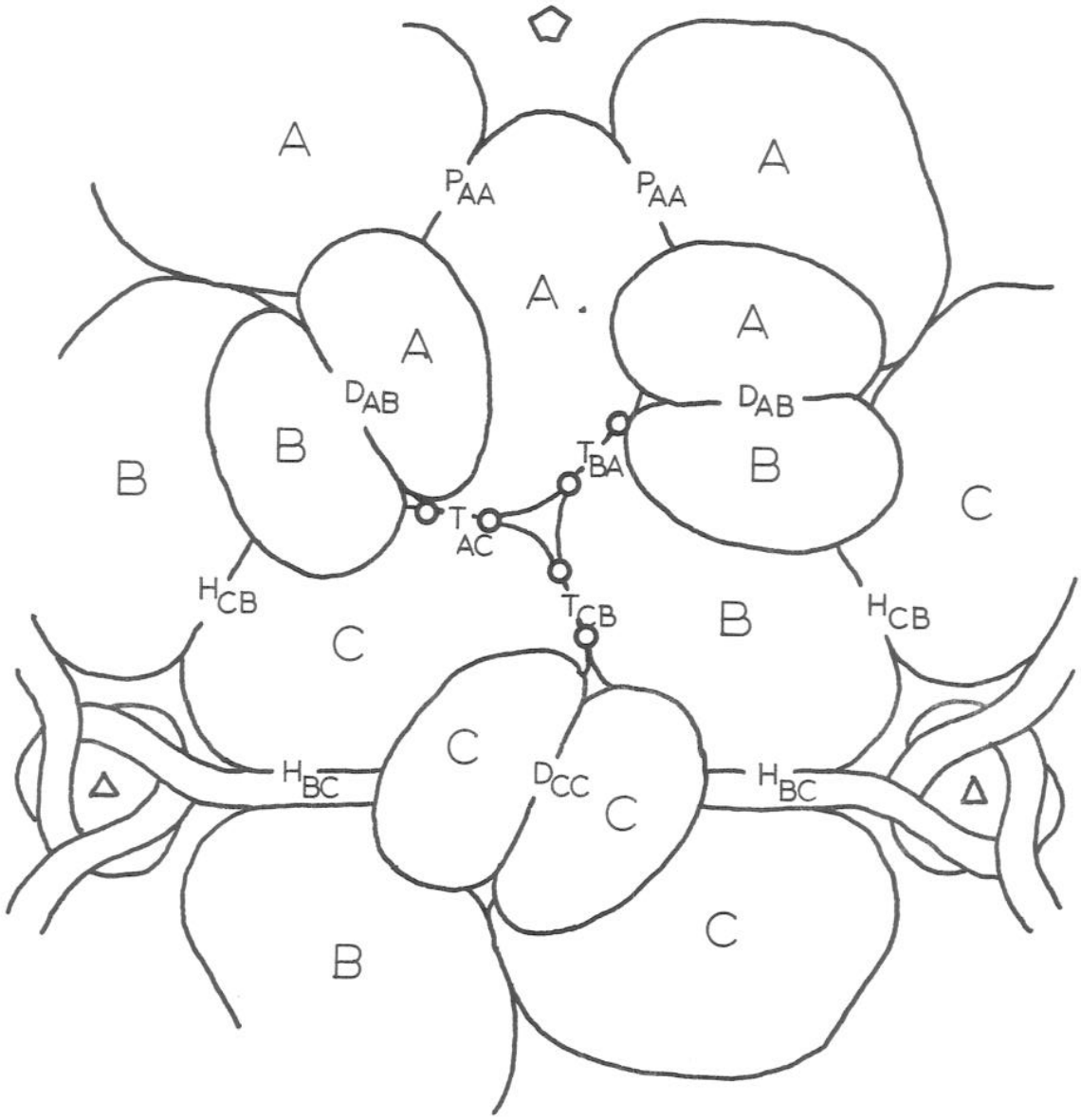

Figure 1.2. The relative location of the subunits of TBSV with respect to the icosahedral asymmetric unit. The quasi equivalent positions are denoted A, B and C. The different kinds of subunit contact are labelled D (dimer), T (trimer), P (pentamer) or H (hexamer), with a subscript of the positions that are related in anticlockwise order, looking upon the particle from the outside. The positions of the calcium sites are denoted by **O**.

