CHAPTER XL

# THE STRUCTURE OF VIRUSES AS DETERMINED BY X-RAY DIFFRACTION*

*Rosalind E. Franklin, D. L. D. Caspar, and A. Klug*

THE PIONEER X-RAY INVESTIGATIONS by Bernal and Fankuchen (5, 9) were among the earliest and most informative physical studies on tobacco mosaic virus (TMV). This work was undertaken on material provided by Bawden and Pirie (5) within a year of the first isolation and purification of TMV by Stanley (89). Likewise when the first spherical virus was crystallized (6), the preliminary X-ray investigations (11) followed soon thereafter. The first X-ray photographs of TMV taken over twenty years ago demonstrated that the individual virus particles are built up of subunits arranged in a regular way (10), but this observation made little impression on the general direction of research into virus structure until fairly recently. Viruses were looked at for some time rather as giant molecules. Physico-chemical studies were concerned largely with characterizing a virus particle in terms of its size, shape, molecular weight, and hydration, while the biochemical investigations emphasized over-all composition and chemical reactivity.

The first approach to the study of substructure in viruses by physico-chemical methods was made by Schramm, who, in work published in detail in 1947 (83), described the alkaline degradation of TMV. The breakdown proceeds in a stepwise fashion until the small, relatively homogeneous A-protein is produced which can be separated from the ribonucleic acid (RNA) of the virus. It was, however, not until 1952 that the end-group analyses of Harris and Knight (51) provided a clear indication that the protein part of TMV consists of a large number of small, chemically identical protein molecules. It is now possible to identify this chemical subunit of TMV with the structural subunit "seen" by X-rays. Substructure has also been revealed, by both biochemical and X-ray analysis, in some other small viruses which contain RNA. This review will be concerned mainly with the structural studies on small RNA-containing plant viruses.

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## The X-ray diffraction method and virus substructure

We cannot here go into the principles of X-ray diffraction techniques, and can hardly do more than mention the method of analysis. The reader wishing to know more about the X-ray analysis of biological structures is referred to the excellent account for the noncrystallographer by Crick and Kendrew (22). X-ray diffraction is particularly useful for studying any structure which repeats regularly in space. The scale of structure which can be investigated ranges from a few angstroms, as in simple atomic and ionic crystals, to hundreds of angstroms, as in virus crystals. X-rays pick out the ordered part of a structure in such a way that the diffraction diagram of a substance is directly related to the structural regularity within it. The order in a structure arises from the regular arrangement of smaller parts (asymmetric units), and it is this arrangement that determines the position of the reflections ("spots") on the X-ray diagram. The important point is that the asymmetric units are identical and all have the same environment.

Crick and Watson (23) have suggested that all small viruses are built up on a framework of protein subunits packed together in a regular manner. This hypothesis provides a simple explanation of the regular shape of small virus particles, and was based largely on the example of TMV. In another paper (24) reasons were presented why one might expect small viruses to be made up of subunits. The infectivity of a virus is carried by its RNA (27, 46) and the function of the protein part is presumably to provide some form of packaging for the specific RNA. It appears that the RNA "codes" the virus protein, and it is reasonable to expect that the RNA may only be able to "code" the protein in the form of small identical molecules. Such protein subunits can then aggregate in a regular way to form the protein shell or coat of the virus.

Regular packing of a number of identical units so that the same kind of contacts between units are used over and over again will necessarily result in a symmetrical structure. This means that a virus particle can be constructed out of subunits in only a limited number of ways since there are only a rather small number of kinds of symmetry possible for a particle. A discussion of symmetry cannot be undertaken here, and for details the reader is referred to the papers of Crick and Watson.

The well-defined symmetry to be expected for virus particles is very favorable for X-ray structure analysis since the symmetry of the particle is indicated in a relatively direct way by the X-ray diagrams. Determination of the symmetry does not indicate what the asymmetric unit looks like. It does, however, tell us how many there are and how they are related and thus gives some indication of the size of the structural subunit and the overall appearance of the virus particle.

A physical limitation of the X-ray method is that a very large number of particles must be examined at one time in order to have enough diffracting material to obtain a photograph. For this photograph to indicate the symmetry of a single virus particle, the particles must be regularly arranged in space, either in a crystal for the spherical viruses, or in an oriented gel ("paracrystal") for the rod-shaped particles like TMV.

The basic problem in X-ray structure analysis is that the diffraction pattern itself does not contain all the information necessary to completely determine the structure which gave rise to it. The ultimate goal of a structure analysis is to calculate (by Fourier synthesis) the three dimensional electron density* distribution in the asymmetric unit to a resolution compatible with the quality of the experimental data. There is, however, no standard or direct method by which this can be done. Each X-ray reflection or spot on the photograph corresponds to a diffracted wave which is characterized by three quantities: direction, magnitude, and phase. Of these, the first two can be determined directly, but in taking the photograph all information as to the phase angle is lost. In order to reconstruct the electron density of the diffracting structure this phase angle must somehow be determined. For complicated structures there is only one generally certain way of doing this. This is the method of isomorphous replacement (48), in which the diffraction pattern is modified by binding a heavy atom, such as mercury, in a regular way to each asymmetric unit. The changes of intensity in the pattern due to the presence of the heavy atom may be used first to determine the position of the heavy atom and then to calculate the phase angles. It should be noted that for the purpose of phase determination it is not necessary to know the chemical nature of the binding of the heavy atom to the virus; all that is required is that it be specific, and that the replacement is isomorphous, i.e., it does not significantly change the structural arrangement of the virus.

Although it now appears that the method of isomorphous replacement affords the only hope for determining the detailed internal structure of a virus, it should be stressed that a great deal of structural information can nevertheless be obtained by fairly direct interpretation of the X-ray diagrams. As discussed above, determination of the symmetry gives considerable insight into the substructure of a virus; by comparing certain parts of the diagram with predictions based on simple models some of the significant morphological features can be deduced, and comparison of the patterns given by different strains can also reveal general structural regularities.

* For most biological structures, which are constituted for the most part of atoms of low atomic number, the electron density is approximately proportional to the mass density.

