FURTHER STUDIES CONCERNING THE BREAKING OF TOBACCO MOSAIC VIRUS*

by

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INTRODUCTION

Most published electron micrographs of tobacco mosaic virus (TMV) have shown variations in its length although there is general agreement that the diameter of the virus is slightly greater than 150 A. In ordinary preparations the most numerous particles have been reported as approximately 2800 A in length, but shorter and longer particles are always present. The detailed study by WILLIAMS AND STEERE[1] on the length of TMV using polystyrene spheres as reference particles gives a value of 2980 A for the length of the most frequently observed particle. These investigators were able to show that with gentle treatment the virus could be prepared as solutions which contained rods over 70% of which had this length. In addition, evidence was presented to show that over 95% of the TMV in solution before mounting for electron microscopic examination was present as rods of 2980 A or multiples of this length. SCHACHMAN[2] has also shown, in a careful ultracentrifugation analysis, that uniform length virus rods can be prepared.

BAWDEN[3] has reviewed the evidence for the existence of rods of lengths less than 2800 A. Some investigators have considered the freezing, grinding and chemical treatment used in the isolation of TMV as sufficient explanation for the presence of short rods. There is ample evidence in the literature that TMV rods are quite fragile. Ultrasonic irradiation will break them into various lengths, apparently first breaking the particles in half[4] and then proceeding on down until a length distribution varying from about 40 to 180 A is obtained[5]. Particles of various lengths are still found after 1 hour exposure to a frequency of about 9 kilocycles. Numerous investigators have studied the disruption of TMV by chemical agents. Thus, certain of the chemical bonds are broken by treating the virus in solutions of strontium nitrate, sodium dodecyl sulfate, in solutions of high pH, and by heat. SCHRAMM AND WIEDEMANN[6] have studied the disruption of TMV by chemical means using both ultracentrifugation and electron microscopy to identify the products. One of the micrographs in their study shows a TMV particle broken *in-a-line* quite similar to those broken by freeze-drying.

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We have reported the breaking of TMV by freeze-drying[7]. In this method the broken pieces are nearly the same length and are found lying in a linear arrangement. Over 75% of 542 linearly arranged pieces found in 30 discrete droplets showed a length of 400 ± 100 A. Despite this rather narrow size distribution we concluded from an analysis of the positions of the breaks that there were not specific points along the rods at which breakage must occur. Rather, the narrow distribution resulted either from the method itself or from weak regions extending over a considerable distance along the particle. The ease with which TMV particles break transversely and the fact that the relative positions of the broken pieces in the original particles are known suggests their value in studies of virus structure. There is already an indication from our micrographs that fine strands join some of the broken pieces. In the present paper we describe experiments concerning conditions under which breaking by freeze-drying occurs and describe some of the biological and chemical properties of the broken pieces of TMV prepared in this way.

MATERIALS AND METHODS

Preparations of rod-shaped biological particles

The TMV solutions were prepared as already described[7]. The 5 strains of TMV were grown for us by Professor R. FULTON of the Department of Horticulture from his stock cultures. These strains were: "C"—Johnson's mild, "D"—Kunkel's aucuba, "E"—an isolate from field tobacco, "HM"—Holmes masked, and "Y 14"—a yellow strain from field tobacco. All of the strains showed differences of symptoms on various plant hosts, and all but Y 14 have been described by Professor FULTON[8]. These strains were isolated and purified following the same procedure as used with the normal TMV.

The potato-X was the necrotic ring spot strain which was cultured in *N. glutinosa* and transferred to tobacco for growing large amounts. This was obtained from Miss K. HELMS working in Professor G. POUND's laboratory in the Department of Plant Pathology.

The isolated bacterial flagella were obtained from Dr. A. BERNSTEIN in Professor J. LEDERBERG's laboratory in the Department of Genetics. They were isolated from *Salmonella paratyphi* B. by the method of UCHIDA, SUNKAWA AND FUKMI[9]. Two additional cycles of differential centrifugation in the Spinco model L centrifuge using distilled water to resuspend the pellets were carried out. Small amounts of flagella from *Rhizobium leguminosarum* (var. Red Clover) were also prepared by the method of WEIBULL[10] in our laboratory.

Insulin fibrils were prepared by several of the methods of WAUGH[11]. The acids necessary for the polymerization of the globular insulin were removed by both a combination of dialysis against distilled water and differential centrifugation or by differential centrifugation alone. The insulin monomer was obtained from Eli Lilly Co. as crystalline zinc insulin.

