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THE POLYHEDRAL FORM OF THE TIPULA IRIDESCENT VIRUS*

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

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

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

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of the T-phages were hexagonal in contour, those of the T-odd phages being particularly regular polyhedra. It has also been found that the contours of some of the "spherical" virus particles are not round but are six-sided. The hexagonal aspect of tobacco ringspot virus is particularly evident[5], while that of wild cucumber, squash mosaic and brome grass mosaic[6], and of bushy stunt[7], is less pronounced. In a more doubtful category is the external contour of turnip yellow mosaic virus. COSENTINO *et al.*[8] concluded that this virus is spherical when frozen-dried, while KAESBERG[6] believed it to exhibit shadows whose shape would suggest that it is polyhedral. Not all so-called spherical viruses of small size ($\sim$ 30 mμ) are found to be polyhedral following freeze-drying. Poliomyelitis virus appears circular in contour, and spherical by implication, after having been prepared in this manner for electron microscopy[9]. On the other hand, an adenovirus has been shown to be distinctly hexagonal in contour after treatment with phosphotungstic acid[10].

There is more than one type of polyhedron that will appear six-sided in contour, and the discovery that the small plant viruses are so contoured does not disclose directly their three-dimensional morphology. KAESBERG[6] has recently attempted to find the polyhedral form of some of the plant viruses, when frozen-dried, by an analysis of shadow shapes. A particular type of polyhedron, if it is regular, should cast a shadow that is uniquely shaped, if it is cast with a specified orientation with respect to the positions of the vertices of the polyhedral particle. Kaesberg concluded that the shapes of the small virus particles investigated by him are best represented by regular icosahedra. Although this conclusion is probably correct, the deduction as to the true polyhedral shape is marginal in its precision, owing to the smallness of shadow detail in comparison with the roughness of the film upon which the shadows are cast.

The difficulty brought about by the roughness of the substrate film is obviously reduced if the particle and its associated shadow are large. The cytoplasmic virus of the dipterous insect *Tipula paludosa*[11,12,13] is a particularly apt object for the determination of three-dimensional shape, since it is large ($\sim$ 130 mμ in diameter), occurs in copious quantities in the diseased larvae, and is readily purified by centrifugation. Centrifuged pellets of the purified virus are iridescent when observed by reflected, white light[14]. For this reason the virus is called the Tipula iridescent virus (TIV).

MATERIALS AND METHODS

Larvae of *Tipula paludosa* diseased with TIV were placed in small bottles of distilled water as soon as advanced morbidity was apparent. After a few days the cadavers disintegrate, releasing the virus particles and cellular debris into the water. The contents of the bottles were centrifuged for about 15 min in a clinical centrifuge to sediment the debris. The supernatant fluid was centrifuged for 30 min in an MSE centrifuge operating at 8000 r.p.m. (about 8000 *g*). This produced an iridescent pellet that was further purified by two more cycles of differential centrifugation in distilled water. Electron microscopic examination showed the final preparation to be essentially devoid of particulate impurities.

Preparations for electron microscopy were first made by allowing the virus suspension, properly diluted, to dry on an albumin-coated collodion film. The purpose of the albumin is to inhibit aggregation of the virus particles upon drying. Subsequent preparations were made by freeze-drying the virus particles. Some of these mounts were micrographed unshadowed, some were shadowed in the usual manner, while others were double-shadowed in two directions differing by 60°, or by 180°, in azimuth.

Polystyrene latex particles, 260 mμ in diameter, were usually added to the preparations before spray-mounting for electron microscopy. These were used as an aid in focussing, as a magnification standard, and as a means of determining precisely the local angle of shadowing. The electron micrographs were secured on a Siemens Elmiskop I.

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RESULTS AND DISCUSSION

As Fig. 1 shows, the Tipula iridescent virus flattens somewhat upon drying out of water. But despite the distortion due to flattening, the particles show a distinctly non-circular contour. Six-sided contours are the most frequent, but occasional five-sided ones are seen. The micrographs secured from such preparations clearly indicate that the virus, in suspension, is not a spherical particle.

When TIV is frozen-dried, and unshadowed, its appearance is that shown in Fig. 2. It is to be noticed that all particles are quite regular in shape, and that the external contour is universally six-sided. This micrograph was obtained with 100-kV electrons to which the virus particles are fairly transparent. When frozen-dried, and unstained, they show no indication of internal structure, in contrast to the structure exhibited in sections of OsO_4-fixed material[13, 14].