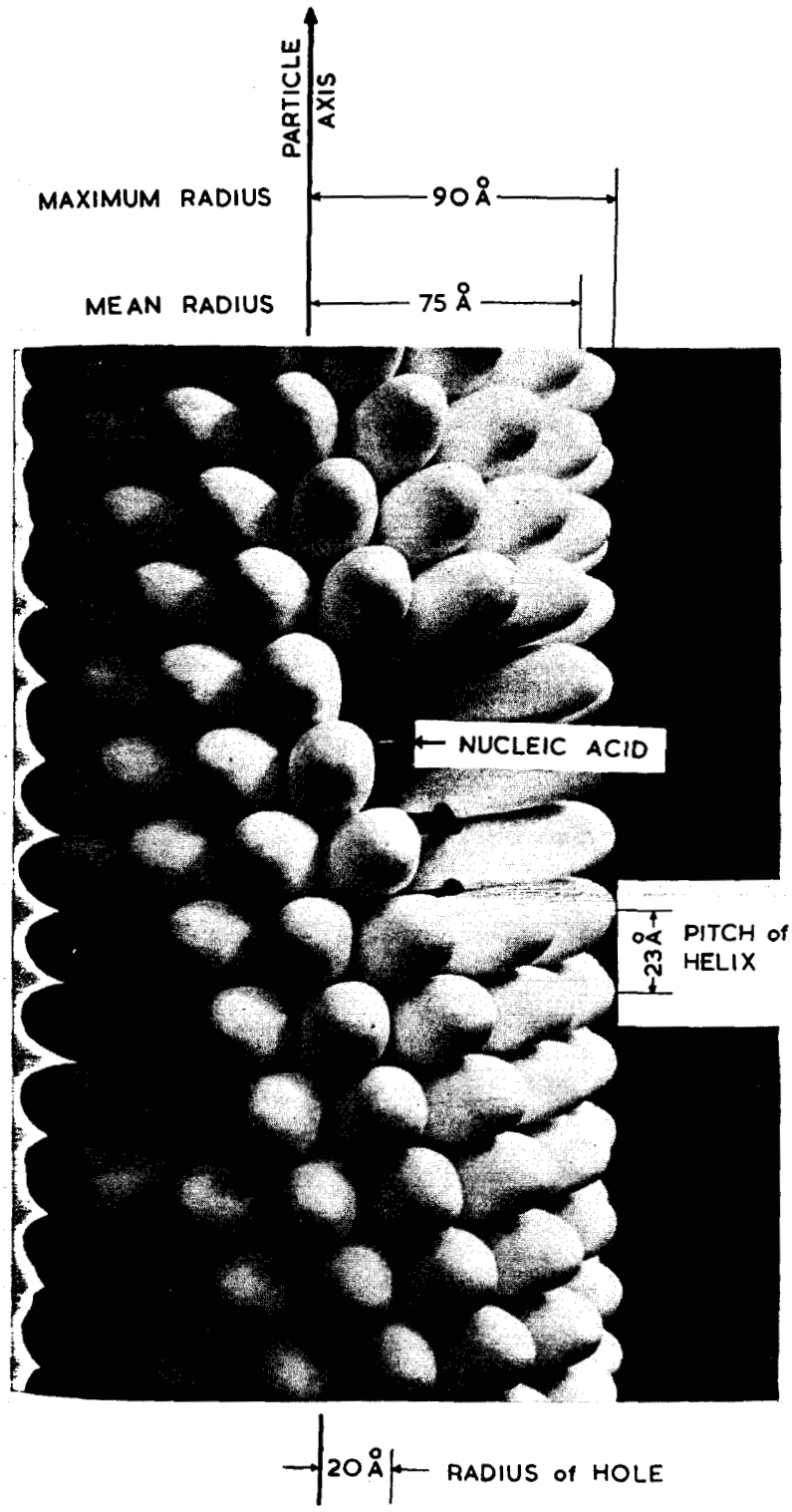



PLATE XL–1.—A model of tobacco mosaic virus, based mainly on the X-ray diffraction studies of Franklin. About one-tenth of the total length of the virus particle is shown.

The virus protein is in the form of a large number of small, identical subunits set in helical array about the particle axis. The structure repeats after 69 Å in the axial direction, and the repeat contains 49 subunits distributed over three turns of the flat, major helix of pitch 23 Å. The shape of the subunits as represented is rather schematic, but is such that the helical array has a hollow core of diameter 35–40 Å and each subunit appears as a protuberance at both the inner and outer surfaces, giving the particle a maximum diameter of 180 Å.

The ribonucleic acid is deeply embedded in the protein array and is in the form of a single long-chain molecule which follows the line of the flat helix, at a radius of 40 Å. Some of the protein subunits have been removed from the model to show the RNA, which is represented schematically by a smooth coil.

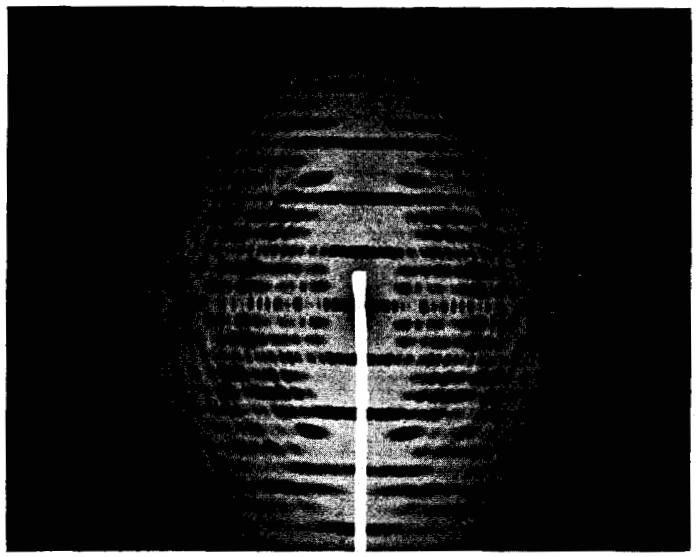


PLATE XL–2.—X-ray diffraction diagram (by Franklin) of an orientated gel of mercury-substituted TMV, common strain, taken with a high resolution focusing camera and crystal-monochromatized CuK$\alpha_1$ radiation. The diagram is very similar to that of TMV itself already published elsewhere (40 43) but is of even higher quality.

The virus particles have their long axes in the vertical direction and the pattern corresponds to the cylindrically averaged intensity diffracted by a single particle.

The horizontal stratification ("layer-lines") corresponds to the axial repeat period of 69 Å in the virus particle. The intensity distribution along a layer line does not consist of discrete reflections as shown by a true crystal, but is continuous with maxima showing up as spots.

The orientation of the specimen is particularly good as shown by the small degree of arcing of the spots on the diagram.

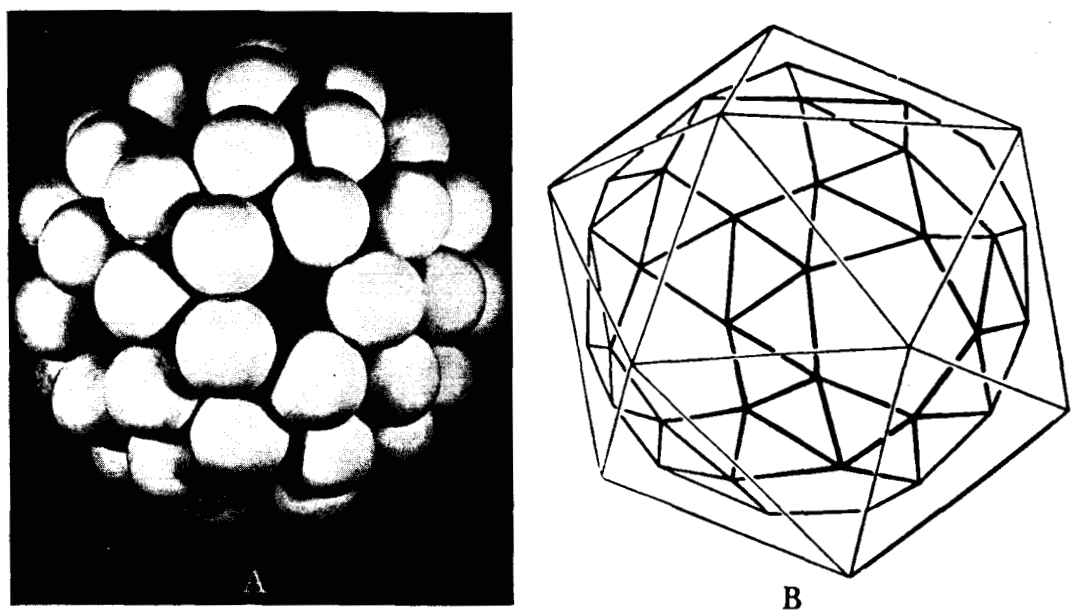



PLATE XL–3.—(*A*) Schematic model illustrating one way in which 60 subunits may be packed according to icosahedral symmetry to form the protein shell of a spherical virus. The subunits are arranged at the vertices of a snub dodecahedron (*B*), which has the same rotational symmetry as the icosahedron in which it is shown inscribed.