The freeze-drying method used for checking for the characteristic breaking

A brief description of the method was published previously[7], and a more detailed description of the apparatus and procedures used is in press[12]. The method consists primarily in freezing droplets containing TMV by spraying a solution onto a cold substrate and then removing the ice from the droplets by sublimation under conditions of high vacuum. In order to vary the pressure of the evacuation system a Cartesian type manostat was used with either a Cenco "hivac" or "presso-vac" mechanical pump. The specimens were shadowed with uranium and examined in a RCA model EMU electron microscope.

The freeze-drying method used for preparing macro amounts of broken rods

Large enough quantities of the broken pieces of TMV for nitrogen, ultraviolet absorption, and infectivity tests were prepared in the following manner: (1) Solutions of TMV prepared as previously described were centrifuged at about 30,000 × *g* for 1 hour leaving most of the "naturally occurring" short rods in the supernatant. Solutions of the pellets were used in the subsequent steps. (2) These solutions, at about 0.10 mg TMV per ml, were sprayed through the small orifice of a large powder funnel by means of a high velocity spray gun onto petri dishes resting on a metal support in a dewar flask. Liquid air was placed in the dewar at a level such that the petri dish was in direct contact with the cold liquid. The large end of the funnel was below the top rim of the dewar. A stream of warm air was directed toward the tip of the spray gun which was below the top opening of the funnel. This was found necessary since spraying was continued for

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several minutes and the current of air from the dewar was cold enough to freeze the solution in the tip of the gun. (3) The thin layer of frozen solution in the petri dish was transferred to large vacuum desiccators kept in the cold chest. Phosphorus pentoxide was placed in the bottom of the desiccators. (4) The frozen preparations were dried by evacuating the desiccators with a mechanical pump or a water aspirator. (5) The dried preparations were dissolved by repeatedly flushing the surface of the petri dishes with distilled water using a small medicine dropper. About 10% of the TMV in the original solution was recovered. Fifty ml of solution containing broken TMV at a concentration of about 0.02 mg per ml were prepared in this manner. (6) These solutions were differentially centrifuged with a Spinco model L centrifuge to separate the various sizes of particles present. (7) After separation, a portion of the solutions was diluted with known volumes of Dow latex polystyrene spheres, sprayed onto collodion covered screens, shadowed, and examined in the electron microscope. Other aliquots of the solutions were analyzed for nitrogen by the micro-Kjeldahl method of JOHNSON[13] and for ultraviolet absorption using a model DU Beckman spectrophotometer. (8) After adjusting the various solutions to contain the same amount of nitrogen, they were assayed for infectivity by the local lesion Latin square method on *N. glutinosa*, *N. tabacum* × *N. glutinosa* hybrid, and *Phaseolus vulgaris* plants. For these assays the solutions were diluted with phosphate buffer. All other solutions were in distilled water.

RESULTS

Mechanism of breakage

By varying the experimental conditions of the spraying and freezing techniques several possible causes of the characteristic breaking were eliminated[7]. In addition, as discussed below, we have sprayed using a low velocity nebulizer and also have varied the pressure under which sublimation occurs.

In the experiments with the nebulizer the usual amount of in-a-line breaking was found both with a metal guide tube chilled to liquid air temperature and at room temperature. This reduced the possibility that the frozen virus was broken by impact with the collodion substrate as was considered earlier.

The sublimation pressure was found to markedly affect the number of broken rods. The pressure of the sublimation system was varied from a low of about 0.03 mm of Hg to that of the atmosphere. The temperature of the specimen gradually approached —22° C from a low of —190° C. Since only distilled water solutions of TMV were used, even —22° C was cold enough to prevent general melting of the frozen droplets. Usually a trap cooled by liquid air served to remove water vapor from the system. In the experiment at atmospheric pressure phosphorus pentoxide was used as a desiccant.

Fig. 1 is an example of TMV prepared at evacuation pressures of 0.1 mm Hg or less. The tangled masses of rods are clearly standing away from the film and cast very long shadows. WILLIAMS[14] has suggested that such masses result from thermal agitation of the particles. Thermal agitation and the vapor stream from the subliming ice cause the individual particles to either leave the substrate entirely or they adhere to the film or to other particles resting on the film. When such three dimensional networks are irradiated with a concentrated electron beam in the microscope, the individual particles are observed to coalesce and the network sometimes completely collapses. However, with care in the adjustment of the beam's intensity such networks can be photographed. (The correspondence of the shadow with the specimen is a convenient check for damage due to the electron beam.) A great many of the individual rods in the network shown in Fig. 1 can be resolved. Note several cases of rods aggregated side by side in the lower left section of the network. In Fig. 1 the few isolated rods in contact with the substrate are not broken. Broken rods lying in-a-line were observed rarely at sublimation pressures below 0.1 mm Hg.

