# HIGH-RESOLUTION ELECTRON MICROSCOPY OF THE PARTICLES OF TOBACCO MOSAIC VIRUS

by

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The rod-like particles of tobacco mosaic virus (TMV) have been photographed with the electron microscope by numerous investigators since the publication of the earliest pictures by KAUSCHE, PFANKUCH, AND RUSKA[1] in 1939. For the next six years there was no striking improvement in the quality of information obtained by electron microscopy of the individual TMV particles, and the information gleaned from the micrographs yielded principally a value for the width of the particles and various values for their lengths. In 1945 the application of the shadowing technique[2] to the electron microscopy of TMV particles allowed information to be obtained about the dimensions of the particles in a direction perpendicular to the plane of the specimen substrate film, and the improved contrast afforded by the technique allowed some detail of surface structure to be looked for. Since the publication of the earliest shadowed micrographs[3,4] some photographs have appeared showing improvement in the amount of detailed structure visible[5,6,7]; but despite the relatively large number of micrographs secured for various purposes, there has not appeared any report of a serious effort to elucidate the detailed structure of the virus particles of TMV by means of high-resolution electron microscopy.

The purpose of the research reported in this paper is to determine whether or not the techniques of modern, high-resolution electron microscopy can add anything new to our concepts of the structure of the "rod-like" particles of TMV. By "high-resolution" microscopy is meant here the securing of micrographs of a degree of resolution (to be discussed in detail later) of 3 m$\mu$ or better. It is realized that some micrographs[8] of special objects on special supports have exhibited resolutions near 1 m$\mu$, but the limit of practical resolution for ordinary biological objects, mounted on thin substrate films, is more likely to be in the vicinity of 2 m$\mu$. It is believed that no micrograph of TMV has been published which exhibits a *demonstrable* resolution within a factor of two of this value.

Actually, the electron microscope has added little to our knowledge of the structure of TMV over that postulated by BERNAL AND FANKUCHEN[9] from X-ray analyses. They found that paracrystals of vacuum dried TMV, previously oriented by rolling or preferential drying, show an interparticulate spacing of 15 m$\mu$, with hexagonal close packing of the particles. Although BERNAL AND FANKUCHEN refrained from explicitly concluding that the diameter of the unit particle is 15 m$\mu$, the close dependence of interparticulate spacing upon water content of the virus gels makes the notion attractive that in the vacuum dried state the particles are actually "solid" and are touching each other.

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BERNAL AND FANKUCHEN also found an *intra*particle unit cell, in the form of a rhombohedron with edge lengths of 87 A, and a *c* (axial) dimension of 68 A. Their schematic illustration shows that multiples of three of these cells would fit exactly into the structure of an intact TMV particle if the latter were in the form of an hexagonal prism with edges 87 A long. They are careful to refrain, however, from drawing any specific conclusions regarding the shape of the cross section of the TMV particles, inasmuch as the X-ray data could give them no information regarding the external morphology of the intact rods.

## EXPERIMENTAL

### A. *Technical Problems of Microscopy*

During the course of the research reported here certain technical difficulties interfering with the consistent attainment of resolutions better than 3 m$\mu$ have been met and partially solved. The requirements of high-resolution microscopy, detailed by HILLIER AND RAMBERG[8], have had to be met, of course, in order that good pictures of test objects could be obtained with the RCA, Model EMU-2D instrument. The difficulties to be described have either not been mentioned in the literature before or have been mentioned without an adequate solution proposed.

1. *Instability of specimen holder.* A serious cause of imperfect images has been found to originate in a motion of the specimen holder itself (with respect to the objective lens), caused by slow temperature changes in the holder subsequent to its introduction into the microscope. The existence of this motion can easily be verified by alternately heating and cooling the holder prior to insertion and observing the consequent image drift in opposite directions. The effect can be minimized by using only one holder, by leaving it in place in the objective lens as much as possible, and by conducting the usual hand-lens inspection for cleanliness as rapidly as possible. Occasionally, of course, the holder must be thoroughly cleaned; in this case it should be subsequently left in the microscope (with the lens current *on*) for about an hour before high-resolution pictures are attempted.

HILLIER AND RAMBERG have pointed out the deleterious effect of the vibration of the specimen stage during the sporadic "bumping" of the oil in the diffusion pump, but they offer no practical solution to the problem. A partial solution makes use of the fact that the oil diffusion pump will continue to maintain an adequate vacuum in the microscope with a heating current far less than its optimum operating current. It is found that the resistor No. 10 in the E.M.U. instrument can be safely set to its extreme counterclockwise value, after an operating vacuum has been obtained in the normal way, and that this setting reduces the effect of the bumping to a value too small to be observed. Experiments have been made with antibumping pellets, such as are used in the laboratory distillation of aqueous solutions, but these appear to have no effect on the normal bumping of the oil diffusion pump.

2. *Specimen contaminations.* Although the effects of so-called specimen contamination have been studied as they particularly relate to the micrography of unsupported particles, such as carbon black, no observations seem to have been reported on the effects of the contamination layer on the high-resolution micrography of small, shadowed particles on ordinary plastic film substrates.

Observations have been made in this laboratory on the effect of the growth of the contaminating film upon the micrography of uranium-shadowed particles of tobacco

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mosaic virus with a view toward evaluating the magnitude of the effect and toward minimizing it. It is found that, to a first approximation, exposure of the specimen to the electron beam causes only a decrease in contrast between the TMV particles and the collodion substrate. Closer examination shows that the result of prolonged exposure is to produce a generally hazy appearance in which the fine detail usually associated with the surface appearance of collodion is lost.

An experiment was performed in order to determine whether very small specimen objects might be coated by a thick contaminating film, despite their approximately normal appearance in shadowed micrographs. A specimen consisting of particles of TMV and polystyrene latex mounted on a collodion film was exposed to the electron beam of the microscope prior to shadowing. The exposure time was estimated to be equivalent to that usually required for searching, checking for drift, focussing, and exposing for the photograph. Fig. 1 shows the appearance of the specimen after shadowing. That the picture is technically satisfactory is shown by the sharpness of the edge of a polystyrene latex particle, but it is evident that the contours of the virus particles have almost disappeared. It is concluded from this type of experiment that the contaminating film covers both the substrate and the small particles with an enveloping film and that in the usual procedure, where the specimen is shadowed prior to the exposure in the microscope, the film is not plainly perceived owing to its low electron scattering power compared with the uranium-shadowed background.

The above conclusion (that one normally "sees" through the contaminating film) is supported by observations made with frozen-dried TMV on collodion substrates and shadowed with uranium. Some freeze-dried TMV rods are found to be in close contact with the substrate, and some are found to be supported by other rods and hence separated from the substrate along their lengths. The former rods appear normal; *i.e.*, they appear sharp unless given a prolonged exposure to the electron beam. The latter rods are enveloped by halos of material of less electron opacity than the TMV nucleoprotein and of a uniform thickness which increases as the exposure to the beam is increased.