Ping-pong balls have been used for convenience in building the model, but do not represent the actual shape of protein subunits. Whatever shape is used in a model of this kind, the geometrical relation between each subunit and its neighbors remains the same for all subunits.

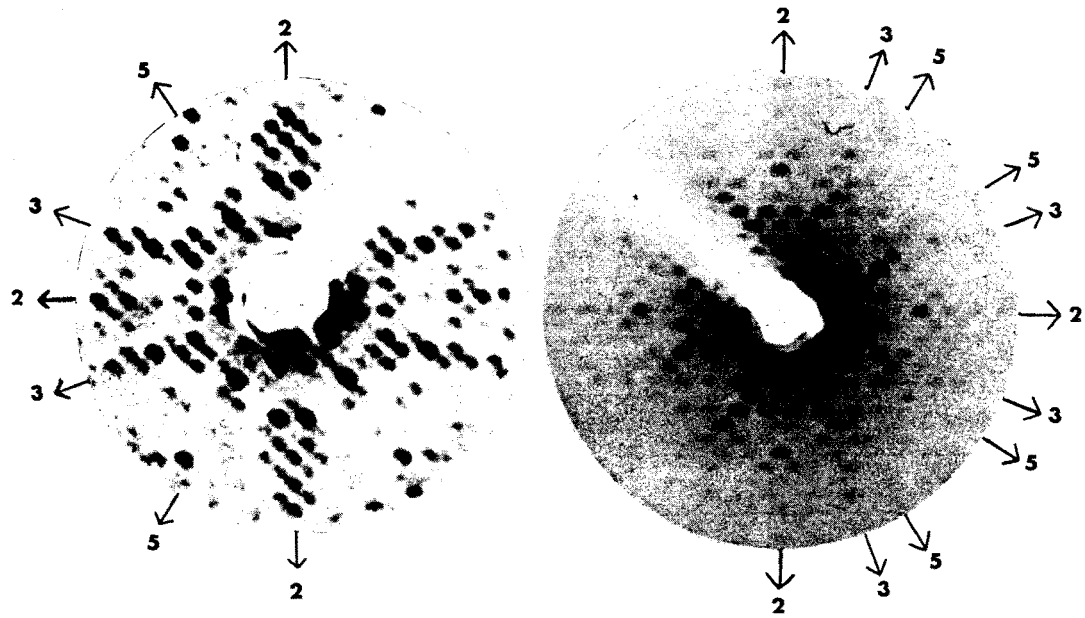



PLATE XL–4.—X-ray diffraction diagrams (precession photographs) of single crystals of (*A*) tomato bushy stunt virus (18), and (*B*) turnip yellow mosaic virus (63), taken in each case with the X-ray beam parallel to the edge of the cubic unit cell. Only the central region of each pattern is shown.

Many such photographs must be taken along different crystal directions in order to record the complete three-dimensional diffraction pattern of a crystal.

The directions of the twofold, threefold, and fivefold rotation axes corresponding to icosahedral symmetry, which lie in the plane of the photograph, are marked by arrows. The twofold axes are parallel to the cube edges of the crystal, but the other two sets do not lie along crystallographic axes. There is a concentration of strong reflections ("spikes") in these directions. There are twice as many such spikes in the TYMV pattern as in the BSV, because the virus particles in the crystal of TYMV are arranged in two different orientations (rotated 90° to each other about the cube edge of the crystal). In the BSV crystal, all the virus particles have the same orientation.

*Tobacco mosaic virus*

TMV is at present the only virus for which the X-ray structure analysis has been carried far enough for us to be able to build a model (Plate XL–1) which gives a reasonably realistic representation of the structure. Most of the information which has gone into the construction of this model has been obtained from the work of Rosalind Franklin. Detailed structure analysis requires X-ray photographs of very high technical quality (Plate XL–2), and these can only be obtained from very well-orientated gels. To illustrate the prospects and difficulties of the X-ray diffraction method as applied to viruses, we shall discuss the studies on TMV in some detail.

*The helical structure of TMV.*—The early X-ray studies of Bernal and Fankuchen indicated that TMV has a regular substructure, but at that time the arrangement of subunits was not clear. In 1954 Watson (91) indicated that the essential features of the X-ray pattern could be accounted for if the virus particle were built up of a helical array of subunits set about the long axis of the particle. In the axial repeat period of 69 Å there would have to be $3n + 1$ (where n is an integer) subunits equally spaced along three turns of the helix. Further X-ray studies by Franklin (30) and Franklin and Klug (41) completely confirmed this helical structure, in particular by showing that certain unusual features of the X-ray diagram (layer-line splitting) could only be accounted for by this type of structure.

The value of n is now known to be 16, so that there are 49 subunits in three turns of the helix or 16⅓ per turn. Direct determination of the value of n proved very difficult and the earlier estimates were too small. This number was determined by Franklin and Holmes (39, 40), using a mercury-substituted TMV prepared by Fraenkel-Conrat, in which a methylmercury group is bound to the sulphur of the single cysteine residue (2, 64) of each protein subunit. The mercury atoms serve as markers for the subunits and are necessarily distributed on a helix with the same axial repeat as TMV. From a detailed quantitative comparison of the X-ray diffraction diagrams of the mercury-substituted TMV and of normal TMV, it was possible to deduce that there are 49 mercury atoms equally spaced along three turns of a helix of radius 56 Å. Therefore there must be the same number of protein subunits in three turns.

On returning to the X-ray diagram of normal TMV itself, and using the recently determined maximum radius of the particle (17, 42, 43), it was found that a self-consistent interpretation of the parts of the diagram due to the outside of the particle is obtained if there are 49 structural subunits in three turns of the helix. Since these independent measurements refer to the structural unit of the virus, and the measurements on the mercury positions refer to the chemical subunit, it appears that the structural and chemical subunit are identical.

Further confirmation of the identity of the TMV subunit demonstrated by chemical and X-ray methods is provided by a comparison of the molecular weight of the structural unit as determined from the number per virus particle with that of the chemical unit given by end group analysis (14, 28, 52, 74) and amino acid composition (64, 73, 80). A recent detailed study of monodisperse TMV preparations by Boedtker and Simmons (13) by a number of physico-chemical methods gives a molecular weight of $39\ (\pm 1) \times 10^6$, and the particle length of these same preparations as measured with the electron microscope by Hall (49) is $3000 \pm 50$ Å. These values agree very well with many of the earlier measurements of the molecular weight (68, 77, 84) and particle length (76, 97). Since there are 49 subunits in a 69 Å length of the virus, there are $49 \times 3000/69 = 2130 \pm 40$ structural subunits in the particle of length $3000 \pm 50$ Å. Five to 6 per cent by weight of TMV consists of RNA (64, 79), thus the molecular weight of the 2130 protein subunits is $37\ (\pm 1) \times 10^6$. Hence the molecular weight of the protein structural subunits is $17{,}300 \pm 800$. This is in complete agreement with the molecular weight of 17–18,000 obtained by the chemical methods. It appears likely from these results that all the protein of TMV consists of small identical protein subunits organized in the helical structure.