which make contacts on all their sides; these contain 170 amino acids and have a largely antiparallel β sheet structure as shown in figure 1.3. There are two short α helices, one horizontal which makes the 2-fold 'dimer' contacts between the S-domains, and one vertical which forms part of the quasi 3-fold ('trimer') contacts. The sheet topology is that of a 'Swiss roll' (Richardson, 1977); four long strands of β sheet appear to be folded in the middle to form the toe of a 'slipper' which points towards the 5- or 3-fold (quasi 6-fold) axis. The C-terminal domain, called 'P' (projecting) contains 110 amino acids and is a 6 strand plus 4 strand antiparallel β 'sandwich' (Richardson, 1977). It is the more regular of the two domains. The PP dimer contacts (marked D_{AB} and D_{CC} in figure 1.2) form a continuation of the β sandwich to four layers, and so are expected to be very tight (see chapter 4).

As they have been so far described, the A, B and C position subunits in the three quasi equivalent positions of the icosahedral asymmetric unit are structurally identical. The conformations of the backbones of these are superimposable to better than 1Å, but there are significant differences in the side chains of the regions of the contacts. The H_{BC} contact is almost flat, while the quasi equivalent P_{AA} and H_{CB} are bevelled (see figure 4 of Winkler et. al., 1977). This gives rise to a cleft underlying the H_{BC} contact that is filled with an additional strand of protein, the arm 'e' of figure 1.2, which is ordered in the C