It has been found that the rate of growth of the contaminating film can be greatly reduced, in a practical manner, by pursuing to the extreme the general observation that the rate is lessened if the specimen film and the supporting grid are not bombarded at the same time by the electron beam. The observations described above were all made with 200-mesh screen of either stainless steel or nickel Lektromesh. An obvious expedient is to try coarser mesh and to utilize only the regions near the center of the mesh opening. Lektromesh* of 80-mesh opening has been successfully tried and is now used routinely. Fig. 2 shows the results of an experiment upon the "growth" rate of some polystyrene latex particles using the coarse mesh. The apparent rate of growth of the particle radius is 8 m$\mu$/min when the electron beam strikes the particle in the vicinity of the nickel grid whereas it is only 0.4 m$\mu$/min when the beam strikes particles near the center of the grid opening. The electron beam intensity in the plane of the specimen in this experiment was sufficient to allow a normal photographic exposure to be made in five seconds at a magnification of 22,000 $\times$ with a beam current of 0.25 amperes. No objective aperture was used. The smaller growth rate of around 0.4 m$\mu$/min allows high-resolution micrographs to be taken without undue haste on the part of the operator.

3. *Stability of specimen films.* It is a common observation that one of the most

* Specified as: Copper-nickel, 80-count, hole size 0.007, open area 31.3%, thickness 0.0037.

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serious technical difficulties in the micrography of film-mounted objects is the instability of the specimen film. This difficulty can readily be separated from thermal and electrical drifts by observing through the focus magnifier ($M = 250{,}000 \times$) the image of an edge of the metal grid, since in a thermally stabilized and clean instrument this image should drift less than 5 m$\mu$/min. Even in uranium-shadowed films of either collodion or Formvar there is generally found a relative motion between film and mesh if the region immediately adjacent to the mesh is observed under a strong electron beam. This motion seems to be due both to a shrinkage of the collodion and to a sliding of the collodion over the metal mesh. It can be reduced by a careful burnishing with fine steel wool of the side of the Lektromesh originally in contact with the electroplating matrix and by subsequently applying the plastic film to this side. The burnishing removes the electroplated "trees" and enlarges the flat area to which the collodion can make intimate contact. Burnishing must be done when 80-mesh screen is used.

It has been found, in common with observations of others, that the films are greatly stabilized by coating them with a layer of SiO about 3 m$\mu$ thick prior to the application of the specimen. Some contrast is lost, owing to the extra film of SiO, but the underlying collodion need be only about one-third as thick as the minimum thickness for unbacked films. As far as can be ascertained, 80-mesh screens and 200-mesh screens, coated with collodion and SiO, exhibit equal stability.

The use of SiO-backed films has brought to light an entirely unsuspected difficulty. If droplets of doubly distilled water are sprayed on such a film, it is found that a residue of material of high electron scattering power is left in each droplet area. As HASS[10] has pointed out, SiO is likely to decompose slightly if evaporated at high temperature and to form a film composed in part of silicon. Since silicon is partially soluble in water, the observed residues are probably due to dissolved and re-precipitated silicon. HASS found that a slow evaporation of SiO is necessary to produce films of reasonably pure SiO. It is now the practice here to form the powdered SiO in a pellet of about five times the volume needed, to place it in a 20-mil tungsten filament wound as a conical helix, and to evaporate slowly only a portion of it at a filament current of 11 amperes. The resulting SiO film does not appear to contain any water soluble material. This difficulty would probably escape attention in preparations formed from gross drops, inasmuch as the area available for dissolution by the water would not be clearly delineated. It also escapes attention even in spray droplets, if these contain polystyrene latex particles, since the precipitated material appears to gather under the edges of the large particles where it is not noticed. Some evaporated films of $SiO_2$ have also been examined and are found to contain water soluble material, presumably silicon.

4. *The focus magnifier and polystyrene latex particles.* The RCA focus magnifier has been found to be extremely useful in checking the image for drift, for vibration, and in obtaining an approximate focus preliminary to photographing a through focus series. This statement is probably valid, however, only if particles such as those of polystyrene latex are present in the specimen. Such a particle has an indefinitely sharp "edge", is opaque up to the edge, and its uniformity of appearance gives the microscopist something always completely familiar to observe in the field of the magnifier. If the magnifier is provided with ocular cross-hairs and a scale, an intolerably high rate of drift of a specimen can be detected in less than a half minute. It is best used, of course, in sufficiently darkened surroundings so that drift and focus can be checked at the illumination level at which the micrograph is to be taken.

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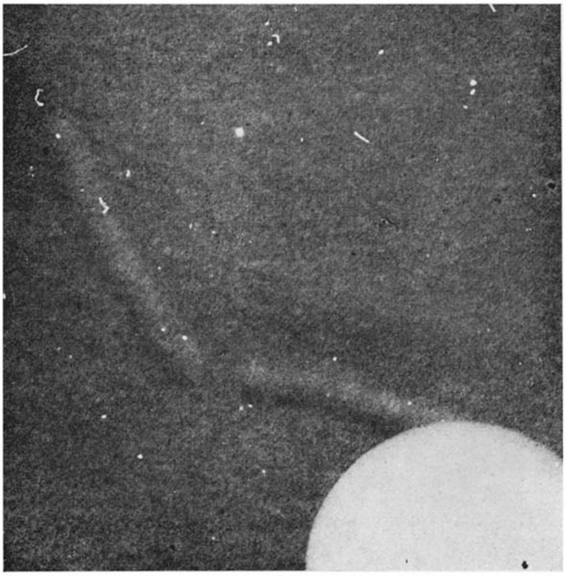
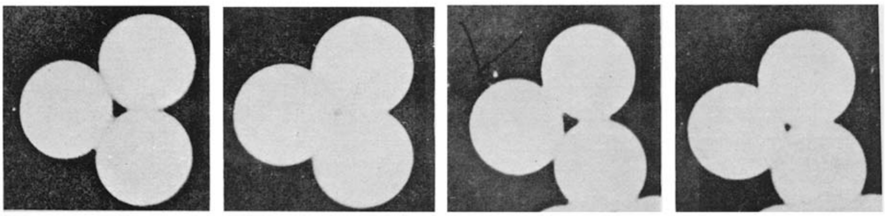


Fig. 1. TMV particles, shadowed after exposure to electron microscope beam. (160,000 ×)

5. *Evaluation of the excellence of micrographs.* It is obvious that, if one is attempting high-resolution electron microscopy, some means of evaluating the excellence of the micrographs must be adopted. Micrographs of inferior quality are likely to exhibit a lack of true, stigmatic focus, a motion of the image during exposure, and/or a fuzziness due to excessive effects of spherical aberration. A critical evaluation should allow the types of defect to be distinguished, even though specimens of widely different character are being photographed. The spherical particles of polystyrene latex appear to be ideal for this purpose, inasmuch as their appearance is independent of substrate smoothness, they are devoid of crystalline image effects, and their peripheral contour is approximately uniform. A glance at a micrograph of one of them will disclose the presence of image motion or astigmatism. These two defects can be distinguished from each other



a b c d

Fig. 2. Polystyrene latex particles (unshadowed) photographed before and after exposure to electron beam, to demonstrate the rate of growth of the contaminating film. (50,000 ×)

a. Particles near edge of 80-mesh grid opening. Brief exposure to beam.
b. Same particles after additional 3-minute exposure to electron beam.
c. Particles near center of 80-mesh grid opening. Brief exposure to beam.
d. Same particles after additional 30-minute exposure to electron beam.