There is of course no structural reason why a helix should contain an exact integral ratio of subunits to turns, and 49/3 is merely the nearest rational approximation. In fact it has been shown (41) that the number of units in three turns is not exactly integral, and that this departure from an integer varies slightly from strain to strain (32), corresponding presumably

to slight differences in the packing of subunits. The effects are, however, very small and correspond to a difference of only one to three subunits in the more than two thousand contained in the virus particle of length 3000 Å.

*Morphology and internal structure of TMV.*— The diameter of TMV has for some time been quoted to be 152 Å on the basis of the X-ray measurements (10) of the interparticle distance in dry orientated TMV preparations. Interparticle distances measured by the electron microscope agree well with this figure, although observers had occasionally been puzzled by the fact that isolated particles seemed to be thicker (95). It is now clear that the maximum diameter of the particle is a good deal greater than 152 Å and that this figure represents the packing distance between particles intermeshed to form a close-packed hexagonal array.

This conclusion was reached by Franklin and Klug (42), who showed that the outer surface of the virus could be represented by a helically grooved model with a mean radius of 76 Å, but with a maximum radius of about 85–90 Å. The helical grooves are only a way of describing the spaces between protruding subunits near the outside of the virus.

The fact that the particle has a maximum radius greater than 76 Å has been definitely shown by the radial density distribution (17) determined by the method of isomorphous replacement as described below. The best estimate of the maximum radial extension of the particle comes from some recent work of Franklin (36) on the binding of osmium to TMV. It is found that osmium atoms attach to TMV at a radius of 90 Å, indicating that there is still some protein at this radius.

To obtain more information on the structure it is necessary, as already mentioned, to resort to isomorphous replacement. This was first successfully accomplished for TMV by Caspar (16, 17) who was able to prepare a lead-substituted TMV. From the changes produced in the equatorial X-ray scattering, it was found that equal amounts of lead are bound at radial distances of 25 and 84 Å from the particle axis, though only an average of about half a lead atom per protein subunit could be bound to each of the two distinct sites in the virus. The location of the bound lead made possible a definite determination of the signs* of those equatorial diffraction maxima which correspond to the cylindrically averaged electron density of the virus. Using these signs and the observed magnitudes, the Fourier transform was calculated to give the cylindrically averaged radial density distribution in the particle. The result is shown by the full curve in Fig. XL–1(*a*).

Caspar's allocation of signs was confirmed by a second successful application of the method of isomorphous replacement to TMV by Franklin (33, 43), using the mercury-substituted TMV prepared by Fraenkel-Conrat. One can therefore have complete confidence in the correctness of the radial density distribution shown in Fig. XL–1*a*. It should, however, be mentioned that the small fluctuations which occur near the axis are of no significance since in this region the effect of experimental error is large.

The most significant features of the radial density distribution are as follows: (*a*) The virus particle has a hole of diameter 35–40 Å extending along the axis; (*b*) The maximum radius of the particle is close to 90 Å; (*c*) There are regions of high density at radii of 25, 40, 66, and 78 Å.

The axial hole of TMV is occupied by water when the virus is in solution, and the inside surface of the virus is as accessible to small molecules as the outside. When TMV is dried from solutions containing small solute molecules, these molecules fill in or line the hole, and in this way Huxley (57) has recently shown the hole directly in electron micrographs.

Electron density carries no chemical label, so the location of the RNA cannot be determined from the density distribution of TMV alone, but studies on nucleic acid-free particles, described below, demonstrate that the very high density region about a radius of 40 Å is due to the RNA. The other well-defined maxima must be due to the configuration of the polypeptide chain which makes up the protein subunit. Although some plausible suggestions regarding the folding of the polypeptide backbone (30, 34, 91) have been made, no definite conclusions can be drawn from the present, limited evidence.

To obtain further information about the struc-

* For this part of the X-ray diagram the phase angles are only 0 or 180°. In this case each diffraction maxima is characterized by a sign, either plus or minus, and a single heavy atom substitution is sufficient to determine the signs.

