THE BREAKING OF TOBACCO MOSAIC VIRUS USING A NEW FREEZE DRYING METHOD*

by

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It is generally accepted that electron microscope specimens made from a preparation of tobacco mosaic virus (TMV) purified by centrifugation consist predominantly of particles which have a length near 3000 Angstroms but that many shorter particles of varying length are also present. There is evidence that the infectivity is associated with the 3000 A particle[1] although BAWDEN and others have considered the possibility that the virus is a smaller unit[2]. In the latter instance, the elongated particles would be linear aggregates of the virus. WILLIAMS[3] has shown that it is possible to make preparations by centrifugation which consist almost wholly of particles of the same length. In his electron microscope experiments he sprayed an appropriately dilute solution onto specimen films and noted that each droplet contained only 3000 A length particles and other particles whose lengths when summed and divided by an integer were equal to 3000 A. It was suggested that the latter particles were pieces of the "monomer" broken in the mounting process. It would be desirable to obtain further information on how the various particles that one sees in the electron microscope are related to each other.

We have observed that many of the particles of a solution of tobacco mosaic virus are broken into smaller units when sprayed onto a cold surface under appropriate conditions. Since the units from each of the broken particles cannot move after the water about them freezes and as the particles are often lying in a line, their relative positions in the original particle are apparent in the micrographs.

METHODS

Preparation of the virus. Three lots of virus at various concentrations were used for spraying. The first mounts were made from an old concentrated virus preparation originally isolated in 1946 by the method of STANLEY[4]. Fourteen months before the spraying experiments, this solution was further purified by differential centrifugation and dialyzed against distilled water. Lot II was from the same stock similarly treated 6 months before use. These two solutions were used to inoculate young *Nicotiana tabocum* var. Havana 38 plants. One month later the leaves were harvested and frozen overnight, ground in a meat grinder while frozen, and put through five cycles of differential centrifugation in a Servall vacuum centrifuge. The low speed step was performed at 7,000 *g* for five

* This work was supported in part by grants from the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation and in part by funds supplied by the National Institutes of Health, Public Health Service.

** Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

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minutes, and the high speed at 45,000 *g* for two hours. The solutions were kept below + 10° C at all times. Distilled water was used for washing and resuspending the pellets. The final preparations were dialyzed against distilled water.

The freeze-drying method[5]. A high velocity spray gun[6] was mounted vertically above a cold chest maintained at temperatures between —22° C and —15° C. Stainless steel or electromesh microscope screens were covered with film and mounted in contact with metal blocks 1 cm thick and 2.5 cm square. One of these blocks was then placed either directly on the floor of the chest, on cakes of solid carbon dioxide contained in a dewar, or on another metal support in a dewar containing liquid air. The height of the liquid air in the dewar was such that the support was awash and the specimen block was in contact with the cold liquid. Solutions of TMV were sprayed at 20 to 30 p.s.i. pressure onto the cold specimen block through a glass tube 50 cm long and 2 cm in diameter. After spraying for 1 to 3 seconds the tube was removed and the specimen block was transferred rapidly from the dewar to a large glass vessel also kept in the cold chest. This vessel was a glass tube fitted with a ground glass joint and a stop cock which allowed a vacuum to be drawn. Phosphorus pentoxide was placed inside this tube to facilitate removal of water from the specimens. The vessel was mounted in an inclined position and surrounded by a small dewar which contained liquid air in order to minimize any possible melting subsequent to spraying while other samples were being prepared. As many as 10 samples have been sprayed during the course of an hour. When all of the desired mounts had been prepared the ground glass joint was closed and a vacuum pulled (usually overnight for convenience) while the tube came to the temperature of the deep freeze chest. Before removing the specimens the tube was warmed above room temperature. The screens were shadowed with uranium, and examined in the RCA model EMU electron microscope.

The freeze, spray-drying method is similar to that suggested by WILLIAMS[7], one important difference being that in his method the collodion film itself is in intimate contact with a cold metal block. His method necessitates stripping the film from the metal and transferring it to screens but it causes the droplets to freeze more rapidly than in our method. Our method, in common with that of WILLIAMS, avoids the danger that the ice in the droplets melts while the specimen dries. Evidence for this is given by the micrographs in this paper and also by the very long, undercut shadows cast by red blood cell ghosts[7] prepared by our method. By contrast, cells prepared from a melted drop exhibit very short shadows characteristic of flattened membranes.