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in a slightly overfocussed exposure. A through focus series of micrographs in which no detectable image motion or astigmatism is present will show on the negative the usual dark border in the underfocussed (long focal length) micrographs and a light Fresnel diffraction ring in the overfocussed ones. A picture in exact focus will show a sharp edge, but the sharpness of this is, of course, dependent upon the angular aperture of the illuminating pencil.

If, for the sake of discussion, a high-resolution micrograph is regarded as one which exhibits a resolution of 3 m$\mu$ or better, the question remains of giving quantitative significance to the figure "3 m$\mu$". This question has been discussed at length in a report of the Committee on Resolution of the Electron Microscope Society of America[11], and several methods of measurement have been suggested. The one that appears most useful,

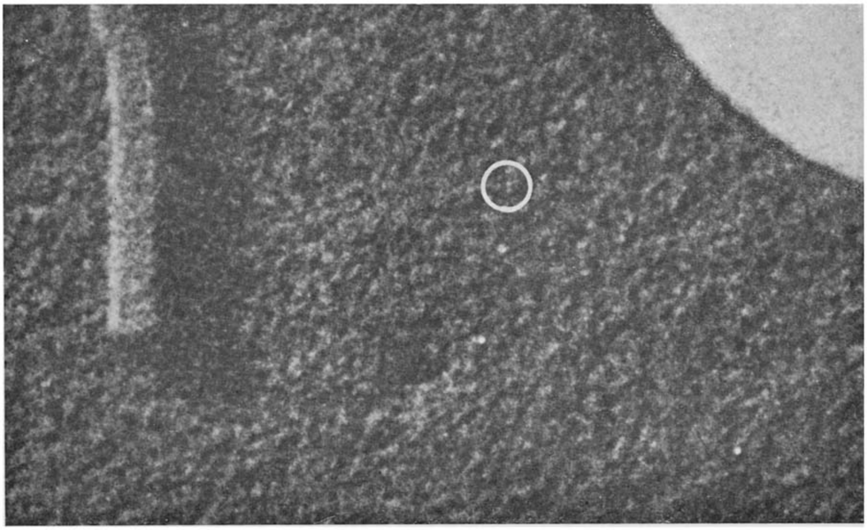

Fig. 3. Micrograph of uranium-shadowed, SiO-coated, collodion film showing appearance of substrate structure. Portions of a polystyrene latex particle, and of a TMV particle, are shown. The two protuberances shown in white circle have their centers 2.0 m$\mu$ apart. (400,000 ×)

in the light of RAYLEIGH's definition of *resolution*, is the measurement of the distance between the centers of two semi-point images which can barely be distinguished as separate. A shadowed collodion or Formvar film, or a SiO-coated film, offers a multitude of point-like images from which to choose pairs of points. Although the resolution inferred from such measurement is always conservative, it has an appealing reality derived from the fact that, on shadowed specimens, this kind of separating ability is exactly what one is demanding of the microscope. Fig. 3 shows an example of the application of the foregoing type of measurement to a collodion surface shadowed with uranium at a 3/1 angle. (The shadow angle designated as "3/1" is defined as: $\alpha = \tan^{-1} 1/3$, where $\alpha$ is measured from the plane of the substrate.) The micrograph is shown at a magnification much too great to be realistic in order to make the distances between image points clearly evident, but it is seen that a resolution of 2 m$\mu$ can be evaluated. It is felt that statements regarding the resolution of micrographs, when the subject is

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of importance, should not be made without a quantitative demonstration of the excellence claimed.

B. *Observations on Tobacco Mosaic Virus Particles*

1. *Preparation.* The TMV used for the micrographs to be discussed is the wild strain grown in Turkish tobacco plants. It was purified by five cycles of centrifugation in which the last three cycles were in doubly distilled water. For most of the preparations the TMV suspension was diluted to a concentration of about 0.005 mg/ml, mixed with a suspension of polystyrene latex particles at a concentration of $3.26 \cdot 10^{10}$ particles/ml, and sprayed upon collodion or SiO-coated collodion films. All shadowing was done with uranium at a thickness of about 0.8 m$\mu$ on the substrate film. The micrographs were mostly obtained from through focus series at an original magnification of 23,000 $\times$ on Eastman contrast lantern slide plates.

2. *Observations*

a. *Normally prepared TMV specimens.* Fig. 4 shows two typical TMV particles shadowed at a 3/1 angle. The features of this and similar micrographs are these: The particles appear not to have any immediately evident surface geometry, although a general impression may be obtained of a cylinder with a rather flat top surface. The apparent width of the particles decreases somewhat as the angle between the axis of the particle and the direction of the shadowing beam (here called the azimuth angle) approaches zero. The detailed surface structure of the particle shows no sign of periodic structure, either transverse or longitudinal, and has a very close similarity to the surface structure exhibited by either collodion or an SiO-surfaced substrate film. This similarity becomes less evident as the azimuth angle approaches 90°, for which angle the accumulation of the uranium on the near side of the particle is a maximum. (The side of the particle nearest the uranium source will be called the "near" side, while the opposite side will be called the "far" side.)

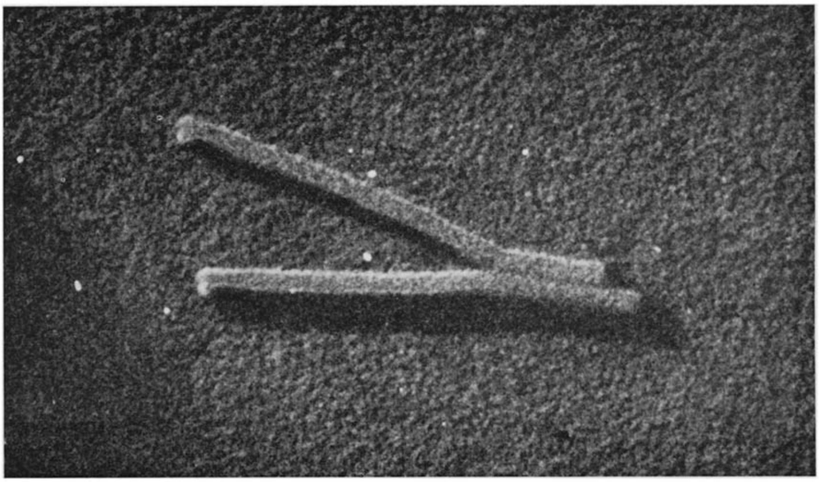

Fig. 4. Two normal TMV particles shadowed with uranium at a 3/1 angle. (200,000 $\times$)