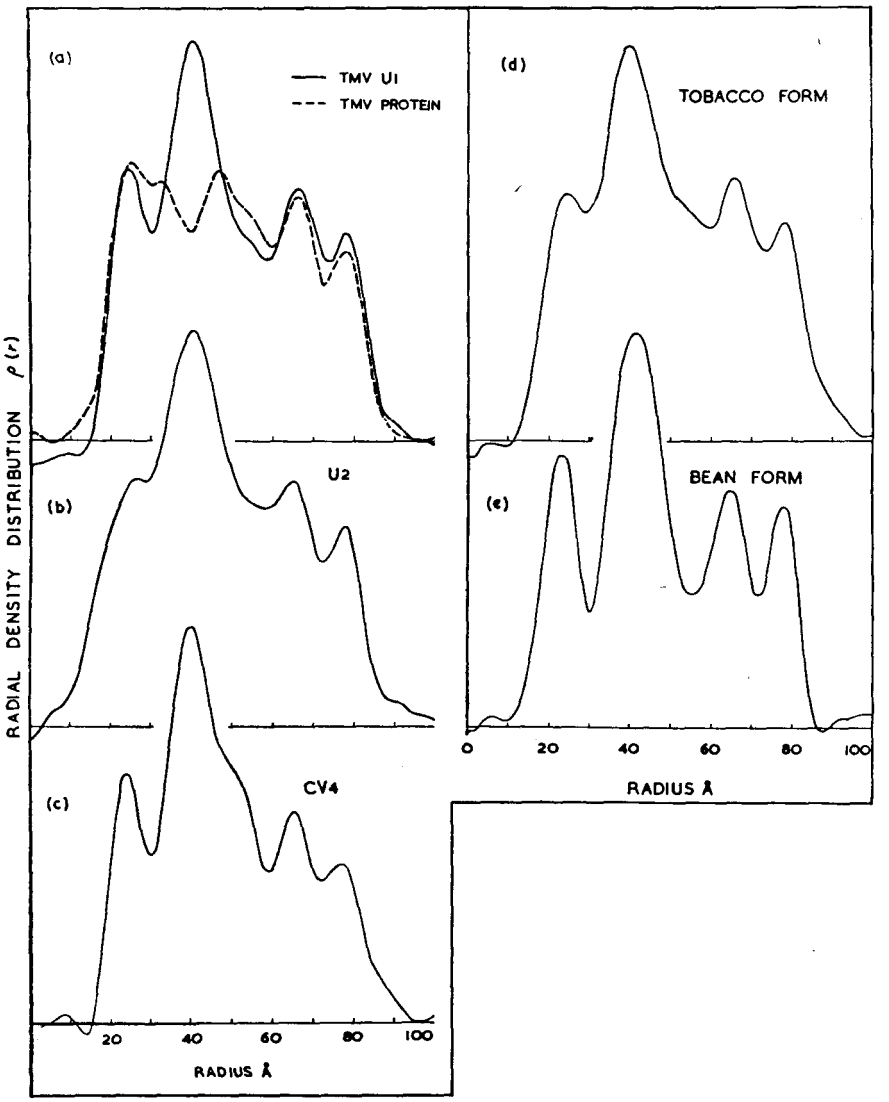



FIG. XL–1.—(*a*) The cylindrically averaged radial density distribution in (i) tobacco mosaic virus, common strain (full curve) (17, 43); (ii) repolymerized, nucleic-acid free, tobacco mosaic virus protein (dotted curve) (33). The curves show the difference between the electron density of the particles and that of water, plotted as a function of radial distance from the particle axis. The high peak at 40 Å in (i) is absent in (ii) and must therefore be due to the RNA in the virus. The actual difference in the 40 Å region is about 10 to 12 per cent, i.e., twice the percentage of RNA in the virus, because the density of RNA above that of water is twice as great as for protein. (*b*)—(*e*) show the radial density distributions (55) in some strains of TMV: (*b*) Strain U2 (87); (*c*) Cucumber virus 4; (*d*) A strain of TMV taken from Nigerian cowpea and grown in Burley tobacco plants (4); (*e*) The same as (*d*), grown in the Prince variety of French bean.

ture of the protein subunits it is necessary to investigate other parts of the X-ray diagram besides the equator. Here, however, the diffraction amplitudes are complex so that phase angles rather than signs have to be determined, and for this purpose at least two isomorphous replacements are necessary. High-quality photographs (Plate XL–2) of the mercury-derivative of

TMV show significant changes in the nonequatorial reflections, but comparable photographs of the lead-derivative have not yet been obtained. It is thus not yet possible to determine the phase angles unambiguously. In any case it would probably be necessary to obtain more than two isomorphous replacements.

Some preliminary work of Franklin on the comparison of third layer-line of the mercury derivative with that of TMV has led to the construction of a map (37) giving a view of the electron density in TMV projected down the basic helix. Because of the uncertainties in the phase determination, the results cannot be regarded as established, but it does seem that certain features (34) shown by the map are probably correct, since they are compatible with the direct interpretation of other parts of the X-ray diagram. It is found that the shape of the protein subunit is such that it protrudes at the inner as well as the outer surface. The internal and external protuberances do not occur opposite one another, thus suggesting that the general lie of the protein subunits is not strictly perpendicular to the particle axis, but somewhat skew to it (see Plate XL–1). Furthermore, the shape of the protein subunits is such that their helical packing results in a set of holes in the virus particle at a radius of 55–60 Å. Inner regions of the virus may be accessible to small molecules through these holes and the external grooves. It is interesting to note that the cysteine, as located by the position of the bound methyl-mercury, lies at about a radius of 56 Å, and thus close to this hole. The fact that the free—SH group of the native virus will react with methyl mercury nitrate (26) and iodine (2, 26), while there is no reaction with parachloromercuribenzoate (PCMB) and other equally large sulfhydryl reagents (2) unless the virus is denatured, may be explained if the spaces between the subunits are acting as "molecular sieves" which accommodate the smaller, but not the larger molecules.

*Strains of TMV.*—It was shown by Bernal and Fankuchen (10), and later by Franklin (32), that different strains of TMV give X-ray diffraction patterns which bear a strong resemblance to one another, though small, measurable differences can be detected. Following the determination of signs of the equatorial reflections of the common strain (17, 33), it became possible to deduce the signs in the different strains as well. The radial density distribution of CV4 was calculated in this way by both Kilkson (59) and by Holmes and Franklin (55), who also investigated three other strains of TMV. The results of Holmes and Franklin are given in Fig. XL–1 (*b–e*) and they show that the structural arrangement of protein and nucleic acid is fundamentally similar in all the strains of TMV and in CV4. The similarities and differences are discussed in full in the original paper, but perhaps the most interesting conclusion concerns CV4, which has been considered (65) as not being a strain of TMV.

The earlier X-ray investigations (10) showed that the packing diameter of CV4 was smaller than that of TMV by about 3 per cent. Fig. XL–1(*c*) shows, however, that the maximum diameter is the same in each case, but that the weight of protein in the outer part of the particle is considerably less in CV4 than in TMV. The closer packing in dried CV4 thus probably arises simply from a deeper interlocking of knobs and grooves. The structure of CV4 as indicated by the X-ray diagrams is as closely similar to that of the common strain of TMV as any of the other strains investigated.

The only other rod-shaped virus besides TMV and related strains that has been investigated by X-ray diffraction is potato virus X. The photographs that have been obtained (10) are not as good as those of TMV, presumably because the sinuous PVX particles are more difficult to orientate. The photographs do suggest, however, that the structure is helical with a pitch close to that of TMV, but that the subunit packing repeats after two instead of three turns.

*The structure of polymerized RNA-free protein.*—Rod-shaped particles of TMV protein freed from RNA may be prepared by breaking down the virus in weak alkali into the low molecular weight A-protein component, which can then be separated from the degraded RNA and subsequently repolymerized by readjusting the *p*H (83). From repolymerized A-protein prepared by Schramm, Franklin (31) was able to obtain a well-orientated gel which gives an X-ray diagram similar to that of native TMV, and which shows that the subunits in the repolymerized protein have a helical arrangement very similar, if not identical, to that of TMV. However, the diagram is less perfect, indicating that there is a small degree of disorder in the struc-

ture. This is probably due to the possibility of slight positional rearrangements of the protein subunits when the RNA is absent.

The existence of different packing arrangements when protein subunits are polymerized to form TMV-like particles in the absence of RNA has been demonstrated by a number of X-ray studies. Franklin (38) examined the polymerized rods formed from an abnormal protein present in infected plants (Commoner's B8 component [21]) and found the X-ray pattern to be significantly different from that of TMV; on the other hand, polymerized rods of abnormal protein (Takahashi's protein X [90]) prepared by Newmark were reported by Rich, *et al.* (81) to give a pattern very similar to that of TMV. Furthermore, an A-protein preparation made and examined by Caspar (19) gave a pattern which corresponds to a mixture of the B8 and TMV types. In view of this latter observation it is probable that the variant pattern obtained from B8 does not necessarily reflect a fundamental difference between this abnormal protein and the A-protein obtained from TMV itself. When gels of these various polymerized protein rods are dried, the axial repeat distance shortens somewhat (31, 38, 81). This shows that the bonding between subunits is rather weak; the intact virus shows no such change, presumably because in this case the protein structure is stabilized by the RNA (31).

*The location and configuration of the RNA in TMV.*—The over-all similarity between the X-ray pattern of the reaggregated A-protein preparations studied by Franklin and that of intact TMV indicates that the main differences between the two patterns are due to the RNA. From the equatorial scattering of the RNA-free protein, she (33) was able to obtain the radial density distribution in the protein particle. This is shown in Fig. XL–1(*a*) where it is compared with the density distribution in the intact virus. The only major difference between the two curves is that the very prominent density maximum at a radius of 40 Å in the virus is replaced by a density minimum in the RNA-free protein, and moreover it corresponds in amount to the quantity of RNA present in the virus (about 5–6 per cent). This shows clearly that the RNA must be located at a radius of 40 Å. Ample confirmation that the structural difference between the virus and the RNA-free protein lies mainly at this radius comes from the nonequatorial regions of the X-ray diagrams.