In order to determine if the degree of contact between the virus and the cold sink had an effect on the cleavage, in one experiment films were mounted directly on metal blocks held in contact with liquid air. The mounts were sprayed in the usual manner and the films with the TMV transferred to screens by a stripping process.

In attempts to determine their effect on the cleavage we have varied the distance from the tip of the spray gun to the screens, the pressure of the nitrogen used to power the gun, the time during which pressure was applied to the gun, the type of film used to cover the screens, the type of specimen screens, the temperature of the mounts, and the concentration of virus.

RESULTS

All of the droplet patterns we examined showed a 3-dimensional, tangled network of rods with numerous rods distributed about the periphery of the network. There was no distinct drop edge (as is seen with unfrozen droplets) and many rods were in only partial contact with the substrate suggesting that the water about them remained frozen as it was removed. Some droplets contained several such masses of rods. Fig. 1 is a portion of a droplet frozen at ice chest temperature. It shows a typical tangled mass of rods very similar in structure to those found also at dry ice and liquid air temperatures. The diameter of rods not in contact with the substrate appears slightly greater than that of other rods.

A striking feature of the specimens prepared at dry ice and at liquid air temperatures (including the experiment with the film in contact with the block) was the high incidence of short particles lying in a linear array as though they were broken pieces from a single rod. In a high percentage of instances the sum of the lengths of the short particles was nearly 3200 A, *i.e.*, only slightly longer than the most common length of unbroken particles in the same drops (3100 A). Few or no broken particles were found in preparations made at ice chest temperatures. Fig. 2 is a micrograph of a droplet

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prepared at liquid air temperatures, showing intact particles of several lengths and also broken particles. The picture is from an area immediately adjacent to a large network. Fig. 3 shows at high magnification a broken rod and also a portion of an intact rod prepared at the temperature of dry ice. This micrograph was prepared from a solution of the older TMV (Lot I) which had a high proportion of particles longer than 3000 A. It shows one of these long particles broken into 11 pieces. These are approximately the size of the broken pieces from shorter particles.

Fig. 4 shows a part of a droplet on collodion film which was in direct contact with a stainless steel block held at liquid air temperature. The number and appearance of the several broken particles is very similar to that found in preparations sprayed onto filmed screens at dry ice and liquid air temperatures. It is apparent from the mass of rods in the center of the micrograph and also from nearby 3-dimensional networks (not shown) that the area in the immediate vicinity of the broken rods was in sufficient thermal contact with the cold block so that the portion of the droplet containing the rods remained frozen while the ice from it sublimed. Another interesting feature of the central mass in the micrograph is the apparent rigidity and strength of the single rod which is holding up several particles many times its own weight.

From an examination of many droplet patterns on the fluorescent screen, it was clear that the other variables previously mentioned had no qualitative effect on the breakage of the rods. The two older lots of virus had a greater proportion of very elongated particles but the size and number of the broken units were similar to those of the fresh preparations which contained predominantly 3000 A length particles.

We measured the distribution in length of 542 linearly arranged broken pieces of TMV rods contained in 30 discrete droplet patterns prepared at either liquid air or dry ice temperature. This distribution is plotted in Fig. 5. We did not measure all particles in the droplets because we then would include particles which might occur naturally in the plant or be produced during the purification process. We further restricted our measurements to units whose added lengths exceeded 1500 A because it was often difficult to decide whether or not the units from shorter particles actually originated from a single rod. The graph shows that we have a narrow, asymmetric distribution of lengths with a maximum occurring at about 1/8th the length of the 3000 A virus particle. Of the particles measured, only 2 were less than 200 A long. Fig. 6 is a plot of the position of cleavage with respect to one end of the rods against its frequency of occurrence for 191 linearly arranged pieces from 28 rods. Only those groups of rods whose lengths when summed were 3200 ± 300 A were included. Since one end of a rod cannot be distinguished from the other, this is a symmetrical curve and consequently only ½ of it is plotted. Although the total number of such broken rods is small, these were found in the group of 542 broken pieces measured in Fig. 5, and should be fairly representative of the total sample. If it is agreed that enough rods have been measured, then it is evident that TMV has no widely separated weak spots for this type of cleavage.

Several possibilities could account for the distribution of Fig. 5 that would not be in conflict with the results shown in Fig. 6. It may be that the TMV particles are only slightly weaker at periodic intervals so that breakage is more likely to occur there but does not invariably do so. Alternatively, a weak zone may extend over lengths of 30 to 40 A so that broken particles would be nearly the same size but not necessarily identical. Either effect, superimposed upon the cumulative errors of measurement inherent in the analysis of Fig. 6, may make the latter distribution meaningless.

