THE NUCLEIC ACID FIBER OF THE TOBACCO MOSAIC VIRUS PARTICLE

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Previous electron-microscopic studies of tobacco mosaic virus have shown that the ribonucleic acid (RNA) forms a discrete substructure within the rod-shaped virus particle. Thus when the virus particles had been treated with a hot detergent solution to remove protein from the rod ends, a thin fiber of RNA could be seen to project from the ends[1]. As the heat-detergent treatment was prolonged and more and more of the protein removed, the exposed RNA, as observed in micrographs of air-dried preparations, remained in the form of a fiber. Its length was about the same as that of an intact rod (3000 Å); whereas its width was between 1/5 and 1/4 that of the rod (150 Å). It is now evident, however, that the RNA fiber observed under the above conditions is distorted from its original configuration in the intact, hydrated virus particle. In the latter, according to X-ray diffraction studies[2], the RNA phosphorus atoms lie 40 Å from the axis of the virus rod. Thus, upon being stripped of protein and dried, the RNA fiber must shrink by a factor more than 2 in width while maintaining approximately its original length.

Interest in the structure and arrangement of the RNA in tobacco mosaic virus has been intensified by recent discoveries which indicate that the ability of the virus particle to cause replication of itself may reside entirely in the RNA[3]. An obvious point of interest is the size of the molecule that possesses this activity. FRAENKEL-CONRAT, SINGER, AND WILLIAMS[4] have stated, on the basis of centrifugation experiments, that the infectious component in their preparations of the purified viral RNA appeared to have a molecular weight about $2.5 \cdot 10^5$. Since the molecular weight of the entire RNA content of a virus particle is at least $2 \cdot 10^6$, their finding would suggest that each virus particle may contain a number of infectious units of RNA. However, GIERER[5] has recently presented evidence indicating that the entire RNA content of a virus particle is a single molecule (presumably a linear polymer of ribonucleotides) and that only the whole, unfragmented molecule can cause mosaic infection.

The electron-microscopic results described here confirm the existence of just one large molecule of RNA in the virus particle. Whether this molecule is the smallest entity that can cause mosaic infection is a question which will not be considered here.

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EXPERIMENTAL

Preparations of purified tobacco mosaic virus were treated at 85° in a detergent solution, as described previously[1], for periods of 15, 40, and 60 seconds. They were then centrifuged in a preparative ultracentrifuge for an hour or more at about 150,000 *g*. The supernatants were discarded, and the pellet materials, resuspended either in water or in 0.1 *M* acetate buffer at pH 7, were sprayed on collodion-covered grids for examination with the electron microscope.

The length of rods and fibres were determined by comparing their images with those of polystyrene latex particles[6] of known diameter. The latter were placed on the side of the collodion grid opposite the sample so that their magnified images would appear in the micrograph fields with the virus particles.

Lengths were obtained from a series of micrographs in which all particles which satisfied the following conditions were measured:

1) that the particle consist of a rod segment of apparent width about 150 Å attached to one or two fibers of lesser width.

2) that the particle appear to be sufficiently free of contact with neighboring particles to be clearly distinguishable from them.

3) that the entire particle appear to be contained in the micrograph field.

It should be noted that the second and third conditions tend to bias toward the selection of the shorter particles since these are less likely to overlap the edge of the field and to be crossed and intertwined with neighbors.

RESULTS AND DISCUSSION

The particles sprayed in water appeared essentially the same as those described previously for the heat-detergent treatment. Those sprayed in acetate, however, were different in that the fibers attached to the rod ends were extended, often to lengths several times that of an intact virus particle. Treatment of the acetate sample with crystalline pancreatic ribonuclease resulted in a disappearance of the fibers. (A similar result noted previously with samples of partially degraded tobacco mosaic virus provided the main evidence that the detergent-resistant fiber was RNA[1].) The distributions of rod lengths were much the same for the samples sprayed in water and in acetate; only the fiber lengths were markedly different. It therefore seems likely that the long fibers of the acetate samples result from an uncoiling or unfolding of the 3000 Å RNA fibers observed in the water samples.