Franklin's results on the location of the RNA required a revision of the obvious interpretation of electron micrographs (53, 85) of partially degraded virus particles in which the exposed RNA appears to form a central core. The X-ray results refer to the intact virus in wet gel specimens which have not undergone any special treatment, and what was observed in the electron micrographs is undoubtedly a collapsed form of the RNA. Indeed, from what is now known of the configuration of the RNA in the virus, one would expect a collapse to take place when the protein in removed, since it is the protein which maintains the RNA configuration in the intact virus.

The location of the RNA at a radius of 40 Å does not by itself indicate how the RNA is arranged in three dimensions in the virus, nor how many chains of RNA there are (43). To determine the configuration of the RNA, it is necessary to compare the nonequatorial reflections of TMV and of repolymerized A-protein, and this has recently been done by Franklin (35). From her work she was able to determine with a high degree of certainty the general configuration of the RNA in the virus.

Fig. XL–1(*a*) shows that the RNA is embedded tightly between protein subunits, so that the chain direction of the nucleic acid molecule must be directly related to the helical arrangement of the protein subunits. Moreover, the perfection of the X-ray pattern of TMV strongly indicates that there is no, or very little, material in the virus that does not follow a helical arrangement. It is, of course, the phosphate-sugar backbone of the RNA which will have the symmetry and not the sequence of bases (23).

Because the symmetry of the RNA conforms to that of the protein there must be an integral number of nucleotides associated in a regular way with each subunit. A critical survey (35) of the phosphorus analyses of TMV (64, 79) gives three phosphorus atoms per subunit as the best integral value. Taking the molecular weight of the subunit as 17,000, this corresponds to a phosphorus content of 0.52 per cent, which is in agreement with reported analyses.

From the number of nucleotides per protein subunit, the known radial location of the RNA, and the helical parameters of the protein subunits, it is possible to predict all possible ways of forming a regular structure for the RNA, be-

cause of the restriction that the distance between successive phosphorus atoms along an RNA chain cannot be greater than 7.5 Å. (The point that this kind of prediction was possible was made in 1955 by Caspar and Watson in an unpublished manuscript.) It turns out that with three phosphorus atoms per subunit there are only two regular ways of doing this. One arrangement is that in which a single RNA molecule follows the line of the main protein helix of pitch 23 Å, and the other involves 16 chains of RNA running at a small angle to the particle axis.

A comparison of the X-ray diagrams of TMV and of the RNA-free repolymerized A-protein shows that the over-all pattern of the strong changes can be accounted for by the single chain structure. Furthermore, the 16-chain structure is definitely inconsistent with some of the changes. The X-ray results are incorporated into the model of the virus in Plate XL–1, where the RNA is represented as a smooth helix of diameter 80 Å and pitch 23 Å. No attempt has been made to show the detailed molecular configuration of the RNA as this is still unknown. Two points are, however, clear. There is only a single strand of RNA, since there is not enough material for a twin-strand structure like DNA (92), and moreover this strand is not fully extended since the P–P distance is only about 5 Å. The kind of chain folding involved remains a problem to be solved. Comparison of the birefringence of TMV with that of the repolymerized protein (31, 38) indicates that the base groups are more nearly parallel than perpendicular to the particle axis, and this is in accord with measurements of the ultraviolet dichroism (78, 86). This indication is compatible with a single chain of RNA following a flat helix, since the bases might be expected to lie approximately perpendicular to the chain direction. It should be noted that since the pitch of the helix is 23 Å, there is no possibility of hydrogen bonding between bases on successive turns of the helix.

It should be stated that the X-ray diffraction method could not detect discontinuities or breaks in the covalent linkages of the RNA chain, if any were present. However, with this reservation, the X-ray results are in agreement with the suggestions from various physico-chemical studies (12, 45, 47, 56, 75) that the RNA in TMV is in the form of a single large unit of molecular weight equal to about $2 \times 10^6$. It is also possible to understand now why the virus has a well-defined length in contrast to the variable lengths (29, 83) found for rods of polymerized A-protein and of abnormal protein, since the length of the RNA chain presumably determines the length of the virus. Perhaps the best confirmation of the conclusion from the X-ray analysis that the RNA is in the form of a single chain comes from the recent electron micrographs of Hart (54) on degraded TMV examined by a method slightly different from that used in his earlier work. A strand of fibrous material is exposed in each partially degraded virus particle, and the extrapolated length is longer than any possible on the multiple-chain models of the RNA.

These results on the configuration of RNA in TMV provide the first detailed picture of the structure of any ribonucleoprotein. In contrast to deoxyribonucleoproteins, where the *in vivo* nucleic acid configuration (93) appears to be the same double helix as for extracted DNA (92), the configuration of RNA in ribonucleoproteins appears to be imposed on it by its packing with the protein. Besides the detailed evidence for TMV, X-ray studies of spherical viruses and microsome particles (44) indicate that there is no intrinsic *in vivo* structure for RNA comparable to the DNA double helix. Extracted RNA appears to have a random coil configuration and may be single stranded (12, 45), yet it can take up a very regular structure in the nucleoprotein complex.

*Spherical viruses*

"Spherical" is not the most apt description of the small viruses that are not long rods, since high-resolution electron microscopy has shown that many are polyhedral in shape (58, 94) and some are significantly longer in one direction than another (3, 67). The existence of substructure in the spherical viruses, as shown by the X-ray patterns obtained from virus crystals, suggests that the virus surface will consist of a regular arrangement of protuberances, which could give the virus a polyhedral appearance. The term "polyhedral" has, however, already been used to describe insect virus inclusions which are structures of an entirely different scale. In the absence of a more acceptable term for the small nonrodshaped virus particles we will continue to refer to them as spherical with the reservation that many may not look much like spheres.

*Spherically averaged structure.*—Before dis-

cussing the diffraction patterns from virus single crystals, which indicate their highly regular internal structure, we shall consider the results obtained by Beeman and his colleagues (69, 82) from low angle X-ray scattering studies on virus solutions, which refer only to the spherically averaged structure. Calculation of the spherically averaged radial density (1) from measurement of the low angle scattering is analogous to the calculation of the cylindrically averaged radial density of TMV using the equatorial scattering from oriented gels, with the significant difference that much less detail in the structure can be resolved in the spherical viruses than in TMV by this method.

The low angle scattering from a number of small plant viruses (69, 82) is very similar to that of a uniform density sphere, although there is some indication of a central hole, particularly for brome grass mosaic virus (1), analogous to the axial hole of TMV. Accurate calculations of the mean particle diameters have been made which are consistent with values obtained by electron microscopy and hydrodynamic methods, if account is taken of the effect of internal hydration on the different types of measurements.