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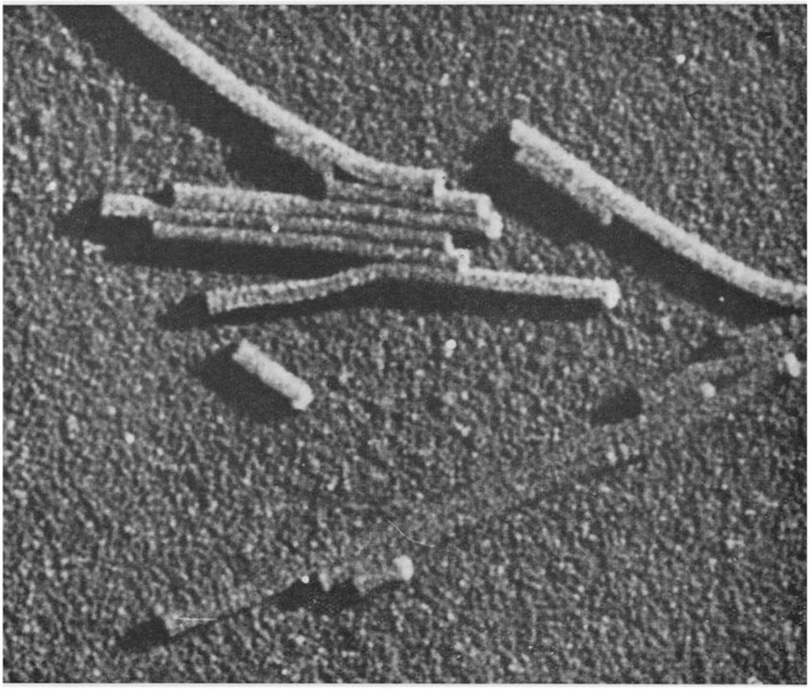

Fig. 5. A group of TMV particles, shadowed at a 2/1 angle. The anomalous features of this micrograph are discussed in the text. (180,000 ×)

Fig. 5 illustrates certain anomalous appearances encountered when one attempts to interpret the fine scale meaning of shadowed specimens of TMV. This is a slightly underfocussed micrograph of normal TMV particles on a collodion SiO-coated substrate film. Attention is particularly drawn to the array of six nearly parallel particles, the upper and lower of which are bent along their length; these are numbered from 1 to 6, starting at the top. Particle 6 shows an anomalous variation in opacity along the length of its mid-line. This mid-line has received the same amount of shadowing uranium along its entire length, but in the right-hand region where the particle received a full uranium coating *on its near side* the opacity of the *mid-line* area is relatively enhanced. It is also to be noticed that the far edge of this particle appears sharp wherever the particle has received a full coating on its near side, whereas particles not having a fully-coated near side (such as particle 5) do not appear sharp on their far edge. Particles 3 and 4 also exhibit an anomalous effect. The left-hand region of particle 3 has been fully coated on its near edge, but the right-hand region has received a coating only on its top surface. The left-hand region has widened slightly on its near edge (due to the accumulation of uranium), but it has also appeared to widen even more on its far side and to overlap partially on particle 4. Particle 4, as a consequence, shows a variation in apparent width; in its right-hand region it is of normal width, but where it has been overlapped by particle 3 it is constricted in width by about 25%. The two long particles at the bottom of the micrograph are also to be noted; they are parallel to the shadowing beam, and it is seen that their surface structure appears almost identical with that of the substrate film.

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The four anomalies shown in Fig. 5 are constantly encountered in electron micrographs, and in summation are briefly these: The mid-line of a particle is unduly opaque if its near edge has received a full coating of uranium; a single particle with large azimuth angle is sharp on its far edge; when two or more particles are in contact, the particle away from the uranium source is not sharp on its far edge; when two particles are in contact, and are shadowed at large azimuth angles, the near particle appears to encroach somewhat upon the particle farthest from the uranium source.

Fig. 6 illustrates the appearance of a short, but otherwise normal, TMV particle when shadowed lightly with uranium at nearly normal incidence. It is to be noticed that the substrate surface has now lost its usual pebbly appearance, as would be anticipated. One would expect the TMV particle to exhibit almost zero contrast also, but instead one sees a thin, opaque line all around the particle. The appearance of this thin, opaque border is like that shown by TMV particles when lightly double-shadowed, as shown by KAHLER AND LLOYD.

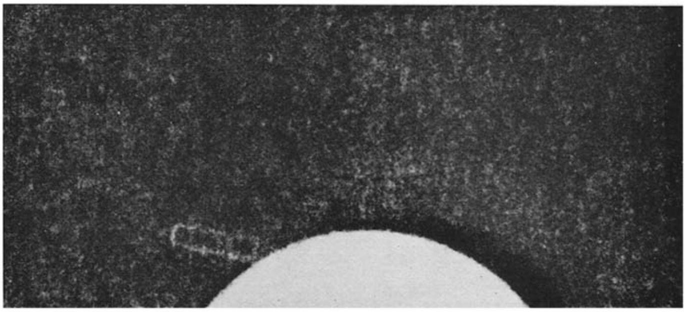

Fig. 6. A short TMV particle shadowed lightly with uranium at normal incidence. A portion of a polystyrene latex particle is visible. (200,000 ×)

b. *Freeze-dried preparations.* Specimens of freeze-dried TMV particles have been prepared in an effort to see whether or not the morphology of the particle is made more clear by the absence of liquid surface tension forces upon drying. The details of the freeze-drying technique will be reported in full in a later article. Briefly stated, however, the technique consists of spraying low velocity droplets from a nebulizer into a space at −60° C, causing the frozen droplets to impinge upon specimen screens at the same temperature, and vacuum drying the droplets at a temperature slowly rising from −70° C to −30° C. Under these conditions most of the dried TMV rods do not lie in full contact with the substrate film, but either stand more or less on their ends or are supported by tangled masses of other rods. It is found that such rods do not exhibit a clearly distinguished surface morphology, and this failure seems to be caused by the circumstance that the rods overlie (without touching) regions of the collodion film which are themselves shadowed. The complexity of the superposition of images of two shadowed surfaces makes interpretation difficult. It was hoped that something of cross-sectional shape would be exhibited by these particles standing nearly on end, but their line-of-sight thickness (approximately 0.3 $\mu$) makes high-resolution microscopy impossible.

Fig. 7 shows a portion of a freeze-dried TMV particle which is in close contact with the substrate. The rod appears about the same as the air-dried specimen shown in Fig. 4.