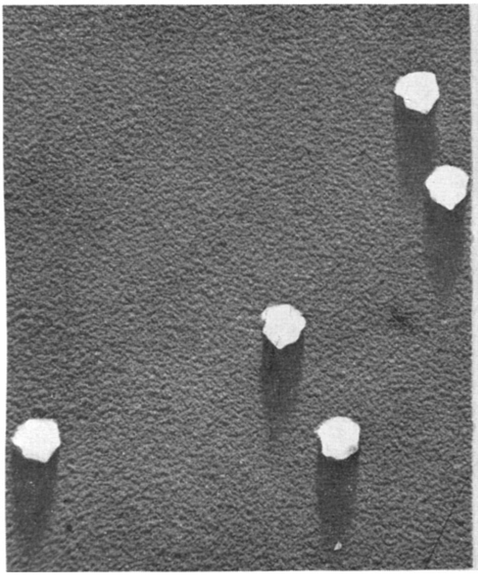

Fig. 1. Electron micrograph of air-dried particles of the Tipula iridescent virus (TIV), showing evidence of non-spherical shapes. × 40,000.

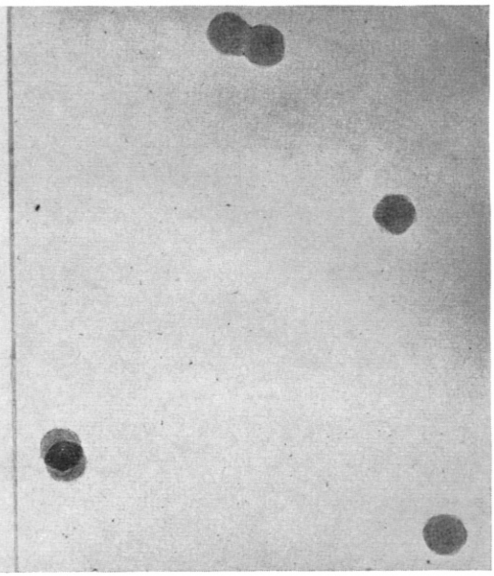

Fig. 2. TIV particles, frozen-dried and unshadowed. Note that the virus particles exhibit six-sided contours. × 38,000.

It may be concluded from the appearance of TIV after drying directly from water, and as frozen-dried, that it is likely that the virus particles have a polyhedral shape in solution and that this shape is one that presents a regular six-sided outline when the particle is resting on one of its facets. There are but two geometrical forms which will present such a regular outline, and one that would very nearly do so. These are the octahedron, the icosahedron, and the rhombic dodecahedron. The virus particles cannot possibly be octahedra, since this polyhedron will cast shadows that are bounded, at most, by three straight edges. As Fig. 4 shows, TIV particles may cast shadows that are five-sided, and are always at least four-sided. A rhombic-dodecahedral model is unlikely, owing to its failure to exhibit a *regular* hexagon in contour. The icosahedral

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model is the most likely one, and the remainder of this paper describes experiments to show that this model is not only likely, but is uniquely determined.

Fig. 3 shows three cardboard models of an icosahedron "shadowed" in three distinctive orientations. In (a) one vertex of the hexagonal contour points directly toward the shadowing lamp. In this case the shadow is four-sided and pointed, the point arising from the shadow of one of the upper vertices. If the model is rotated through 60°, the orientation is that shown in (b). The extremity of the shadow is now cast by the edge that connects two upper vertices. The shadow is five-sided and has a snub end. The particle in (c) is oriented so that a vertex does not point directly at the light source; the shadow is unsymmetrical, as shown. In each photograph the

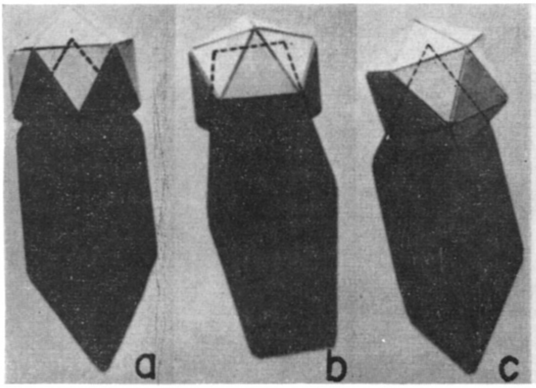

Fig. 3. A cardboard model of an icosahedron, "shadowed" in three orientations by an ordinary light-bulb.