For those viruses which have associated with them closely related RNA-free protein particles, for example turnip yellow mosaic virus (TYMV) (72) and wild cucumber mosaic virus (WCMV) (88), a comparison of the low angle X-ray scattering from the virus and protein particles provides significant information about the distribution of protein and RNA in the virus. Markham (71) has shown by physico-chemical methods that the serologically related virus and protein particles of TYMV have the same diameter and surface properties, and he suggested that in both of them the protein is in the form of a spherical shell. Low angle scattering studies (63, 82) have confirmed that the protein particle, of mean external diameter about 280 Å, is essentially a spherical shell of mean thickness 35 Å. More detailed results have been obtained by Anderegg (1) for WCMV by comparison of the density distributions for the top and bottom components of this spherical virus (Fig. XL–2). It is clear that most of the RNA is centrally located and most of the protein goes to make up the protective shell of these viruses.

*Symmetry and substructure.*—In order to study substructure within the protein and RNA parts of the virus it is necessary to work with

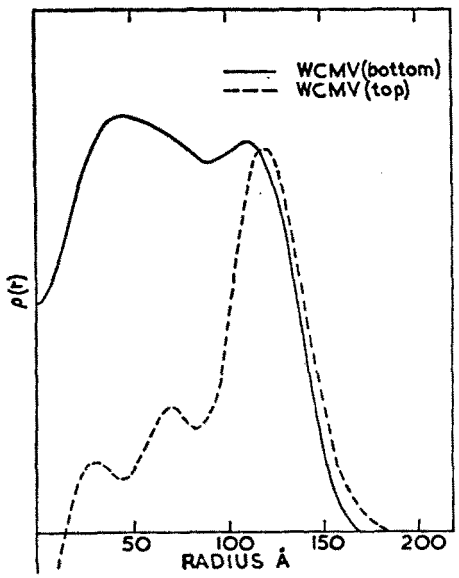


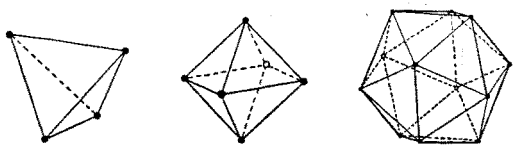

FIG. XL–2.—The spherically averaged radial density distributions of the top and bottom components of wild cucumber mosaic virus (1). The former contains no RNA, thus it can be concluded from the difference between the two curves that the RNA in the virus is centrally located and that most of the protein is in an outer shell.

The virus itself (solid line) appears to have a central hole, but this and other details seen in the density distributions must be regarded with caution, because of the limited resolution, which is a good deal lower (50 Å) than that of the curves in Fig. XL–1 (10 Å). The overall features are, however, significant.

(We are grateful to Dr. J. W. Anderegg for providing this diagram.)

single crystals. X-ray patterns have been obtained from six different crystalline viruses (18, 20, 25, 62, 70) which suggest that, like TMV, they are built up of regularly arranged subunits. Only bushy stunt virus (BSV) and TYMV have yet been studied in any detail, and even for these two viruses our knowledge is less advanced than for TMV, mainly because up to now no isomorphous replacement has been achieved. We shall therefore not discuss the spherical viruses in the same detail as we did TMV.

The X-ray techniques and methods of analysis differ a good deal in detail from those used for TMV. In place of the difficulty of preparing well-orientated gels there is now the problem of growing and mounting crystals large enough for diffraction studies. As in the case of TMV, special theoretical methods (60) have to be developed



*Tetrahedron*
3 two-fold axes
4 three-fold axes

*Octahedron*
3 four-fold axes
4 three-fold axes
6 two-fold axes

*Icosahedron*
6 five-fold axes
10 three-fold axes
15 two-fold axes

FIG. XL–3.—Regular polyhedra in which the respective arrangements of rotation axes represent the three types of cubic symmetry possible for a spherical virus. In each case the three-fold axes are in directions perpendicular to the faces. Note that a virus particle having one of these types of symmetry does not necessarily look like the regular polyhedron chosen to represent the symmetry (cf. Plate XL–3).

to deal with the special problems of symmetry not encountered in the X-ray analysis of crystals of simpler organic substances and proteins.

We have already discussed how regular packing of subunits leads to a symmetrical structure. In a helix, each unit is related to the next by a rotation plus a translation, and the length of a helix, such as TMV, is not determined by its symmetry since the structure can repeat indefinitely along a line. For a particle of finite extent, built up of subunits regularly packed about a central point rather than along a line, only rotational symmetry is permissible if the subunits consist of optically active molecules. Only three general types of *point group*, or finite collection of symmetry axes, are possible (22, 23): first, those containing a single $n$-fold rotation axis*; second, those possessing, in addition, twofold axes perpendicular to the main axis; and third, the cubic point groups. It is the latter which are of particular interest in connection with spherical virus structure since subunits arranged with cubic symmetry will all be equidistant from the center of the point group, and will thus form an approximately spherical or polyhedral shell. Three kinds of cubic symmetry are possible for a virus particle and the arrangements of rotation axes can be illustrated by the tetrahedron, octahedron, and icosahedron (Fig. XL–3); the number of asymmetric units required by each of these three point groups is, respectively, 12, 24, and 60. Plate XL–3(*A*) illustrates one way in which 60 units (ping-pong balls) can be put together to form an approximately spherical particle using icosahedral symmetry. The ping-pong balls are arranged in positions corresponding to the vertices of the snub dodecahedron illustrated in Plate XL–3(*B*). By changing the shape of the subunits, a more polyhedral-shaped particle could be produced.

In the twenty years following the first crystallization of a spherical virus a number of X-ray measurements (7, 8, 10, 11, 15, 25) of interparticle distances and packing arrangements of viruses in crystals were made. The first photographs of a single crystal were obtained by Crowfoot and Schmidt (25) from a derivative of tobacco necrosis virus, and shortly afterwards Carlisle and Dornberger (15) obtained photographs from single crystals of BSV. However, it was not until the systematic studies of Caspar (18) on crystals of BSV, and of Klug, Finch, and Franklin (62, 63) on crystals of TYMV that evidence regarding particle symmetry was obtained. Cubic symmetry has been definitely established for these two plant viruses, but the results, although already very informative, represent only the beginning of the investigation of internal structure.

The choice of crystalline BSV for the first of these studies proved very fortunate since all the particles have the same orientation in the crystal, and thus the X-ray diagram relates directly to the diffraction from a single virus particle. The X-ray patterns (Plate XL–4[*A*]) show that the crystals have tetrahedral symmetry which implies that the individual virus particles have at least this symmetry and must therefore be made up of 12 identical subunits. The virus may have higher symmetry (but not lower) than that of the crystal. Examination of the distribution of strong reflections in the X-ray patterns (18) indicates that the virus particles have fivefold as well as the threefold and twofold rotation axes required by the crystal symmetry; this suggests that the virus has icosahedral symmetry which would require that it be made up of 60 identical structural subunits. It is not possible to con-

* An $n$-fold rotation axis can be represented by a wheel with $n$ equally spaced identical spokes; $n$ is necessarily an integer.

clude, on the basis of the present evidence, whether this symmetry applies to both the protein and the RNA backbone, or whether all the 60-protein subunits indicated are chemically identical.