Some typical features of the acetate samples appear in Fig. 1. Most of the longer rods (lengths greater than 500 Å) have fibers attached at just one end. The frequent occurrence of two fibers on the shorter rods (Table I) can be accounted for by supposing that in these cases the rod has been degraded at both ends by the heat-detergent treatment, whereas the longer rods with just one attached fiber may be supposed to have been attacked at just one end. In support of this it may be noted that the number of particles with two fibers relative to the number with one fiber is much less for the 15-second treatment than for the 40- and 60-second treatments. None of the

TABLE I

FRACTION OF FIBER-BEARING PARTICLES HAVING 2 FIBERS

Sample sprayed in acetate buffer

Rod lengths (Å)	*Treatment*		
	15 seconds	*40 seconds*	*60 seconds*
0–500	0.18 (4/22)	0.95 (19/20)	0.82 (31/38)
500–1500	0.06 (4/64)	0.13 (2/15)	0.26 (11/42)
1500–3000	0.08 (2/26)	0 (0/4)	0 (0/1)

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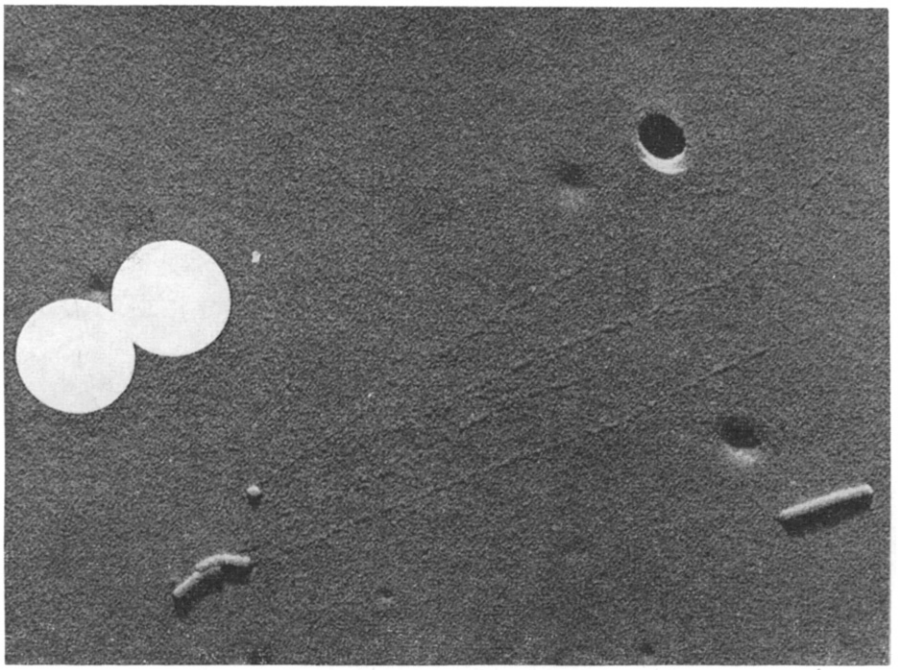

Fig. 1. Electron micrograph of tobacco mosaic virus treated with hot detergent solution and sprayed on grids in acetate buffer. Particles consist of nucleoprotein rod segments of width 150 Å and various lengths attached to thinner fibrous structures. The latter are believed to be single polynucleotide strands whose presence can be detected at this magnification (× 60,000) only by virtue of an adhering contaminant material. Note their discontinuous appearance, which suggests that they are held together by a member too thin (less than 20 Å) to be resolved in the micrograph. The large white disks are the images of polystyrene latex particles whose known diameter is used as a reference in measuring distances in the micrograph fields.

observed particles had more than 2 attached fibers, nor did the fibers appear to be branched.

The thickness of the fibers in the acetate samples cannot be estimated from the electron micrographs. Their irregular and discontinuous appearance suggests that they are resolvable in the micrographs only because of adhering contaminant material (perhaps detergent or denatured protein). This material may have become associated with the RNA merely by drying against it on the grid. The total invisibility of the fiber in the spaces between "beads" of adhering material would indicate that its thickness is less than 20 Å.

The increase in observed fiber length accompanying the replacement of water by acetate buffer as the suspending medium can be readily reversed. Thus, if the particles are removed from acetate by ultracentrifugation, resuspended in water, and sprayed on grids, they then appear the same as those originally sprayed from water. One interpretation of this observation is that the RNA fiber can expand and contract reversibly with changes in the ionic strength of the medium. Another interpretation is that the differences observed between the water and acetate samples arise while the spray droplets are drying on the collodion surface of the grids. From the final arrangement of the particles relative to the drop patterns in which they lie, it is

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apparent that their positions and orientations have been determined by such factors as adhesion to the collodion and surface tension forces. These same factors, modified by different ionic strengths in the media, might be responsible for differences in the final extension of the RNA fibers.