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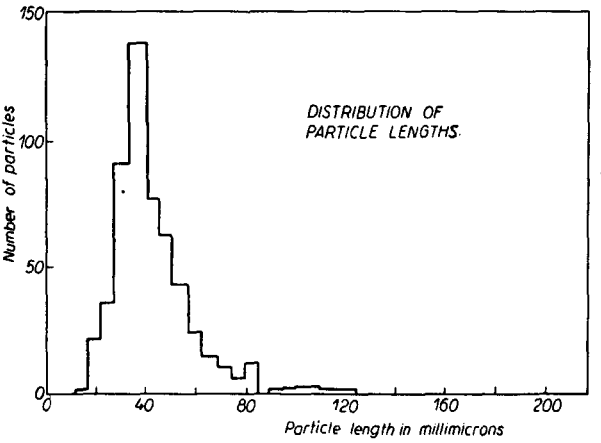

Fig. 5. The distribution in length of broken units of tobacco mosaic virus rods.

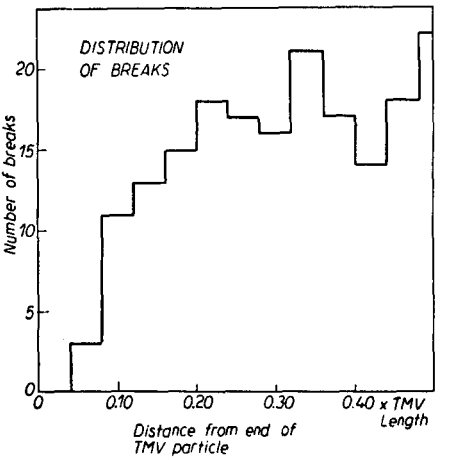

Fig. 6. The position of virus rod cleavage with respect to one end of the rods plotted against its frequency of occurrence.

The fact that the broken pieces which come from a single virus particle are not in contact suggest that a movement has occurred during the breaking process. To estimate the magnitude of this movement we have measured 10 broken particles selected at random. Table I gives for each particle the number of broken pieces, the sum of the lengths of the pieces (L), the distances between the extreme ends (D), and the ratio D/L. Clearly neither D nor D/L are constant. For comparison we have also measured at random 10 unbroken particles. It is readily seen that the lengths of unbroken particles are approximately the same as the sums of the lengths of the broken particles. The distances between the far ends, however, are much greater; in the case of the ten broken rods of Table I this distance ranged from 16 to 75% greater than this sum.

TABLE I

REPRESENTATIVE DIMENSIONS OF BROKEN AND INTACT TOBACCO MOSAIC VIRUS PARTICLES

Number of broken pieces	*Distance between far ends (D)*	*Sum of lengths (L)*	*Ratio D/L*	*Unbroken particle lengths*
8	5220 A	3300 A	1.55	2600 A
9	4760	3340	1.42	3280
6	4590	3290	1.39	2900
7	4150	3120	1.33	3300
8	5740	3280	1.75	3060
7	3710	3180	1.17	3100
7	3530	3100	1.14	3000
6	3360	2540	1.32	4800
9	4350	3400	1.28	3040
7	3670	3220	1.14	1900

Mechanism of breakage. The cause for the breaking of the virus particles remains obscure; however, it occurs only at very low temperatures. It may be that there is a contraction of the virus particle with respect to the substrate during freezing or drying

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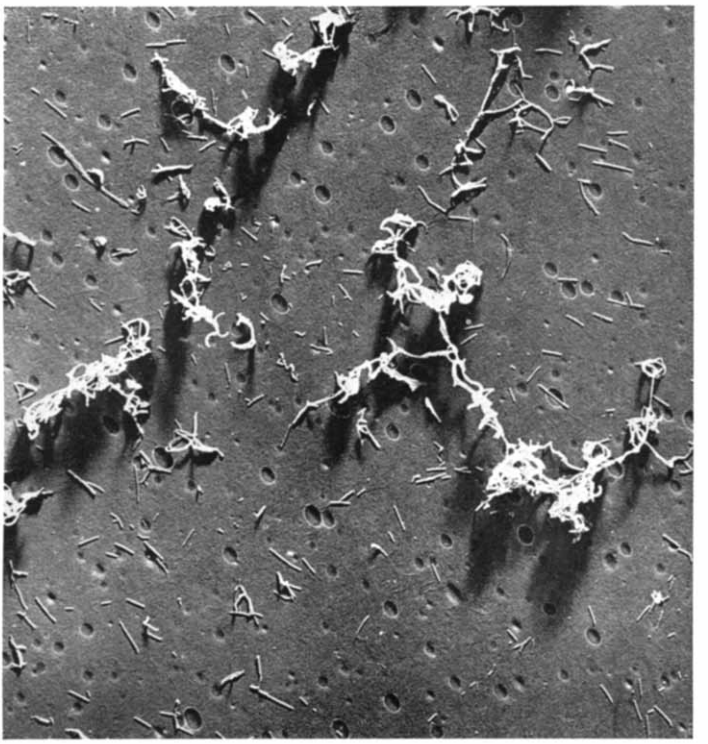