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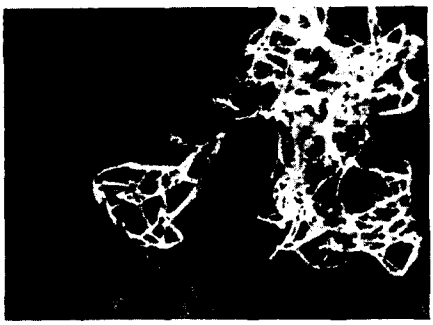

Fig. 1. An electron micrograph of a droplet containing TMV prepared by freeze-drying. Sublimation pressure was 0.03 mm Hg. Note that many of the individual rods in the 3-dimensional mass can be resolved. Magnification is 30,000.

Fig. 2. An electron micrograph of a droplet containing TMV, frozen and then sublimed at 1.0 mm Hg. The rods in the 3-dimensional network have aggregated so that they cannot be resolved easily but the network still casts a very long shadow characteristic of frozen-dried preparations. Magnification is 35,000. Micrograph prepared by Y. CHEN.

Fig. 3. An electron micrograph of a droplet containing TMV frozen in the usual way but sublimed under poor vacuum. It is an example of the appearance of the networks when pressures of about 11 mm of mercury are used. Note the almost complete aggregation of the networks. Magnification is 35,000. Micrograph prepared by Y. CHEN.

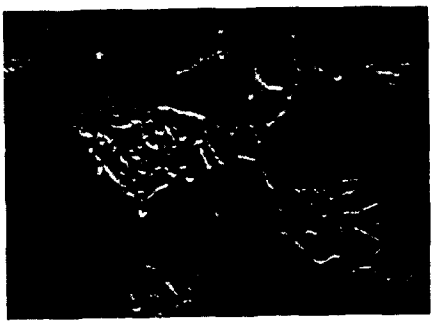

Fig. 4. An electron micrograph of a droplet containing TMV showing piles of broken rods and some individual rods with pieces lying in a linear arrangement. This is an example of the appearance of TMV when conditions of sublimation are such that some melting occurs or drying is carried out at atmospheric pressure. Magnification is 35,000.

Fig. 2 shows another network of rods prepared at about 1.0 mm of Hg. At this pressure we usually find fairly good resolution of individual rods in the 3-dimensional network and there are many examples of rods broken in-a-line.

Fig. 3 is an example of a portion of a droplet containing TMV prepared at 11 mm of Hg. At this pressure the network has coalesced to form a solid mass of TMV with the individual rods indistinguishable from possible extraneous contamination.

Fig. 4 is an example of the results obtained when the frozen droplet of TMV is either prepared under extremely poor drying conditions, or allowed to partially melt before completely subliming the ice. Results such as this were obtained in a drying experiment at atmospheric pressure at —10° C. No networks are present and most of the broken particles are found in low piles of short pieces. An occasional example of rods broken into pieces having a linear arrangement was found. Under such poor

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drying conditions, the possibility of at least local melting cannot be excluded although the temperature was never above the melting point of pure ice. The presence of some particles broken in-a-line and of a great number of short rods indicates that the same breaking force was operating as under the better drying conditions. It is probable that the broken pieces were piled up by liquid produced by melted ice at some subsequent stage or resulted from the complete collapse and breaking of the three dimensional masses.

The examples of breaking shown in Fig. 1, 2, and 3 were obtained with TMV preparations dried under conditions which did not allow melting and in many cases rods broken in-a-line were seen within a few thousand Angstroms of three-dimensional masses in which the rods are not broken. This is evidence that the breaking does not result from melting or from surface tension effects.

The breaking may be due to migratory recrystallization of the ice surrounding the rods. This recrystallization phenomenon has been studied by MERRYMAN AND KAFIG[15] who noted that ice crystal size was a direct function of the vapor pressure. If within a few minutes, the temperature of the frozen specimens approached $-22°$ C from the low of $-190°$ C, the solid droplet would have a higher vapor pressure and larger crystallites would form. The growth of the crystals by migratory recrystallization would be enhanced by the longer periods of time necessary for sublimation with the poor vacuums used in our experiments. Such a phenomenon might cause both the coalescing of the three-dimensional networks and the breaking of the individual rods. It does not, however, account for the narrow length distribution of the broken pieces which must depend upon the mechanical properties of the virus particles.