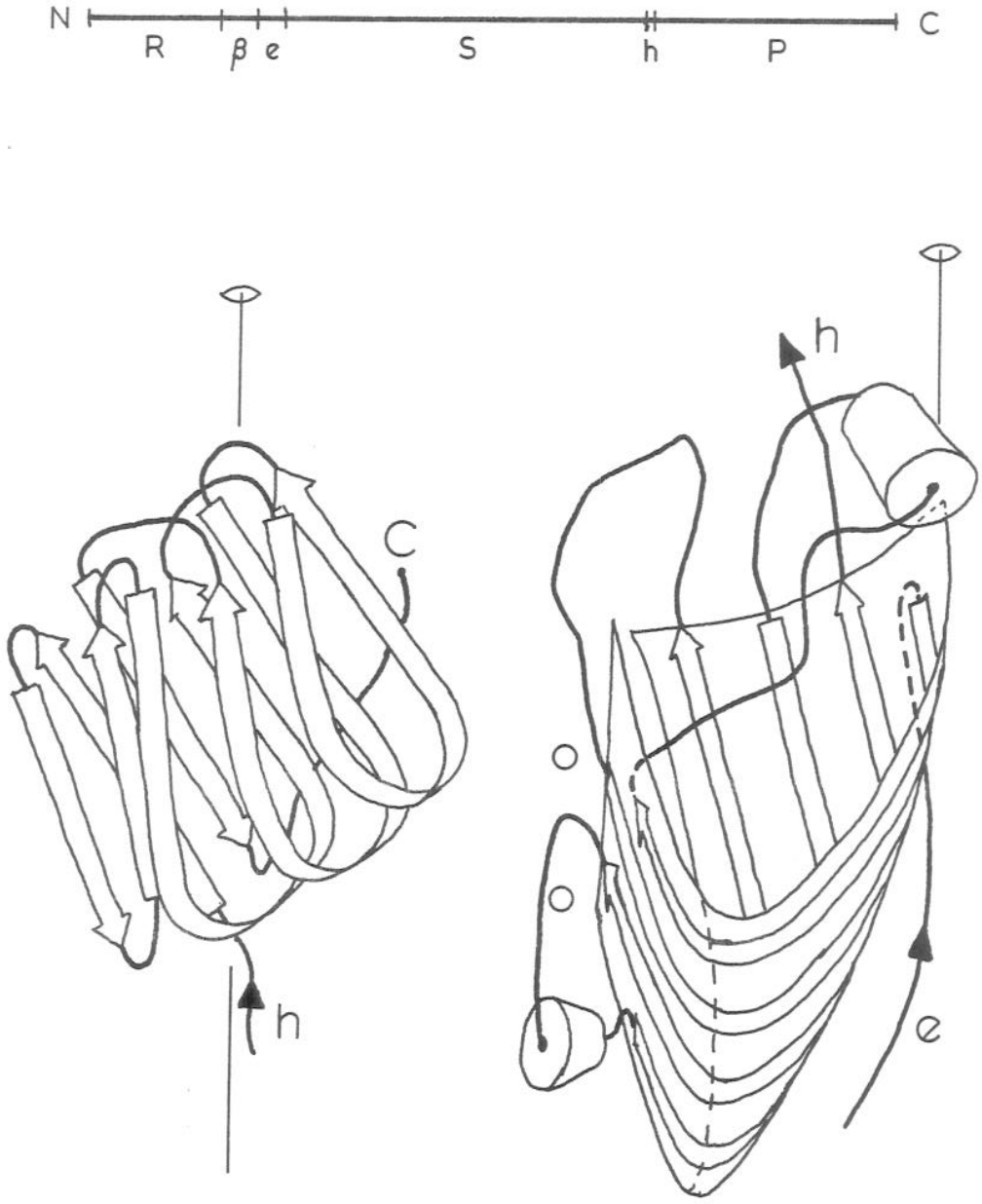

Figure 1.3. Schematic of the organisation of the TBSV coat protein polypeptide into its various domains (top) and cartoons of the chain fold in the P- (left) and S- (right) domains. The ribbons represent strands of β sheet and the cylinders represent α helices. The P-domain is viewed perpendicular to the plane of its 2-fold contact. The S-domain is viewed from the direction of the 5- or 3-fold axis. The location of the calcium sites in the trimer contact region are denoted by ○.

position subunit only. This extra feature terminates with an unique 'β annulus' structure, lying on the 3-fold axis, with alternating strands of a β pleated sheet contributed by the three C position subunits around that axis. The structure contains a total of about 60 amino acids and so is large enough to be considered as an extra domain. There are also slight locational differences associated with the quasi equivalence that place the A position S-domains slightly further from the virus origin, for instance, accounting for their visibility in the electron microscope.

The remainder of the polypeptide chain is spatially disordered in all three quasi equivalent positions of the subunit. This corresponds to 70 amino acids of the C position and 100 amino acids of the A and B position subunits. A 77 amino acid piece from this portion of the protein has been isolated after cyanogen bromide cleavage, it being the only water-soluble fragment. S. C. Harrison has shown that most of this fragment is resistant to proteolysis, chasing into a sharp electrophoresis band of molecular weight about 6,000, and implying that it exists as a well-defined domain. This has been called the 'R' (for RNA binding) domain and its structure is currently being sought. It is quite likely that this accounts for the shell of protein seen in the small angle scattering experiments to lie between 50 and 80Å radius. A crude volume estimate suggests that about 120 R-domains would fill this shell; the 30Å distance to the outer shell could be spanned easily by the

disordered arms of the A and B position subunits.

Two divalent cation sites per subunit have been located by Hogle and Harrison (1981) by examination of difference maps calculated for data collected from crystals soaked in EDTA at low pH. They lie between the S-domains along the trimer ('T') contacts, fairly close to the quasi 3-fold axis (see figure 1.2). The implication of this for the virus expansion mechanism will be discussed in chapter 4.

1.1.5 Related Virus Structures.

Of the group of viruses of interest, two others have been solved at high resolution: SBMV (Abad-Zapatero, et. al., 1980) and TCV (J. Hogle, unpublished). Both have T = 3 surface lattices, with 180 subunits per virion, and an extremely high degree of structural homology in their S-domains; the inner radii of all three virus capsids are identical. Both related structures include the ordered arm and β annulus in the C position and not the A or B positions. On the outer surface, TCV has a β annulus that is only half the size of that of TBSV, while SBMV appears to have an α helix inserted into the S-domain at residue 206 in the TBSV sequence; this lies parallel to the inner surface of the virus.