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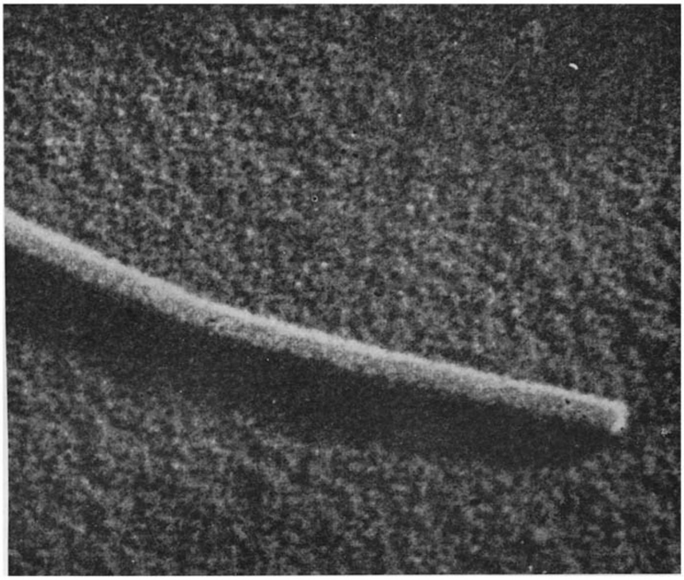

Fig. 7. A portion of a long, freeze-dried TMV particle in contact with substrate surface. (200,000 ×)

This is an example of several such photographs obtained, and the conclusion is that the rods do not distort appreciably upon air drying out of an aqueous suspension. The results described below offer confirmation of this hypothesis.

c. *Micro-incinerated particles.* It was thought barely possible that controlled incineration of the TMV particles might reveal some sort of internal structure, such as the arrangement of the nucleic acid constituents. The TMV was mixed with colloidal gold before spraying to provide indicator particles which would not melt during the incineration, and was sprayed upon SiO films. The incineration was performed both in air and in a vacuum, with no perceptible difference resulting. Incineration times were ten minutes, with approximate temperatures of 180° C and 250° C for the specimens shown in Fig. 8. It is seen that the effect of heating is to cause the particles at first to flatten and widen, an effect similar to what would be called melting in grosser structures. Higher temperatures cause the virus material to be evaporated, and at temperatures higher than reported here (400° C) nothing observable is left of the TMV particles. It is readily apparent that incineration of the virus particles adds nothing to an interpretation of external or internal structure.

d. *Height-to-width ratios.* The use of particles of polystyrene latex on all specimens makes possible a precise determination of the local angle of shadowing, since it is well established that the particles are spherical in shape and suffer no observable distortion upon drying. Some care must be taken in calculating the local angle of shadowing when the angle $\alpha$ is large. A simple determination of shadow length made by measuring the distance from the end of the shadow to the *center* of the latex particle will not suffice. Instead, the correct reference point is the point of tangency on the particle surface of the line drawn from the end of the shadow to the uranium source, and this point does not lie on the vertical line through the center of the particle. All shadowing angles specified in this paper are local angles, measured with the aid of the latex particles.

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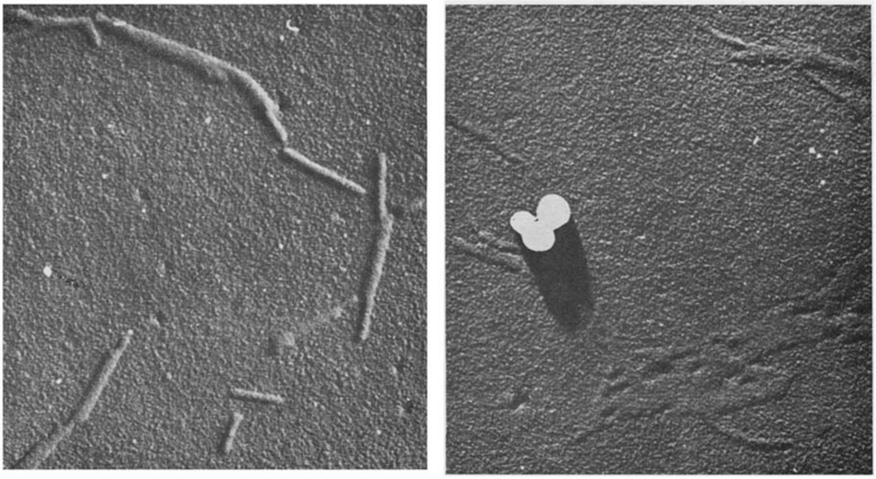

a b

Fig. 8 Microincinerated TMV particles, shadowed at a 2/1 angle.
a. 10 minutes incineration at 180° C.
b. 10 minutes incineration at 250° C. Opaque particles are of colloidal gold. (40,000 ×)

The distinction between local and general shadowing angles is a serious one, when high accuracy is demanded, or, in any event, when SiO-coated films are used. It is found that in the case of 200-mesh screens, filmed with collodion, the local angle may vary by as much as 20 to 40% over one grid opening, while the variation can be as great as a factor of two for SiO-coated films. The variations are even greater, of course, for the 80-mesh screens.

The ratio of height to width of the TMV particles is ascertained in the following way: In those electron micrographs containing images of several TMV particles, a few particles can be found whose shadowing azimuth is approximately 0°. The width of these particles is measured in any arbitrary scale system. Particles are then selected whose shadowing azimuth is 90°, and the length of the shadow is measured, in the same scale units, from its outer edge to a point on the virus rod midway between the center line and the edge. (The reason for this latter choice of reference line will be made clear later). Since the shadowing angle is known for spherical latex particles usually lying not more than a micron or so from the TMV particles, the ratio of height to width of the latter is determinable with fair accuracy.

Twenty-five particles from both freeze-dried and air-dried specimens have been measured for their height-to-width ratios. The numerical average of the calculated ratios is $0.98 \pm 0.06$ with no evident dependence upon the method of drying. It is realized that the probable error stated is apt to be an understatement of the degree of absolute inaccuracy since the chances of systematic error, owing to diffraction effects, are great when one is measuring such small dimensions.

e. *Preparation by sonication.* When a concentrated suspension of purified TMV is examined by electron microscopy, occasional particles are seen which are extremely short, of the order of 20 m$\mu$ in length or less. If particles can be produced which are even shorter, the chances are great that the surface tension forces during drying will

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cause such particles to dry out on end, with the particle axis normal to the plane of the substrate film. It has been found[12] that high intensity sonication, at about 9 kilocycles, breaks TMV particles efficiently, and if the sonication is continued for over an hour, almost all the particles are broken. This result is made evident, grossly, by the disappearance of streaming birefringence in a suspension of TMV particles which exhibit a high degree of birefringence prior to sonication.

Material has been prepared by sonication, and the resulting suspension mixed with polystyrene particles and sprayed upon the microscope specimen films. The micrographs exhibit some variety of forms: There are fragments of rods lying on their sides, an occasional fragment so short (about 15 m$\mu$) that it appears to have a square cross-section, and many objects whose *approximate* cross-sectional shape is circular. The last type of particle is believed to be representative of those which have dried out in the end-on position, since their numbers increase with prolonged sonication, their diameters are all about 15 m$\mu$, and some of them are so short that their thickness in the line of sight is less than 10 m$\mu$.