In general, the shorter rods were seen to have the longer fibers attached to them. For the samples sprayed in water, it was evident that the rod length and length of exposed fiber were so related that the over-all length of the particles tended to be about the same as that of an intact virus rod. In order to study the corresponding relationship for the acetate samples, a series of electron micrographs was made for each of the three periods of treatment and the length of rod and fiber were measured for all suitable (see EXPERIMENTAL) particles in the micrograph fields. For particles with two fibers, the fiber length was taken to be the sum of the two individual lengths.

The results of the length measurements (summarized in Table II) show that for each period of treatment the average fiber length increases with decreasing rod length. Such a relationship is to be expected, because the RNA fiber can be uncoiled or unfolded only after it has been released from its chemical bonds to the protein of the rod.

TABLE II

MEAN FIBER LENGTH PER PARTICLE (Å)

Sample sprayed in acetate buffer. Number of particles observed in each category is given in parenthesis.

Rod lengths (Å)	*Treatment*		
	15 seconds	*40 seconds*	*60 seconds*
0–500	16,900 (22)	18,900 (20)	10,400 (38)
500–1500	14,500 (64)	10,400 (15)	6,100 (42)
1500–3000	8,400 (26)	5,200 (4)	1,200 (1)

As the rod is shortened by disintegration of protein, there is a progressive release of RNA, which can then be extended into the form shown in the micrographs of the acetate samples. If the segment of rod left after treatment contains RNA and protein in the same proportion as in a whole virus particle, then the length of RNA fiber attached to it will be proportional to the length of rod destroyed.

From the above considerations, if a rod of initial length 3000 Å is degraded to length R, the extended length of fiber, T, attached to it should be given by $T = k(3000 - R)$, where the proportionality constant, k, represents the factor by which the RNA fiber extends after its release from the rod. Thus, if a virus preparation consisting entirely of 3000 Å rods were degraded systematically as described above, without any additional breakage of the rods or fibers, the ratio $F = \frac{T}{(3000 - R)}$ would be constant for all particles and equal to k. These conditions cannot be realized, however. While the virus sample may initially consist largely of 3000 Å rods, the rods are so fragile that many of them are broken merely in the course of drying on the grids[7]. The fibers exposed in the treatment, being thinner than the rods, may be even more subject to breakage while drying. Both rods and fibers would also be expected to undergo some random breakage during the heat-detergent treatment.

The ratio F will therefore vary; however, from the definition of F, it is obvious that any breakage—either of the rod before treatment, or of the rod or fiber after

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treatment—will have the effect of *reducing* this ratio. The maximum attainable value of F is then equal to k, the extension factor for the RNA. From the histograms of F values (Fig. 2) computed from the rod and fiber measurements, it is therefore possible to infer a lower limit for k. If the conservative value of 11 is taken, and the few values that exceed this are discounted as accidents of measurement, the length of the intact, extended RNA fiber is inferred to be at least (11) (3000) = 33,000 Å. This value was approached by the greatest of the measured fiber lengths. In all, there were 11 particles with fiber lengths exceeding 26,000 Å, of which the longest observed single fiber measured 27,500 Å. The greatest total length for particles with two fibers was 30,500 Å.

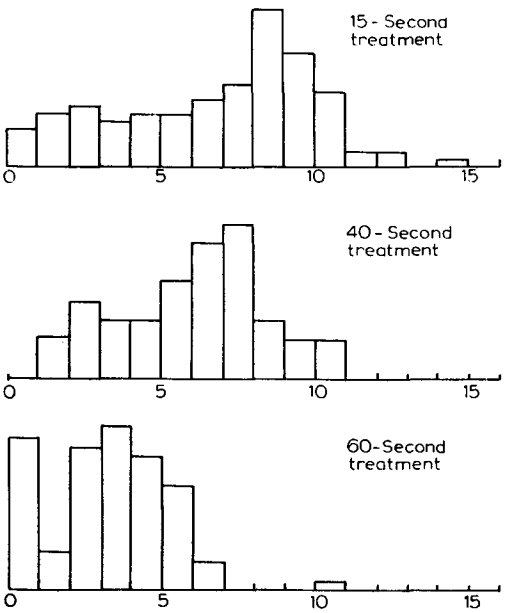

Fig. 2. Histogram indicating the relative numbers of particles having values of F = fiber length/(3000–rod length) lying between the successive integers. Breakage of the rods and fibers during the heat-detergent treatment is evidenced by a displacement of the distributions toward lower F values with increasing period of treatment. If there were no breakage, the ratio F would presumably be the same for all particles and equal to the factor by which the RNA fiber is extended after its release from the protein.