Fig. 1. A portion of a droplet containing tobacco mosaic virus frozen at ice chest temperature 8000 ×.

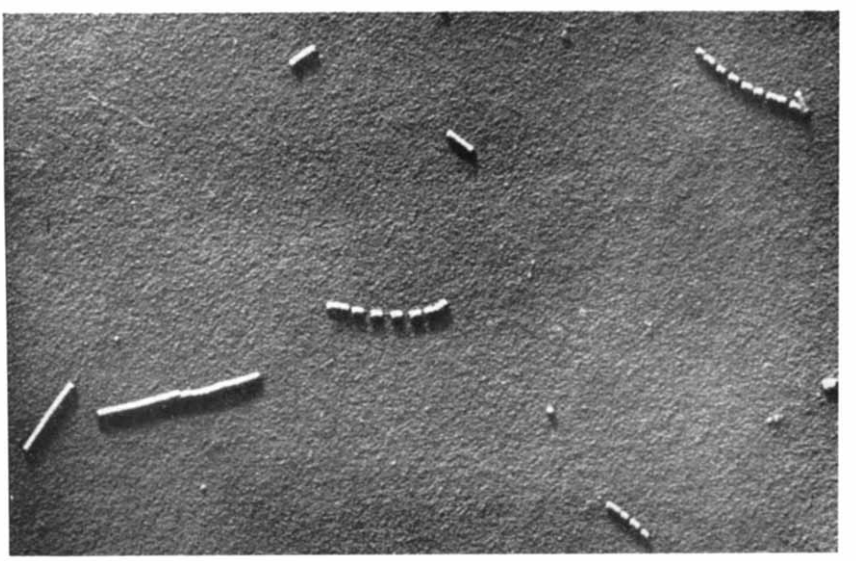

Fig. 2. A portion of a droplet containing tobacco mosaic virus frozen at liquid air temperature 36,000 ×.

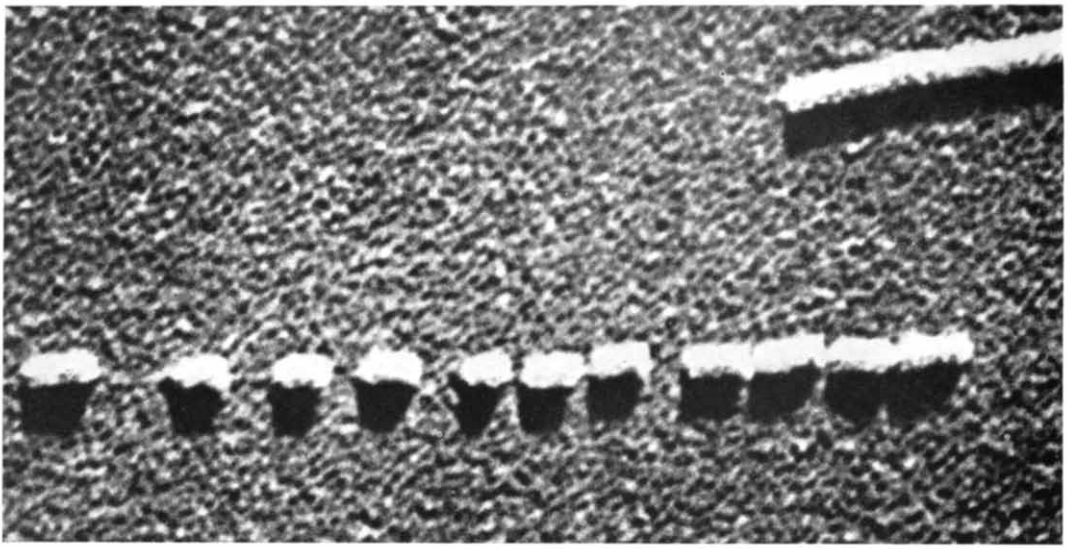

Fig. 3. A portion of a droplet containing tobacco mosaic virus frozen at dry ice temperature 260,000 ×.