Close examination of high quality micrographs of the end-on particles (to be called "platelets") shows a small variety of cross-sectional shape; about 20% of them exhibit

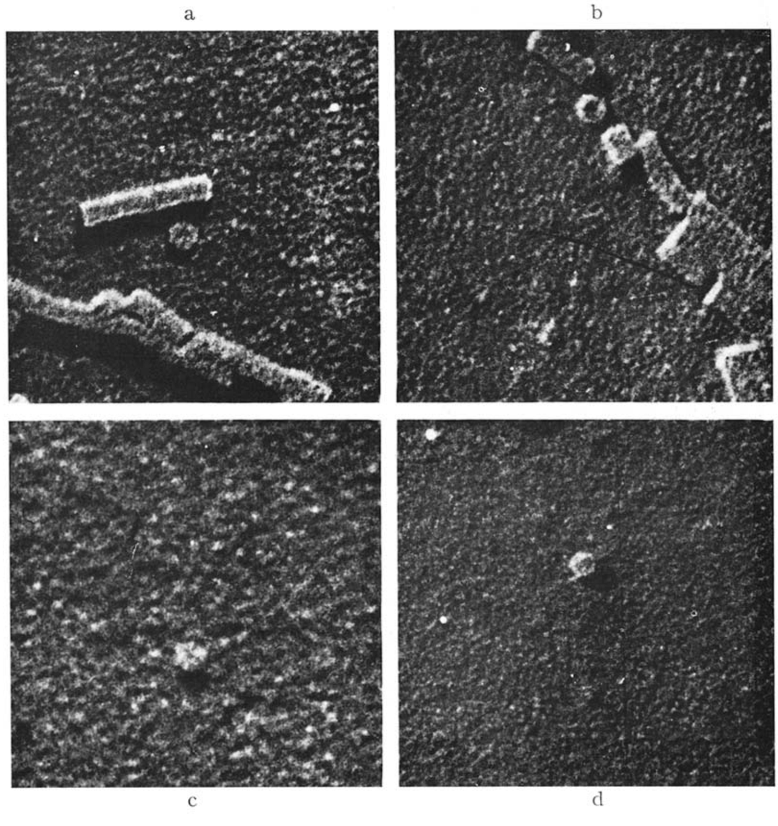


Fig. 9. Four examples of hexagonal platelets obtained by prolonged sonication of TMV particles. The very thin platelet in micrograph *c* is almost perfectly hexagonal, while the thicker one shown in *d* is noticeably distorted

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a distinct hexagonal cross-section; about 60% of them show a distorted hexagonal shape, with one or two of the edges of the hexagons shorter or longer than the remaining edges; the remainder are either of a distorted circular shape or else of a bizarre shape, such as half a hexagon or half a distorted circle. In no case have platelets been found whose cross-sectional shape is a square, except the occasional forms mentioned above, but in these cases the thickness of the particle is always found to be 15 m$\mu$. It is believed that these square-appearing rods are simply fragments lying on their sides and of an axial length equal to their width. In no case have smoothly circular platelet cross-sections been seen.

Fig. 9 shows four examples of hexagonal and near-hexagonal shapes from four different specimen screens made from three different sonicated preparations. Perfect hexagonal figures seem not to be obtainable, although the extremely thin hexagon shown in Fig. 9c approaches perfection.

Measurements have been made of the thickness of the platelets. Since the breaking of the original TMV particles is apparently clean, and presumably takes place at regions of minimum mechanical strength, there is a faint expectation that some natural cleavage lengths might exist and give rise to a discontinuous distribution of thickness of the end-on particles. The thickness of 35 of the platelets (defined as particles which appear hexagonal or roughly hexagonal, and which have a diameter of 15 m$\mu$) has been measured by use of the technique described above. The shadow length measured is from the end of the shadow to the *edge* of the particle on the far side. It is thus assumed that the top surface of the end-on fragment is not sharply convex. The thinnest fragment found is 4 m$\mu$ thick, the thickest found is 18 m$\mu$, and there seems to be an approximately Gaussian distribution of thicknesses between these two limits.

## DISCUSSION

### A. *The Normally Intact Rods*

Normal TMV rods, either air-dried or freeze-dried, exhibit the same surface appearance, and this appearance seems to contribute little to an understanding of the rod structure. The surface appears not to be indefinitely smooth, but has the generally pebbly appearance of the collodion, or of the collodion-SiO, background. A moderately underfocussed image which has drifted 3 m$\mu$ or so during the exposure always shows striated transverse structure, but the impression of any striated structure, either transverse or longitudinal, decreases rapidly as the excellence of the micrographs increase. It is concluded that no periodic surface structure is presently demonstrable on TMV particles, but that there may be a disordered, small scale surface irregularity.

The height-to-width ratio of approximately 1.0 for both air-dried and freeze-dried specimens bears on speculations about the existence of water of hydration in these particles. Either one must assume that very little such water exists, or else that the particle is so rigid that it retains its shape despite the volume shrinkage resulting from the evaporation of the water of hydration. Other small objects such as bacteriophage particles, papilloma virus particles, and the very small spherical particles obtained from cell homogenates do exhibit considerable flattening upon drying. At present tobacco mosaic virus is the only virus whose particles have been shown to suffer little or no flattening upon air-drying.

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### B. *The Hexagonal Platelets*

The first question concerns the reliability of the observations: Do particles shaped like short hexagonal prisms really exist on the specimen films. The observations are rendered difficult by the small dimensions involved, but simple lack of resolution would tend to make true hexagons look like discs, rather than *vice versa*. Motion of the image during exposure, or lens astigmatism, will make a truly disc-like cross-section (like bushy-stunt virus) appear vaguely hexagonal, but in such a micrograph all end-on particles would appear somewhat hexagonal. The existence of images varying from nearly perfect hexagons to fairly true discs, *on the same micrograph*, is the strongest evidence that hexagonal prisms truly exist. As a matter of fact, the original excellence of hexagonal shape of the platelets must surely be better than the micrographs exhibit. Small amounts of low molecular weight contamination will tend to dry around the particle edges and render them non-regular; the unilateral shadowing will always distort the shape somewhat; and the effects of spherical aberration and diffraction in the microscope image will tend to round off angular corners.

This demonstration that most of the very thin plates are short rod fragments standing on end, and that some of the platelets, at least, are thin hexagonal prisms, helps to elucidate the structure of the intact TMV rod. There is, of course, the dilemma as to why not all the end-on fragments are hexagonal, aside from the explanations offered in the preceding paragraph. It is proposed that only a fraction of the broken-off plates withstand forces of cavitation during sonication, and that, for those that do, the effects of the distortions of drying upon such very small objects would detract from perfect regularity of shape. The distortion referred to here need be only very slight indeed (of the order of 1 to 2 m$\mu$), and its effect would not be detectable in the height-to-width ratios of the intact particle, as discussed above. Other hypotheses are possible, of course, to explain the failure of all fragments to be hexagonal prisms, the most attractive of which is that the intact TMV rod is not a true hexagonal prism along its entire length and that fragments of various cross-sectional shapes are broken off in the sonication.

Aside from consideration of hexagonality, the existence of such extremely thin platelets, bounded by approximately planar sides, is amazing. During the examination of dozens of micrographs containing several hundred fragments of TMV rods, a non-transverse break has been seen only once. There apparently exists in the rod a direction of easiest breakage, analogous to the direction of easiest cleavage in single crystals, and the mechanical anisotropy is so great that cleavage by sonication always takes place parallel to this direction. This anisotropy evidently extends to low orders of size, since the thinnest fragments found are only about 4 m$\mu$ thick and have a particle weight of approximately 640,000 g/mole.