The outer surfaces show some even more striking differences. SBMV has no P-domain at all, but has an

additional α helix protruding near to the quasi 3-fold axis, that is involved with the trimer contacts, and probably increases their strength. TCV has a P-domain of about the same size and shape as TBSV; it appears to have the same β sheet topology but a very different structure at the detailed level, as its atomic coordinates do not superimpose very closely. In both SBMV and TCV there is a deletion of the 'Schutt door stop' (Winkler, et. al., 1977) at the extreme outside of the S-domain that is the point of interaction with the P-domains in the C position subunits of TBSV; this is understandable because of the differences in the P-domains among the viruses. The structural differences in the outer surfaces of these viruses probably account for their serological differences.

1.2 Virus Expansion.

There is very little published about the expanded state of TBSV or TCV, so we will look at the data for BMV first and then discuss the generalisation to TBSV.

1.2.1 Expansion of BMV.

The phenomenon of virus expansion was first observed by Incardona and Kaesberg (1964) for BMV. For this virus, the transition is induced by pH alone, and so several physical properties of solutions of the virus were examined as functions of pH, including sedimentation velocity,

diffusion coefficient and viscosity. They showed, by use of the Svedberg and Scheraga-Mandelkern equations relating the various coefficients, that the high and low pH states were accurately described by spheres of a fixed molecular weight (4.7×10^6) but different hydrodynamic radius (138Å at pH 6.0, 149 or 159Å at pH 7.2 depending on the calculation). All these values were for data extrapolated to infinite dilution. They concluded that the phase transition was structural, involving no change of aggregation state. From solution x-ray scattering, by means of Guinier plots, they showed that the expanded state had a radius of about 157Å, which agrees well with (although is not strictly comparable with) the hydrodynamic radius. Electron microscope studies, using an internal catalase crystal standard for accuracy, subsequently showed spherical particles with radii of 136 ± 6Å at pH 6 and 150 ± 7Å at pH 7.5 (Luftig, et. al., 1967), again in good agreement.

Incardona and Kaesberg (1964) also discovered the anomalous titration behaviour of BMV that is associated with the phase transition. Two protons per subunit were released when the low to high pH transition was made near pH 6.7. Bancroft (1970) postulated, by analogy with arguments for tobacco mosaic virus (Caspar, 1963), that this might be due to a pair of opposed carboxylic acid groups that could hydrogen bond together and so titrate near pH 7 instead of pH 4. Bancroft et. al. (1967) found exactly the same behaviour for the sedimentation of the related CCMV, and

found, in addition, that Mg^{++} ions prevented the transition to the expanded state. They proposed that this was due to the formation of a salt bridge link between the charged carboxyl groups, which would be stable at all pH values, and have the same structural effect as the hydrogen bonded low pH state.

More recently, small angle neutron diffraction experiments on BMV have concluded that the expansion is not just a simple two state transition (Chauvin, et. al., 1978A). The compact state is well defined, having a radius of 134 ± 1.5Å under all conditions, but the expanded state shows apparent polymorphic behaviour at pH 7.35. In the absence of Mg^{++}, the radius is 154 ± 1.5Å regardless of the ionic strength; with 10^{-2}M Mg^{++}, the radius drops to 145 ± 1.5Å and this value is <u>increased</u> by the additional presence of salt, the radius being 150 ± 1.5Å in 0.2M KCl, 0.01M Mg^{++}. This last result may be due to a competitive effect between the K^{+} and Mg^{++}.

Chauvin et. al. (1978A) also found that the radius change arising from the expansion was not completely reversible. Recontracted particles were found to have an outer radius of up to 140Å, instead of 134Å. Although this result and the previous one suggest that there a number of expanded states, possibly even a continuous distribution of them, another interpretation would be that there is a relatively small component of the protein (analogous to the R-domain of

TBSV) that can vary its radius from the centre of the particle over a wide range, depending on the prevailing conditions. In this interpretation, the failure of BMV to regain the original radius upon recontraction would be attributed to the trapping of this mobile component outside the particle (see below).

Durham et. al. (1977) performed careful titration experiments on BMV and found a distinct hysteresis loop in the anomalous titration curve. The uptake of protons (contraction) takes place at a lower pH value than the release (expansion), suggesting that some major structural change was taking place (see chapter 7). They found the same result for TCV, but not for turnip yellow mosaic virus (TYMV), which does not show the anomalous titration effect.