TYMV crystallizes with a more complicated cubic arrangement of the virus particles (8, 63) than does BSV; the particles occur in two different orientations, so that the unit cell (the repeating unit of the crystal) contains eight times as many virus particles as the simpler unit cell of BSV. The additional complexity of the crystal has proved an advantage in analyzing the symmetry of the virus particles, although without the evidence already obtained from BSV this symmetry might not have been recognized. The fivefold symmetry in BSV was recognized by the concentration of strong reflections in certain directions; in the X-ray patterns (Plate XL–4 [*B*]) from TYMV crystals there are twice as many "spikes" of strong reflections, due to the two different orientations of the particles in the crystal. The analysis of these patterns (63) shows that TYMV, like BSV, has icosahedral symmetry. This, however, is not the complete story. The larger unit cell of TYMV gives rise to more reflections than does a BSV crystal, and these additional reflections show that the symmetry of the particles is not completely icosahedral. The most likely explanation of this result is that though the protein may be made up of 60 identical subunits, there are a smaller number of structural subunits for the RNA (perhaps 12). As for TMV, symmetry will only apply to the RNA backbone, and, even if it is a continuous chemical chain, it can be folded to give structural subunits.

The RNA-free particles associated with TYMV crystallize in the same form as the intact virus (7, 72). This provides an exceptional opportunity to study both the substructure of the protein and RNA in a spherical virus, as has already been done for TMV and repolymerised A-protein. This comparison is only in its earliest stages (61), but it does seem to confirm the earlier suggestion that the protein shell of the virus may be made up of 60 identical subunits, while the RNA may have a lower symmetry.

End-group analyses on TYMV (50), as well as for BSV (66), indicate that there are at least 120 protein molecules in the virus and that therefore the structural subunit (asymmetric unit) may contain two chemical subunits. It is interesting to note that there is a very close structural similarity between BSV and TYMV, in spite of the considerable difference in composition and mass of the particles. On the basis of the present X-ray analyses the molecular weights of the protein structural subunits are, respectively, 125,000 and 50,000 for BSV and TYMV. The structural correspondence between these two significantly different virus particles is probably not fortuitous, but is most likely a reflection of the fact that this type of cubic symmetry is a very efficient way for nature to build a compact particle out of smaller protein subunits.

On the basis of Crick and Watson's (23, 24) suggestions discussed earlier, we might expect that many small spherical viruses are built up by using cubic symmetry, and will thus contain some multiple of twelve identical protein subunits arranged to form a spherical shell. Evidence for cubic symmetry in tobacco ring spot virus (20) has recently been obtained; and there is at least an indication of some kind of symmetry, which may be cubic, in coxsackie (20), a small animal virus similar in many respects to the small plant viruses. All small virus crystals need not have cubic symmetry, as the recent X-ray studies of Magdoff (70) on southern bean mosaic virus (SBMV) indicate. Although the crystals are not cubic, there is evidence for subunits in the individual virus particle. If more asymmetrically shaped viruses, such as alfalfa mosaic (3), are built up of subunits, these units could not possibly be arranged according to cubic symmetry. The virus particle could still have one of the lower types of rotational symmetry mentioned earlier.

It should be noted that cubic symmetry need not be confined to the small RNA-containing viruses only. Particles of tipula iridescent virus (TIV), a DNA-containing insect virus of over one hundred times the weight of small RNA-containing viruses, have been shown by Williams (96) to be regular icosahedra; thus, morphologically at least, this virus has the same symmetry as small plant viruses such as BSV and TYMV.

## Conclusions

X-ray diffraction is at present the only method available for studying the internal three-dimensional configuration of viruses and other highly organized, biological structures in the hydrated native state. Detailed X-ray diagrams obtained from viruses tell us that the virus par-

ticles have a highly regular internal structure, and moreover give a good deal of fairly direct information about their size, shape, and symmetry. However, the only way we can yet be reasonably certain of extracting the wealth of detailed structural information contained in the diffraction patterns obtained from virus crystals or paracrystals is by studying virus derivatives structurally related to the intact virus, in particular, heavy-atom substituted virus particles. For the preparation of these modified viruses, we depend on the assistance of virologists and biochemists.

Even with a number of suitable isomorphous heavy-atom derivatives of a given virus, there is an ultimate limitation to the detail that can be "seen," which is determined by the perfection of the virus structure. The X-ray diagrams tell us that although one virus particle is very much like all other particles of the same strain, there are small differences in the positions of the atoms, and perhaps even in the exact chemical composition, between one particle and another. However, in viruses such as TMV, BSV, and TYMV, these differences are very small and, to a resolution of about 3 Å, one particle is identical with all its sister particles. Hence, although we may never expect to determine the positions of all the atoms in a virus particle, we can hope eventually to determine the configuration of the polypeptide chain(s) in the virus protein subunit, the molecular arrangement of the ribonucleic acid, and its structural relation to the protein in the intact virus. Some of this information has already been obtained by Franklin for TMV, but for the spherical viruses the analysis of detailed structure has only just begun.

The investigations of substructure in both TMV and the crystalline spherical viruses have already made possible some generalizations about the way in which small viruses are put together. In particular, it seems likely that the parts are made by a type of subassembly process before being assembled to build the virus. The forces holding together the protein subunits in the virus particle are like those between globular protein molecules in a crystal. The configuration of the RNA is determined by its regular packing with the protein. These generalizations are probably applicable to all small RNA-containing viruses and may also apply to other particulate nucleoproteins.

## A tribute to Dr. Franklin

*By W. M. Stanley*

Dr. Rosalind Franklin of London, who was scheduled to present the above paper at the symposium, died on April 16, four months before the meeting took place. In a way this paper can be regarded as a memorial tribute to Dr. Franklin from the co-authors.

Dr. Franklin was born July 25, 1920, in London and received her Ph.D. degree from Cambridge University in 1945. She has the distinction during her rather short lifetime of having made great contributions to two quite different areas of research: first, in the study of coke and coal and then later on, as you well know, in studies on the structure of viral nucleoproteins. She moved to Birkbeck College at the beginning of 1953 and started her work on tobacco mosaic virus, using the techniques she had developed earlier, and made notable advances in the study of this virus. She first verified and refined Watson's helical hypothesis for the structure of this virus and then made very important contributions having to do with the location of its nucleic acid. By using tobacco mosaic virus containing substituted mercury atoms as markers she was able to locate key points in the viral structure. She developed a scale model of the tobacco mosaic virus molecule and this was one of the central features of the virus exhibit at the Brussels World Exposition.

Dr. Franklin's life, I think, is an example of complete devotion to scientific research. She was a woman of great intelligence and wide culture and her main interest was devoted to discovering the ever more complex and significant patterns underlying the processes of nature. In addition to this, she was essentially an international courier of good will and scientific information. She visited several active virus laboratories, including, of course, those at Cambridge and Rothamsted in England, and the laboratory at Tübingen. She was quite well at home on the continent, having worked earlier for some years in Paris. She made several trips to the Virus Laboratory in Berkeley. She discussed her work with many investigators and took materials of interest to her from all of these areas and succeeded in a way in blending certain aspects of the scientific research of these centers together into a coherent picture.

I think one of her most outstanding characteristics was her courage. It is now known that she was quite aware of the fatal nature of her last illness. Those of us who were fairly close to her knew little about it; she never spoke about it; but she continued right on to the last to work and plan as though her life were to continue. She died within a few minutes of the time that her last scientific paper was due to be read at a conference of the Faraday Society. Her death has certainly cut off a life of great promise, but she had already done work which will insure for her a notable place in the history of biological science.

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