### C. *Inconsistency in the Appearance of Intact and Sonicated Particles*

An examination of the intact rods in micrographs such as shown in Fig. 4 fails to impart any distinct impression that such rods are shaped like hexagonal prisms. At shadow angles less than about 3/1, and with an azimuth angle of less than 45°, the rods appear to have a reasonably flat upper surface with only a hint of a generally convex shape. In a discussion of the possible reasons for this anomalous appearance, it is necessary to recall that visual impressions of shadowed surfaces may sometimes be too literal. All that is actually observed on shadowed surfaces are variations of opacity,

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and the literal interpretation is that these variations are produced only by variations in the angle which the local surface makes with the shadowing beam. This interpretation can be false for at least two reasons: The opacity of the particle itself may be a major contributor to the total opacity; the thickness of the uranium film may not be uniquely determined by the geometry of the shadowing process.

In the case of TMV shadowed with a film of uranium 0.5 to 0.8 m$\mu$ thick the contribution to the total opacity by the rods themselves is negligible, as can be seen by observing the extreme faintness of TMV rods in the shadows on the far side of the large polystyrene latex particles. If an *exact* correlation exists between the thickness of the uranium film and the local shadowing angle, the apparent surface contours of objects as thin as the rods of TMV could be literally accepted. In particular, this condition would result in zero contrast for TMV rods shadowed at $\alpha = 90°$. To be sure, a variation in the thickness of the uranium film would exist over the rod surface, with the mid-line receiving the most uranium, but this would be exactly compensated by the variation in "optical path" which the electrons experience in going through the film. If the uranium film is spread with a more uniform thickness than straight geometry would provide, the result would be that the edges of the particle (where the electrons go through the film almost tangentially) will appear relatively opaque. If the far sides of a rod of approximately hexagonal shape are considered, and if it is assumed that the two facets on the far side of the rod (which should be in complete shadow) have received enough uranium, the result could be a flat-appearing rod with the far edge quite sharp and opaque. This effect could be brought about by spreading of the uranium film, or by back-scattering in the vacuum chamber, and if as much as 25% of the film thickness at the mid-line is present on the two far facets, the result would be an apparently flat-surfaced particle.

The evidence from Fig. 5 and Fig. 6 would indicate that the uranium film does not have a thickness solely in accord with geometrical considerations. Back-scattering of uranium atoms appears to be ruled out by a consideration of the third item in the paragraph summarizing the detailed observations from Fig. 5. It appears that the most satisfactory explanation of the anomalies exhibited by Fig. 5 and Fig. 6 is that the uranium film has somehow spread from the region on the near side of the particle (where the uranium deposit is initially two to four times as heavy as on the midline) across the mid-line and over the surfaces of the two far facets.

A spreading of the uranium film could be brought about by at least two mechanisms: a surface diffusion of the uranium atoms over an immobile substrate, and a diffusion of some sort of adsorbed film which carries with it the uranium. At present the experiments do not provide a choice between these mechanisms, but the more likely one appears to be the latter. The shadowing equipment used in this work uses an untrapped oil-diffusion pump as well as the usual mechanical pump. As shown earlier by WILLIAMS AND BACKUS[13], the adherence of a metal film to glass is strongly influenced by the type of oil used in the diffusion pump, and this fact strongly implies that there is a film of adsorbed oil between the glass and the deposited metal film. It is also well known that if a glass surface, initially hydrophilic, is merely placed in a vacuum system and exposed to the vapors of the pumping units for a few minutes, it will be changed to a hydrophobic surface. Further evidence that an adsorbed oil film is present on the entire specimen is provided by the close similarity in surface appearance between shadowed substrate films and TMV rods shadowed at zero azimuth angle. This similarity

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in surface detail has also been found in this laboratory for particles of bushy stunt virus and rabbit papilloma virus, and it seems unlikely that all three of these virus particles would have a real surface structure nearly identical with that of collodion. The average thickness of an adsorbed oil film need be only about 1 m$\mu$ to provide for the size of the observed discrete mounds, since even the larger mounds have a diameter of only about 3 m$\mu$ and a thickness of about 1.5 m$\mu$.

It is suggested that the anomalies described for Fig. 5 can all be explained by the existence of an initially adsorbed film of oil on the TMV particles and on the substrate. In particular, the queer "cricket-bat" appearance of the left-hand end of particle 3 is explained by migration of an oil film entirely across this particle and partially over the near facet of the adjoining particle 4. This film would be strongly and locally heated on the near side of TMV particles by the heat of condensation of the uranium atoms and would be caused to migrate away from the near side. The migrating oil would presumably be impregnated with uranium and would have considerable opacity to electrons. In order to account for the observation that this assumed oil film shows some shadowed structure (like the two small mounds shown in Fig. 3) it is necessary to assume further that the oil film collects in small droplets and that the process of migration effectively stops before the uranium shadowing is completed. Experiments have been started to determine conclusively whether or not a semi-mobile oil film exists on the specimen surface. If it is found not to exist, possible creeping of the uranium film itself must be investigated. The conclusion to be drawn from this discussion of shadowing anomalies is that *the appearance of normal TMV rods, lying on the substrate film and shadowed with uranium, has little bearing upon the details of their actual surface contours.* The behavior of the uranium film would not cause thin objects of truly circular or square cross-section to appear hexagonal, however, and it is believed that the observed hexagonality of the thin platelets can be accepted literally.

It might be expected that something can be learned of the surface contours of the TMV rods by observations of the shadows themselves, as well as by the previously discussed observations of the shadowed particles. One type of observation is that of the shape of the shadow cast by the extreme far end of a particle when the shadowing azimuth angle is between 0° and about 45°; this should be different for rods with square, cylindrical, and hexagonal cross-sections. Unfortunately, the micrographs appear to give no consistent impression of this shape, owing to the rough surface of the substrate film upon which the shadows fall, and probably owing to the failure of the TMV rods to have always perfectly formed ends. One observation has been rewarding, however, and that is the degree of "undercutting" shown by the intersection of the shadow and the end of the particle when the shadowing azimuth is about 45°. (The left-hand ends of particle 4 in Fig. 5 shows this intersection well.) A rod with square cross-section will exhibit no undercutting; the edge of a cylinder will be undercut to the mid-line; while that of a hexagonal prism will be undercut half-way to the mid-line. The observations show fairly consistently a partial undercutting—enough to rule out a square cross-sectional shape, but not sharply enough defined to rule out the possibility of a cylinder with a large area of contact with the substrate. The value of 0.98 for the height-to-width ratio of the particles, however, makes it unlikely that they are cylinders with a greatly flattened bottom surface.

It is theoretically possible to distinguish a hexagonal prism from a cylinder by measurement of the width of the shadow, from the shadow edge perpendicularly to the

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particle edge, as the shadowing azimuth changes from 0° to about 20°. Calculation shows, however, that in the case of rods only 15 m$\mu$ in diameter and with a 2.5/1 shadowing angle, the maximum difference expected between the widths of the shadows of the kinds of particles is only about 2.5 m$\mu$—too small a distance to be measured reliably.

In conclusion, it can be stated that the observations make it extremely unlikely that the cross-sectional shape of a TMV rod is that of a square, and that the observations in no case clearly support the possibility that the rods are cylindrical. On the other hand the observations of hexagonal platelets and of partially undercut shadows, as described above, make it most probable that the shape of the intact particle of tobacco mosaic virus is that of an elongated hexagonal prism.