The inferred minimum length for the extended fiber suggests strongly that it consists of a single polynucleotide chain. A double-strand structure of that length would appear necessarily to contain more RNA than is found in a virus particle. Thus, for a polynucleotide chain, the maximum extension consistent with known lengths and angles for the covalent bonds has been determined with a molecular model and found to be 7.0 ± 0.2 Å per nucleotide residue. If the RNA content of a virus particle were arranged in this form, its total length would be 57,000 ± 13,000 Å*. If it consisted of two equal strands, their length could be at most (57,000 ± 13,000)/2 = 35,000 Å. Any intertwining of these strands to permit hydrogen bonding between their

* In calculating this length, the molecular weight of the RNA has been taken to be 2.5 ± 0.5 millions, and that of the average nucleotide residue has been taken to be 307[8].

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bases would almost certainly result in a structure of less than the 33,000 Å minimum length indicated by the present data.

With what is known about the structure of tobacco mosaic virus, it is possible to consider and compare some of the conceivable ways in which the RNA might be arranged within the virus particle. The protein of the virus comprises about 95 % of its mass and is in the form of several thousand identical, or nearly identical, subunits arranged along the single path of a helix of pitch 23 Å[9]. According to the best evidence available[10], there are $16^1/_3$ protein subunits per turn of this helix. Since all of the subunits (except those at the rod ends) have apparently equivalent positions and orientations relative to the axis and to neighboring subunits, it will be assumed that this same kind of symmetry exists in the mode of attachment of the protein to the RNA; that is, that the same sites on all protein subunits lie along the path of the RNA fiber[11]. There are then just three general ways (Fig. 3) here denoted by *a*, *b*, and *c*, in which a single RNA strand of molecular weight between 2 and 3 millions could be arranged in the particle.

The simplest, and perhaps the most plausible path for the RNA is (*a*) a helix which follows the protein helix at a radius of 40 Å. For such a path (23 Å pitch, 40 Å radius, 3000 Å coiled length), the total length would be 33,000 Å, a value in fortuitously

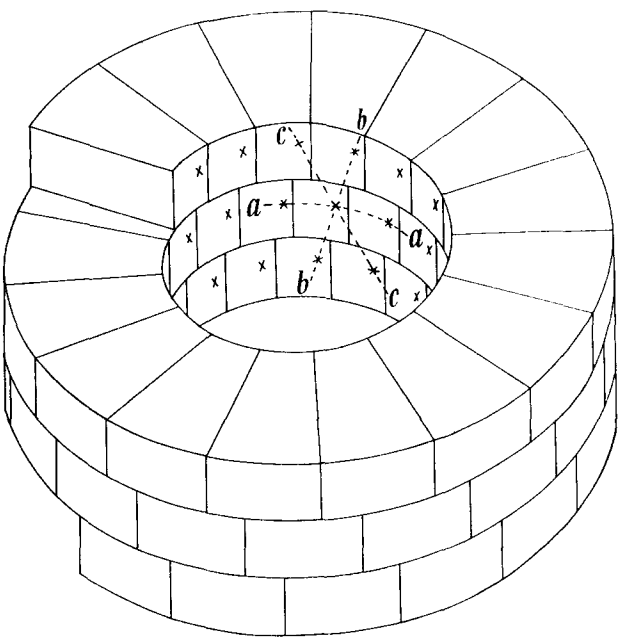

Fig. 3. The 3 kinds of path, *a*, *b*, and *c*, that a ribonucleic acid fiber might follow in the tobacco mosaic virus particle. The sector-like blocks represent the protein subunits of the virus, which comprise about 95 % of its mass. The virus particle contains some 2100 of these subunits arranged helically, as shown in this short segment, with $16^1/_3$ subunits per turn. The protein subunits are believed to be identical, and the same several bonding sites on each are thought to attach to the nucleic acid fiber. One of these bonding sites is here represented by an "X" located at an equivalent position (arbitrary) on each of the blocks, at the correct radius (scaled according to the helical pitch and the diameter of the particle) for the ribonucleic acid phosphorous atoms. The "X" marks have been connected by 3 line segments *a*, *b*, and *c*, illustrating the three kinds of path which the RNA fiber might follow.