1.2.2 Expansion of TBSV.

Durham et. al. (1977) showed for BMV that the hysteresis and anomalous titration effects described above are substantially reduced in the presence of 27mM Ca^{++}. This is in agreement with the stated findings for Mg^{++}. They also performed the experiment on TCV and found that there was no proton release at all when a Ca^{++} concentration of 1.5mM was present. The conclusion is that TCV has a tighter binding constant for Ca^{++} by at least two orders of magnitude (2 pCa units). Another significant difference is that native preparations of BMV do not have any divalent cations bound

(Incardona and Kaesberg (1964) did not control this variable), whereas those of TCV contain bound Ca^{++} and require treatment with EDTA before expansion effects can be observed.

The expansion of TBSV can be seen in small angle x-ray scattering patterns of concentrated solutions of the virus in the two states. There is a 10% shift inwards of the diffraction minima, indicating a 10% radial expansion of the outer surface of the virus (see Harrison, 1969). Another feature that may be significant is that the diffraction pattern of the compact virus has clear rings visible out to 10 Ångstroms resolution, whereas that of the expanded state does not; this could indicate that the expanded state has a distribution of particle radii due to its increased flexibility.

The two states of TBSV have been examined using ^{31}P nuclear magnetic resonance techniques by Munowitz et. al. (1980). The ^{31}P linewidth is considerably narrower for the expanded state, even though the reduced particle tumbling rate would be expected to show the opposite trend. The interpretation is that the RNA in the compact state is immobilised relative to the viral capsid, whereas at least a fraction of this becomes detached in the expanded state, and behaves more like RNA in solution.

Expanded TBSV is highly sensitive to proteases, just as was shown for BMV (Pfeiffer and Hirth, 1975).

S. C. Harrison (unpublished) has shown that there is hysteresis in the pH response of the radius of TBSV by small angle x-ray scattering, but also that the recontracted state can retain its sensitivity to proteolysis by chymotrypsin, when the recontraction is performed rapidly. However, when the expanded virus is dialysed against recontraction conditions so that the transition takes place slowly, it loses its proteolytic sensitivity. The rapidly recontracted ("quenched") state has a somewhat lower pH threshold for further expansion than the native virus, so is clearly not the identical state. Even reintroduction of the Ca^{++} does not return it to the original state. Cleavage mapping experiments (S. C. Harrison, unpublished) have shown that the points of chymotrypsin cleavage are in the 'arm' region of the coat protein (see figure 1.3), so the conclusion must be drawn that the quenched state has its arms protruding from the protein shell, probably trapped in the gaps that must form when the S-domains part during expansion. Both the quenched and expanded particles are also sensitive to Staph. protease, which makes a single clean cut at Glu 36. This is well inside the region considered to belong to the R-domain, so the implication is that the R-domains as well as the arms are extruded from the virus in the expanded state. The stochiometry of this cleavage suggests that only 1/3 to 1/2 of the arms are extruded, and certainly not all of them, which may be showing a differential preference among the three quasi equivalent positions of the arm.

TCV is a better experimental system to work with for these investigations into the expansion mechanism because it can be dissociated into subunits (see below), which can be used as controls for proteolysis rates. It is hoped that a better understanding of the physical chemical nature of expansion will result (P. Sorger, work in progress).

1.3 Virus Assembly.

The results of attempts to perform in vitro assembly and disassembly of the group of viruses of interest may reflect on how a structure of the expanded state is to be interpreted. As with the expansion experiments, more work has been done with BMV and CCMV than TBSV or TCV.

1.3.1 Assembly of BMV and CCMV.

CCMV and BMV coat protein can be prepared by dialysing the virus against 1M NaCl at pH 8, and isolated by centrifugation (Bancroft et. al., 1967). The protein sediments at 3S, indicating that it is in an aggregated state, and is not in the form of monomers. It is not clear whether this fraction contains dimers or trimers (Adolph and Butler, 1974). The protein preparation was dialysed against a large range of buffers of different pH (3.8 to 7.5) and ionic strength (0 to 1.0M) to produce a phase diagram of the aggregation. The most interesting feature is that there are

no states between the 3S and 50S states corresponding to the protein and the complete capsid respectively; hence no intermediates are stable for long enough to be seen as a sedimentation band. The same general picture is true for BMV (Pfeiffer and Hirth, 1974). In both cases, polymerised states larger than the capsid are seen at low ionic strength. No self-assembly into the expanded state is observed: all attempts at pH > 6.0 , I > 0.3 yielded no aggregated states at all. Thus it seems unlikely that the expanded state of these viruses is an assembly intermediate.