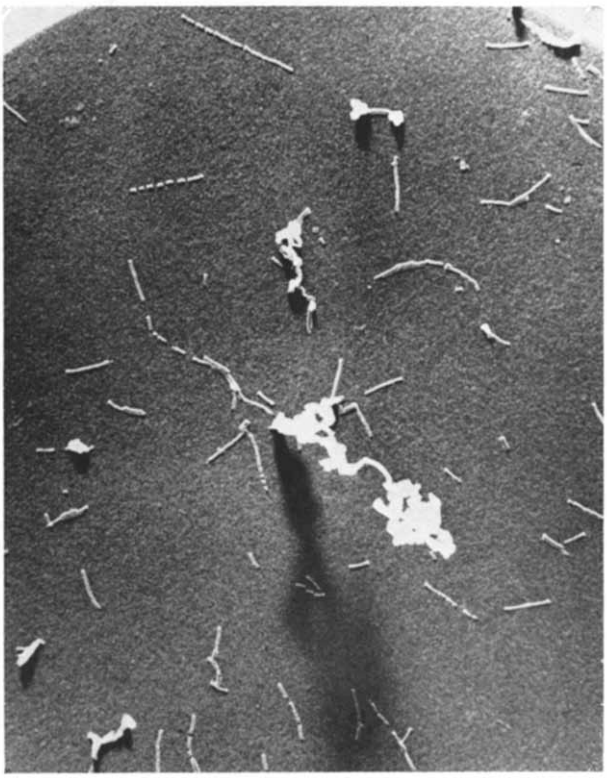

Fig. 4. A portion of a droplet containing tobacco mosaic virus in which the substrate was in direct contact with a metal block held at liquid air temperature 18,000 ×.

and as this takes place the rods break when they adhere to the substrate along a part of their length. One would not necessarily expect that the distance between the far ends of the broken rods would be the same since there could be movement before a portion of the rod adheres sufficiently strongly to cause breakage. The adherence might be one of freezing to the substrate. If the contraction were due to loss of internal hydration by freezing or drying it would be necessary to assume a change in length of somewhat more than 50% as the water of hydration was removed. Hydrations of this magnitude are not in conflict with views concerning the amount of internal hydration of other viruses[8]. Thermal expansion or contraction of the substrates is ruled out as a cause for the breakage because it is far too small to account for the large spaces between the broken units. Another possible cause of breakage might be that the virus particles are shattered upon impact with the substrate which is relatively inelastic at these low temperatures.

SUMMARY

An electron microscope study shows that many of the particles of a solution of tobacco mosaic virus break into smaller units when sprayed onto a cold substrate under appropriate conditions and subsequently dried. The broken units have the same diameter as the original rods, a fairly narrow distribution of lengths about 400 A, and a linear arrangement. The freeze-drying methods and the resulting micrographs are described in detail and possible causes for the breakage are suggested.

RÉSUMÉ

Une étude au microscope électronique montre qu'un grand nombre de particules d'une solution de virus de la mosaïque du tabac se rompent en unités plus petites, lorsqu'on les étale, dans des conditions convenables, sur un substrat froid et qu'on les sèche. Les particules résultantes ont le même diamètre que les bâtonnets initiaux; leurs longueurs se distribuent étroitement autour d'une moyenne de 400 A; leur arrangement est linéaire. Les méthodes de lyophylisation et les micrographies obtenues sont décrites en détail et les causes possible de la fragmentation sont envisagées.

ZUSAMMENFASSUNG

Eine Untersuchung mit dem Elektronenmikroskop zeigt, dass viele der Teilchen einer Tabakmosaikviruslösung in kleinere Einheiten zerfallen, wenn die Lösung unter angemessenen Bedingungen auf einem kaltem Substrat zerstäubt und dann getrocknet wird. Die zerfallenen Einheiten haben den gleichen Durchmesser wie die ursprünglichen Stäbchen, eine ziemlich einheitliche Verteilung der Länge (ungefähr 400 A) und sind linear angeordnet. Methoden zur Trocknung durch Einfrieren und die sich ergebenden Mikrobilder werden eingehend beschrieben und es werden Vermutungen über die möglichen Ursachen des Zerfallens gegeben.

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Received April 8th, 1953