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close agreement with the inferred minimum length of the extended RNA fiber. The distance spanned by the RNA between equivalent points of attachment on adjacent protein subunits would be 15.5 Å, or more than twice the maximum internucleotide distance. The total number of nucleotide residues per protein subunit would therefore be 3 or 4, corresponding to molecular weights of $2.0 \cdot 10^6$ and $2.6 \cdot 10^6$, respectively, for the RNA fiber. A number greater than 4 would require a molecular weight greater than 3 million for the RNA.

If the number of nucleotides per protein subunit is 4, then two additional arrangements of the RNA would be consistent with the assumed symmetry. In these arrangements the fiber would again follow a helix of radius 40 Å, but the pitch would be much longer than that of the protein helix. If the protein subunits are numbered consecutively according to their positions along the path of the protein helix, the two paths now considered for the RNA fiber are those which connect equivalent points on the subunits numbered m, $m + 16$, $m + 32$, $m + 48 \ldots$ for b; and m, $m + 17$, $m + 34$, $m + 51 \ldots$ for c. (m is the subunit number at some arbitrary starting point.) The distances along these paths between equivalent points of attachment are: (b), 23 Å; and, (c), 26 Å. Thus, either distance could be spanned by 4 nucleotide residues. Of the total number of protein subunits in a rod, only a small fraction would be encountered in traversing the entire rod length by a b or c path. In order for the RNA fiber to be bonded to all protein subunits, the fiber would have to bend sharply at the rod ends and pass back and forth along the rod, spanning its length by each of the 16 or 17 separate equivalent paths of type b or c, respectively. The total path length would be 49,000 Å for a b arrangement, or 55,500 Å for a c arrangement.

The true arrangement of the RNA fiber in the virus particle is probably determined by the configuration of bonding sites on the protein subunit. Thus, in the assembled particle, each protein subunit would constrain 3 or 4 adjacent nucleotide residues at its own bonding sites and so direct the rest of the fiber toward the bonding sites on two neighboring subunits. From this consideration, the conditions which would have to be met for a type a arrangement appear less stringent then for types b or c. For the latter arrangements, the bending of the fiber at the rod ends would seem to place some additional requirements on the configuration of bonding sites in the protein subunits. Also, in the b and c arrangements, as the fiber folds back and forth along the rod, the sequence of chemical groups in the sugar-phosphate "backbone" would be reversed in direction; the configurations presented by both orientations of the fiber would therefore have to be sterically acceptable to the protein subunit.

ACKNOWLEDGEMENTS

I wish to thank Prof. WENDELL M. STANLEY for the hospitality extended to me at the Virus Laboratory, University of California, Berkeley, where this work was done. I am also very grateful to Prof. ROBLEY C. WILLIAMS for his helpful suggestions and to Mrs. GLADYS E. PEREZ-MENDEZ for her excellent technical assistance.

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Received December 18th, 1957

THE POLYHEDRAL FORM OF THE TIPULA IRIDESCENT VIRUS*

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In the early days of the investigation of virus morphology by means of electron microscopy it was generally concluded that virus particles had forms that could be generally divided into three categories: (1) non-spherical but otherwise symmetrical (rods, prolate spheroids, bread-loaf shapes), (2) non-spherical and unsymmetrical (tad-pole shapes), and (3) spherical. After the advent of shadowing it became apparent that, as observed in dried preparations, the heads of the tad-pole shapes, the bacteriophages, were flattened ellipsoids, while the "spherical" viruses were flattened spheroids. It was correctly concluded that the flattening of the forms was due to the forces of surface tension, and means were sought to eliminate this source of morphological artifact.

Two techniques were particularly developed for electron microscopic use, the critical-point method[1] and an adaptation of freeze-drying[2]. Sensitive test-objects, such as red-cell membranes, were found to appear as undistorted spheres when prepared by either of these methods, and it was generally concluded that the two techniques were quite useful in the preservation of three-dimensional morphology.

The first observations of viruses, the T-even bacteriophage, prepared for electron microscopy by the critical-point method paved the way for a re-appraisal of virus forms[3]. The bacteriophage heads were seen to possess a distinct polyhedral shape the general appearance of which was that of a hexagonal prism with pyramidal ends. Subsequently, observations of frozen-dried material[4] disclosed that the heads of all

* Aided by Research Grant No. C-2245 from the National Cancer Institute of the National Institutes of Health, United States Public Health Service.

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