While the experiments described above gave some insight into the nature of the breaking of TMV, it was thought desirable to determine whether the phenomenon was a gross artifact of the freeze-drying process. Other biological particles of the approximate size and shape of TMV rods were therefore prepared under the same conditions which break TMV. Although only rather minor differences in chemical composition are known among the various strains of TMV, these virus rods were tested for breakage since there have been reports of differences in the distribution of lengths of some strains. This difference would presumably be due to a variation in the ease of breakage of the particle. All five strains described earlier were found to break in the same manner as the normal TMV. Potato-X virus is slightly smaller in diameter than TMV, and is usually found as somewhat longer and more "flexible" rods (although many particles of varied length are always present after the usual isolation procedures). Solutions of this rod-shaped particle were found broken in the same linear arrangement when frozen-dried under the conditions which break TMV. While the nucleic acid content[16] and X-ray diffraction patterns[17] of potato-X virus have been studied, not enough is known about these factors to warrant a further comparison with TMV.

The bacterial flagella which we examined have diameters of the same order of magnitude as that of TMV; various lengths were present presumably due to disruption by the isolation method[10,18]. Upon examining many droplets of solutions frozen-dried under the conditions which break TMV we found no examples of flagella which were broken in-a-line. It might be noted that only protein has been found (with minor traces of other materials) in those samples of flagella subjected to chemical analysis[10].

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When insulin fibrils were prepared under the conditions known to break TMV, no examples of rods broken in the linear arrangement were seen. This was surprising since FARRANT AND MERCER[19] have found examples of cleavage in preparations dried in macro droplets at room temperature. However, the spaces between the broken pieces of insulin fibrils found by these authors were much smaller than those usually found in broken TMV.

From the results of these experiments on different types of rod shaped particles it is apparent that the characteristic breaking is a property of normal TMV, five strains of TMV, and potato-X virus and is not an artifact common to all slender rod-like protein particles prepared by the freeze-drying procedure.

Properties of the isolated broken pieces of TMV

The solution obtained after step 5 contained a mixture of three classes of rods as follows: (A) elongated rods (longer than about 3000 A), (B) normal rods (about 3000 A), and (C) short rods (about 400 A). The relative number of each was determined by spraying the solution using the representative spray droplet technique of BACKUS AND WILLIAMS[20]. Broken pieces whose lengths fell between normal and short rods were counted with their nearest counterparts. This was done for simplicity since their number was small.

Differential centrifugations were carried out with the Spinco model L centrifuge at two speeds which gave average centrifugal forces of 80,000 and 140,000 $\times$ g for 1 and 2 hours respectively. The former centrifugation should remove a considerable fraction of normal and long rods from the supernatant while the latter should remove a considerable fraction of short rods as well. Table I shows the results of analyses on the supernatants of these centrifugations. The controls were portions of the original TMV solution which was also used for spraying. The optical densities (OD) listed in the second column were measured at 265 mμ through a 1 cm path of the solutions containing the amounts of nitrogen per ml listed in the first column. The third column is the ratio of OD/micrograms of N contained in the solutions. The fourth column lists the average of local lesions of the test solutions over the average number of lesions in the untreated virus. Both the control and the test solutions were adjusted to contain the same quantity of nitrogen. The last three columns record the number of the rods of the three lengths over the number of polystyrene spheres found in the same droplets. They are totals of several droplets (3 to 5). The dilutions for these countings were different than for the local lesion assays so no comparisons of the number of rods per local lesion were made.

The results show that all of the solutions contain some rods of 3000 A length and the treated solutions gave much less infectivity than the untreated. The decreased number of lesions found with the centrifuged control is in accord with the results of the several investigations on both "naturally"[2, 21] occurring short rods and sonically broken rods[4]. It would appear that the broken pieces are not infective.

The absorption of TMV at 265 mμ is primarily due to the purines and pyrimidines of the nucleic acid of the virus. The ratio of OD/micrograms N per milliliter is thus a measure of the relative amounts of nucleic acid and protein since both types of material contain approximately the same percentage of nitrogen. These ratios (in column 3) indicate that at the lower speed either free nucleic acid or nucleoprotein of small dimensions is present in the supernatant after centrifugation. The precision

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of the measurements involved in this ratio is no better than ± 20%; therefore, only the large increase in the ratio obtained with the frozen-dried solution centrifuged at 80,000 × *g* is significant. It is possible that additional material absorbing at 265 mμ was sedimented at the higher speed.