CCMV coat protein can be reassembled with its own RNA or with that of BMV or broad bean mottle virus (BBMV), with complete retention of the infectious and nuclease resistance properties (Bancroft, 1970). The full pH and ionic strength dependence has not been investigated, however. Jacrot (1975) showed that the positions of the hysteresis loop in titration experiments of CCMV and in vitro assembled CCMV capsids are very different, much more so than the difference between native and recontracted TBSV, as described above. This indicates that these species expand at different pH values and implies that the RNA also has a role in the transition.

1.3.2 Assembly of TCV and TBSV.

Attempts to disassemble TCV (Leberman and Finch, 1970) in high salt, high pH Tris or ethanolamine solutions

yielded 4S and 46S sedimentation bands, both lighter than the 120S band of the expanded particle. Electron micrographs and a preliminary x-ray crystal analysis showed that the 46S band corresponds to a T = 1 'small particle' of TCV containing 60 coat protein subunits and no RNA. The fully dissociated state (corresponding to the 4S band) has been shown by cross-linking studies to consist of dimers of the subunits connected by their P-domains (Golden and Harrison, 1981). The conditions for obtaining small particles were at that time poorly defined, but have since been refined by S. C. Harrison. The critical step is the removal of the N-terminal arm by proteolysis in the dissociated state. Close to 100% yields of small particles are then possible. The T = 1 particle is a very reasonable aggregation state of the TCV coat protein: all the 60 subunits are in the A conformation and the P-domains form dimers along the particle 2-fold axes (see figure 1.2). The fact that TCV does <u>not</u> form small particles when it possesses its arm suggests a function for that arm: it acts to block the pentamer polymerisation that would lead to T = 1 particles. We must therefore look to the arm for the first step in the mechanism of assembly of T = 3 particles from dimers.

No conditions have been found under which TBSV can be disassembled other than by denaturation of the protein itself. TCV reconstruction from separate RNA and protein dimer fractions has been achieved (D. Kimmelman, P. G. Stockley and S. C. Harrison, unpublished). The

fractions are mixed under high salt, high pH conditions and first dialysed against low pH, then against low salt buffers. Calcium is not found to be necessary for reassembly.

1.3.3 Size Determination.

The most important question to be answered concerning the assembly of spherical viruses with T > 1 surface lattices is that of the mechanism that is used to ensure precise control of the triangulation number, T, while the subunits are packing together. The subunits must adapt the correct detailed conformation of the particular quasi equivalent positions they occupy. Presumably the subunit in solution has a favoured conformation. If the pentamer-forming conformation were preferred, there would be a strong tendency to form just T = 1 particles; if the hexamer-forming conformation were prefered, flat sheets would probably result. It is also possible that the favoured conformation is neither of these, being, say, equally distant (in conformation space) from both; if this were so, a random mixture of pentamers and hexamers might form, but there is still no guarantee of the proper closure of the particle, and it seems likely that many errors would occur. Particles in electron micrographs of native virus preparations have never been observed with assembly errors.

Harrison et. al. (1978) proposed a specific

mechanism by which TBSV can assemble perfectly into T = 3 capsids. Under disassembly conditions, the TCV subunit is a dimer in solution, with the contact between the P-domains, and so presumably is the TBSV protein when it is first synthesised inside the cell. In this state, the β annulus cannot have been formed; it is reasonable to expect this to be the next step in assembly. The result of dimers aggregating in threes to form β annuli is a polymerisation of a highly crosslinked kind. The interaction between the β annulus and the base of the S-domain will constrain the geometry of the aggregation to that of a pentagonal dodecahedron. This hypothetical intermediate contains the 60 C position subunits of the final capsid in their correct positions, with the overall size and T = 3 geometry determined. The assembly then proceeds by the addition of five dimers to every pentagonal face. These are forced to adopt the A and B positions of the final structure.