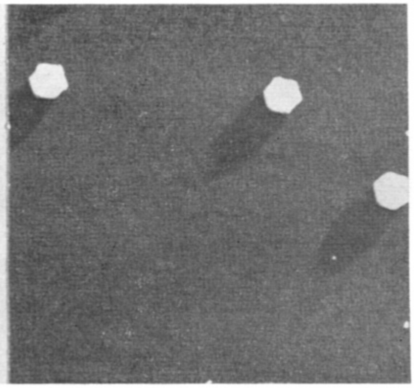

Fig. 4. Three frozen-dried TIV particles, shadowed with gold–manganin, and exhibiting the three forms of shadows shown in Fig. 3. × 37,000.

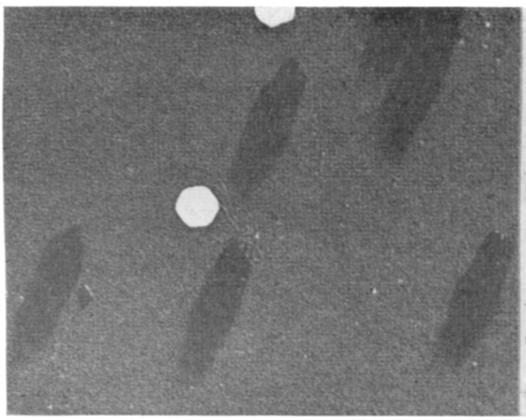

Fig. 5. Shadows cast by frozen-dried particles of TIV. The particles have been rubbed off the substrate film subsequent to shadowing, thereby disclosing the complete shadow. It is seen that each shadow has a center of symmetry and is similar in appearance to those cast by an icosahedral model and extended by dotted lines (Fig. 3). × 41,000.

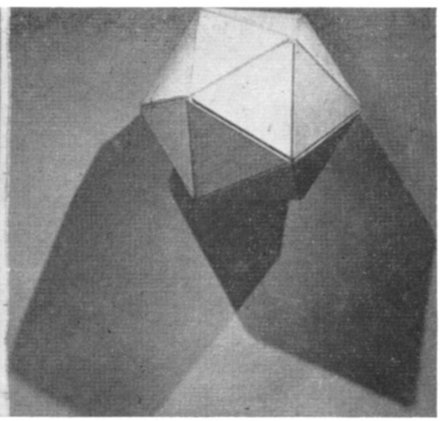

Fig. 6. A model of an icosahedron, shadowed by two light sources separated 60° in azimuth and oriented such that an apex of the hexagonal contour points directly toward each light source.

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extension of the shadow under the model is sketched in dotted lines, and it is evident that the complete shadow has a center of symmetry.

In Fig. 4 is shown the appearance of three frozen-dried TIV particles that happened to be oriented in ways such as to show the three types of shadows illustrated by the models. While this micrograph has been selected for convenience in presentation, the relations shown here between shadow shape and particle orientation are universally encountered. It is also possible to exhibit the appearance of the *complete* shadows, as is shown in Fig. 5. Here the virus particles have first been shadowed and then removed from the specimen film by gentle rubbing with the finger. It can be seen that, at least for three of the shadows, there is a shape possessing a center of symmetry.