TABLE I

CHEMICAL AND BIOLOGICAL PROPERTIES OF ISOLATED BROKEN TOBACCO MOSAIC VIRUS RODS

Solution	*Nitrogen recovered in supernatant micrograms per milliliter*	*Optical density at 265 mμ*	*OD/N × 10³*	*Local lesions**	*Number of rods / Number of spheres*		
					longs	*actives*	*shorts*
Frozen-dried virus centrifuged for 1 hour at 80,000 × *g*	8.0	0.391	49	35/1655	1/61	4/61	142/61
Control untreated virus centrifuged for 1 hour at 80,000 × *g*	6.0	0.120	20	438/1645	3/83	22/83	5/83
Frozen-dried virus centrifuged for 2 hours at 140,000 × *g*	8.1	0.171	21	27/2230	0/57	2/57	96/57
Control untreated virus centrifuge for 2 hours at 140,000 × *g*	5.2	0.072	14	58/1620	2/88	10/88	15/88

* The local lesion assays are ratios of the average number of lesions on half leaves of plants inoculated with the test solution over the average number on the other half inoculated with a normal preparation of TMV. Assays were conducted on glutinosa, glutinosa-tobacco hybrid and bean plants with similar results.

DISCUSSION

The results presented here add to the evidence that TMV readily breaks transversely to its long axis. Freeze-drying, under relatively poor sublimation conditions, the "chemical" breakage of SCHRAMM and his collaborators, and sonic oscillations all appear to produce short pieces. Since a wide range of lengths can usually be found in normal TMV preparations, it is likely that this length distribution is produced in the purification procedures due to the presence of natural salts or added buffers, by mechanical disruption and by freezing of the leaves prior to isolation.

Since we have not been able to break insulin fibrils and flagella as we have TMV, it may indicate that in the latter case only a relatively few strong bonds need to be disrupted. It might be noted that recent X-ray diffraction studies by WATSON[22] and infrared dichroism experiments by FRASER[23] are in agreement as to the orientation of the polypeptide chains of TMV. Both investigators have found that their results could be best interpreted by assuming that the chains are preferentially oriented perpendicular to the longer axis of the rod. On the other hand, KOLTUN, WAUGH AND BEAR[24] found that the polypeptide chains of insulin fibrils have their axes predominantly parallel to the fibril axis. No extensive investigation of the X-ray diffraction patterns of the several strains of TMV or of potato-X virus has been carried out, but the conclusions drawn by BERNAL AND FANKUCHEN[17], were that the patterns of the latter and the strains they examined were somewhat similar to normal TMV. This difference in the orientation of the peptide chains in the virus

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nucleoprotein as contrasted to the fibrous proteins may be an important factor affecting their susceptibility to the type of cleavage that we have observed with TMV and other virus nucleoproteins but not with fibrous proteins as insulin fibrils or bacterial flagella.

The breaking of the rod shaped plant viruses seemed to occur during the sublimation process. Breakage occurs most frequently under conditions which promote migratory recrystallization and the possibility that this may be a factor in causing the breaking has already been discussed, but one other possible explanation remains. It may be that there is a contraction of the virus particle during drying and as this takes place the rods break whenever they adhere to the substrate along a portion of their length. The adherence might be one of freezing to the substrate. The contraction may be due to the sublimation of the water of hydration of the virus. However, we cannot then account for the large spaces between the pieces because it is known from diffraction diagrams that no large structural changes take place in going from wet gels to dry preparations[25, 22].

Direct evidence is now available that the nucleic acid is in the interior of TMV[26]. In addition, the light-scattering study of NORTHROP AND SINSHEIMER[27] of nucleic acid liberated from TMV by heat gave a molecular weight of the nucleic acid which corresponded to the weight of the entire nucleic acid contained in the virus rod. They concluded that all of the nucleic acid is continuous in the rod. Recently the virus causing turnip yellow disease was found to be a shell of protein surrounding a core high speed supernatants of the fractionated broken TMV rods is due to the release of nucleic acid from the core of TMV. With procedures for preparing large enough amounts of broken pieces this hypothesis could be further tested. The present study confirms the results of other investigations to the effect that short pieces of TMV are not infective.

SUMMARY

Electron micrographs of tobacco mosaic virus prepared by freeze-drying under progressively poorer sublimation conditions show corresponding differences in the appearance of the 3-dimensional networks of TMV and in the number of broken rods found lying in a linear arrangement. Possible explanations for the breaking are discussed. Since neither bacterial flagella nor insulin fibrils break under the same conditions, it is concluded that the breaking is not a gross artifact of freeze-drying.

Isolated broken pieces of tobacco mosaic virus in milligram quantities were prepared and studied. The short pieces do not appear to be infective. The results of ultraviolet absorption measurements on supernatants of differentially centrifuged solutions of the broken pieces suggest that nucleic acid may be liberated during the breaking process.

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