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## SUMMARY

High-resolution electron microscopy of the particles of tobacco mosaic virus has been undertaken in an attempt to elucidate the details of the surface contours of these objects.

Some techniques have been developed to aid in the consistent attainment of resolutions better than 3 m$\mu$ in micrographs of shadowed virus particles. These developments bear upon the problems of mechanical stability of the specimen, and of contamination of the specimen in the electron microscope. The routine use of polystyrene latex particles is discussed in connection with the problem of the evaluation of the excellence of electron micrographs.

Observations have been made of the surface structure of the particles of tobacco mosaic virus in normal preparations, and in freeze-dried, incinerated, and sonicated preparations. It is concluded that no evidence exists of periodic surface structure, either transverse or longitudinal. Sonicated preparations provide thin platelets which have been broken cleanly in planes transverse to the axis of the rods. When these platelets are observed perpendicular to their planes, they exhibit hexagonal cross-sections of varying degrees of perfection of figure.

The failure of the normally observed rods of TMV to appear hexagonal is discussed. Evidence is given which indicates that some sort of creeping of the shadowing uranium film exists, and that consequently the surface appearance of rods lying on their sides on the substrate film has little bearing upon their true contours. It is suggested that a film of adsorbed oil from the vacuum pumps of the shadowing apparatus is the cause of the apparent surface mobility of the uranium atoms.

It is concluded that the shape of the normal rods of tobacco mosaic virus is that of an elongated hexagonal prism.

## RÉSUMÉ

Les auteurs ont appliqué la microscopie électronique à haut pouvoir de résolution aux particules du virus de la mosaïque du tabac afin de tenter d'élucider les détails des contours de la surface de ces objets.

Certaines techniques ont été développées, permettant d'obtenir régulièrement des résolutions meilleures que 3 m$\mu$ pour des micrographies ombrées de particules de virus. Ces améliorations portent sur des problèmes de stabilité mécanique des spécimen dans le microscope électronique. L'emploi usuel de particules de latex de polystyrène est discuté par rapport au problème de l'évaluation de la qualité des micrographies électroniques.

Les auteurs ont observé la structure de la surface des particules de virus de la mosaïque du tabac dans des préparations normales, séchées à l'état congelé, incinérées ou traitées aux ultrasons. Ils en concluent qu'il n'existe aucune preuve à l'appric d'une structure de surface périodique transversale ou longitudinale. Les préparations traitées aux ultrasons montrent des plaques minces formées par des cassures nettes dans des plans transversaux par rapport à l'axe des bâtonnets. Lorsqu'on observe ces plaques perpendiculairement à leurs plans, elles montrent des sections hexagonales plus ou moins parfaites.

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Les auteurs discutent le fait que les bâtonnets du virus de la mosaïque du tabac (TMV) en semblent pas hexagonaux. Certains faits indiquent que le film d'uranium employé pour ombrer la préparation rampe de quelque sorte, ce qui fait que l'apparence de la surface de bâtonnets couchés sur leurs côtés sur le film de substrat dépend peu de leurs vrais contours. Les auteurs suggèrent l'idée qu'un film adsorbé d'huile de la pompe à vide de l'appareil à ombrer soit la cause de l'apparente mobilité de surface des atomes d'uranium.

On conclue que les bâtonnets normaux de virus de la mosaïque du tabac ont la forme d'un prisme hexagonal allongé.

## ZUSAMMENFASSUNG

Teilchen von Tabakmosaikvirus wurden mit Hilfe der Elektronenmikroskopie von hohem Auflösungsvermögen untersucht um Einzelheiten der Umrisse der Oberfläche dieser Objekte aufzuklären.

Einige Methoden wurden entwickelt, welche erlauben regelmässig eine bessere Auflösung als 3 $m\mu$ in Mikrographien von schattierten Virusteilchen zu erhalten. Diese Verfahren beziehen sich auf Probleme der mechanischen Stabilität der Spezimina und auf deren Verunreinigung im Elektronenmikroskop. Die gebräuchliche Anwendung von Polystyrenlatex-Teilchen wird erörtert und zwar im Zusammenhang mit dem Problem der Schätzung der Qualität der Elektronenmikrographien.

Die Oberflächenstruktur von Teilchen von Tabakmosaikvirus wurde in normalen Präparaten, sowie in im gefrorenen Zustand getrockneten, in veraschten und in beschallten Präparaten beobachtet. Es wird geschlossen, dass für eine periodische, Transversal- oder Longitudinalstruktur kein Beweis besteht. Beschallte Präparate zeigen dünne Plättchen, welche deutlich in zu der Achse der Stäbchen transversal liegenden Flächen abgebrochen worden sind. Beobachtet man diese Plättchen senkrecht zu ihren Flächen, so zeigen sie mehr oder wenige perfekt hexagonale Querschnitte.

Die Tatsache, dass normal beobachtete Stäbchen von Tabakmosaikvirus (TMV) nicht hexagonal erscheinen, wird erörtert. Es werden Beweise angeführt, die darauf hinweisen, dass der schattierende Uranfilm irgendwie kriecht, und dass deshalb das Aussehen der Oberfläche von seitlich auf dem Substratfilm liegenden Stäbchen wenig mit deren wirklichen Umrissen zu tun hat. Die Möglichkeit wird geäussert, dass ein Film von adsorbiertem Öl aus der Vakuumpumpe des Schattierapparates für die scheinbare Oberflächenbeweglichkeit der Uranatome verantwortlich sein könnte.

Es wird geschlossen, dass die normalen Stäbchen von Tabakmosaikvirus die Form eines länglichen hexagonalen Prismas haben.

## REFERENCES

[1] G. A. KAUSCHE, E. PFANKUCH, AND H. RUSKA, *Naturwissenschaften*, 27 (1939) 292.
[2] R. C. WILLIAMS AND R. W. G. WYCKOFF, *J. Appl. Physics*, 15 (1944) 712.
[3] R. C. WILLIAMS AND R. W. G. WYCKOFF, *Proc. Soc. Exptl Biol. Med.*, 58 (1945) 265.
[4] R. C. WILLIAMS AND R. W. G. WYCKOFF, *Science*, 101 (1945) 594.
[5] H. KAHLER AND B. J. LLOYD, JR., *J. Appl. Physics*, 21 (1950) 699.
[6] R. W. G. WYCKOFF, *Electron Microscopy*, (1949) Interscience Publishers, New York.
[7] *Life* (Magazine), 30, No. 17 (1951) 72.
[8] JAMES HILLIER AND E. G. RAMBERG, *J. Appl. Physics*, 18 (1947) 48.
[9] J. D. BERNAL AND I. FANKUCHEN, *J. Gen. Physiol.*, 25 (1941) 147.
[10] G. HASS, *J. Am. Ceramic Soc.*, 33 (1950) 353.
[11] W. G. KINSINGER, *et al.*, *J. Appl. Physics*, 17 (1947) 989.
[12] G. OSTER, *J. Gen. Physiol.*, 31 (1947) 89.
[13] R. C. WILLIAMS AND R. C. BACKUS, *J. Appl. Physics*, 20 (1949) 98.

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