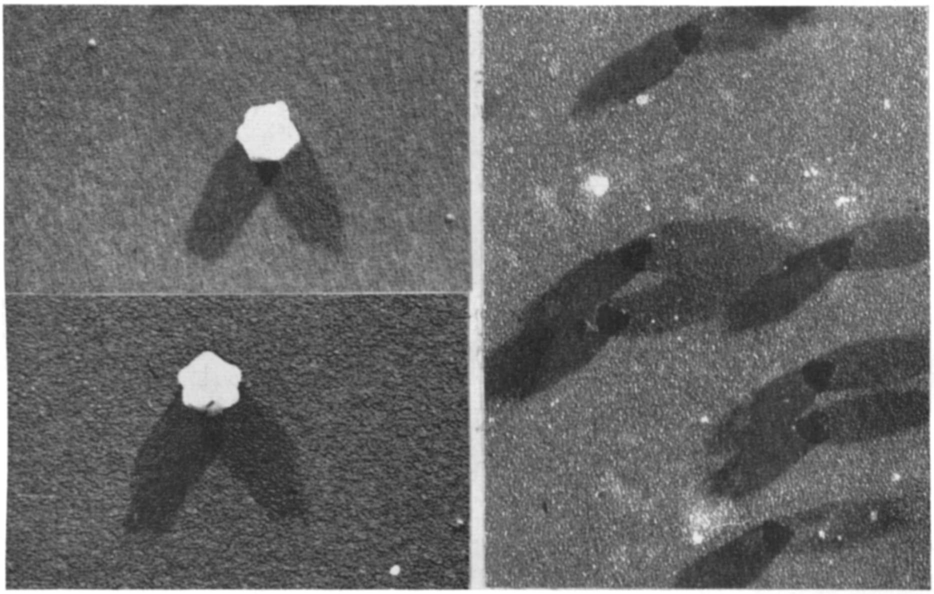

Fig. 7a. A frozen-dried, doubly-shadowed particle of TIV, oriented similarly to the icosahedral model shown in Fig. 6, and exhibiting similar shadows. × 61,000.

Fig. 7b. A doubly-shadowed particle of TIV with an apex of the hexagonal contour oriented 30° from the direction toward either shadowing source. × 61,000.

Fig. 8. Shadows cast by TIV particles by two shadowing sources about 180° apart in azimuth. The particles have been rubbed off subsequent to shadowing. The darkest areas of the shadows should have a shape identical with that of the facet of the TIV particle that was in contact with the substrate film. Note that these areas are three, sided, and have approximately the shapes of equilateral triangles. × 32,000.

A somewhat more critical test of the aptness of the icosahedral model comes about from the application of double shadowing, since now the same particle has to disclose its shape as seen from two aspects. If the shadowing sources are 60° apart in azimuth, as seen from the position of the virus particle, and if the particle orientation is such as to combine the effects shown in Fig. 3a, b, the double shadow will have the appearance shown by the model in Fig. 6. A particle of TIV double-shadowed with this orientation is seen in Fig. 7a; it is evident that the similarity of the shapes of its shadows to those of the model is quite close. If a virus particle is double-shadowed with its orientation differing by 30° from that shown in the model, it is to be expected that

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the two shadows will be unsymmetrically shaped, and that one will be the mirror image of the other (Fig. 7b).

It has thus been shown by shadow analysis that an icosahedral form for frozen-dried TIV is not inconsistent with the shapes of the shadows cast. Another test of the uniqueness of the model, however, can be applied. If the particles are shaped like icosahedra their facets will all be equilateral triangles. This conclusion cannot be tested by electron microscopy of the whole particle, owing to its high opacity relative to that introduced by the shadowing metal. But when the virus particles are shadowed from two sources, ideally 180° apart, with the shadowing angle quite oblique, the only portion of the substrate film that will not have received any shadowing material is that representing the area of contact between the particle and the film. If the icosahedral model is a correct one, this area should be an equilateral triangle. Fig. 8 shows the appearance of the substrate film after TIV particles have been double-shadowed and rubbed off. As can be discerned, the areas of contact are the blackest areas on the micrograph, and these have generally a triangular shape. Some of them are fair approximations to equilateral triangles. It is believed that this observation, coupled with those previously described, unambiguously demonstrates that frozen-dried particles of Tipula iridescent virus are icosahedral in shape. Since air-dried particles show a somewhat similar morphology, it is also most likely that the virus particles are icosahedra when in suspension.

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SUMMARY

The particles of the cytoplasmic virus of *Tipula paludosa*, named Tipula iridescent virus (TIV), are remarkable for the association of large size (130 mμ) with a high degree of uniformity of size and shape. When air-dried they have an appearance that suggests a polyhedral morphology. Following freeze-drying the TIV particles are uniformly hexagonal in contour. Owing to their large size it is possible to secure a knowledge of their polyhedral shape by analysis of the shadows cast by the particles. Both single and double shadowing have been employed, and a comparison made with the shapes of shadows cast by model polyhedra. It is concluded that the shape of the Tipula iridescent virus is that of an icosahedron.

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