MORPHOLOGY OF THE SEVEN T-BACTERIOPHAGES¹

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Received for publication April 9, 1953

The development of a new freeze-drying technique for the preparation of biological specimens for electron microscopy (Williams, 1953a) has led to the reexamination of the *Escherichia coli* phages T3 and T7 and to the observations that these supposedly spherical, tailless phages have short, stubby tails and a generally hexagonal cross-section (Fraser and Williams, 1953). These findings, coupled with observations on a few other viruses (Williams, 1953b) which indicate that the sizes previously reported might be substantially erroneous, have suggested the desirability of a complete electron microscope reexamination of the T-phages. In the present work all seven of the phages have been prepared under substantially identical conditions, examined biologically to establish their identity and viability in the final stages of purification, and photographed in the electron microscope following both air-drying and freeze-drying. The morphological details of the frozen-dried specimens are different significantly from those which have been observed using previous techniques.

METHODS

Cultures. The original stocks of the T-phages were obtained from M. Delbrück. For purposes of the present work each type of phage was grown in a synthetic medium containing glycerol and "casamino acids" as carbon sources and the usual assortment of inorganic salts (Adams, 1950). The only differences in the conditions of growth were necessitated by the requirement of T5 for extra calcium in the medium and the finding that the plaque titer of T5 seems to drop rapidly if aeration of the culture is prolonged after most of the bacteria have lysed. The phages were purified by differential centrifugation. It was found that the speeds which are required for T3 and T7 (15,000 rpm for one hour in the 40-rotor of the Spinco Model L ultracentrifuge) are

excessive for the rest of the set and result in the loss of the tails (and viability) of a large proportion of the phages. In general the minimum speed which will form a pellet should be used. We have found that one hour at 10,000 rpm for T2, T4, and T6, 11,000 rpm for T1, and 9,000 rpm for T5 is satisfactory. The high speed cycles were alternated with low speed cycles of 5,000 rpm for five minutes. In all cases the phages were resuspended in a minimum volume of their original supernatant fluid and the mixture was diluted slowly with whatever solution was desired. Generally we have used an isotonic ammonium acetate buffer for air-dried preparations and an isotonic ammonium benzoate buffer for frozen-dried preparations, but have found that at least some of the phages, as described later, can be resuspended in redistilled water provided that care is taken to dilute slowly. This is not safe with T5. The final purified suspensions were plated for viability in all cases—we can generally account for 80 to 90 per cent of the original titer—and were examined for identity by such biological tests as were indicated for the phage in question; i.e., the observation of the usual properties of host range and plaque type where applicable (Adams, 1950), and quantitative inactivation with antisera to distinguish T3 from T7, to distinguish the even-numbered phages from each other, and to identify T1 and T5.

Preparation for electron microscopy. Specimen mounts of both air-dried and frozen-dried viruses were prepared for electron microscopy. The material to be air-dried, suspended in either isotonic ammonium acetate or in distilled water, was deposited upon the collodion-film microscope grids in the form of minute droplets (Backus and Williams, 1950) from a Vaponefrin Nebulizer. The material for freeze-drying was suspended in either isotonic ammonium benzoate or in distilled water and was frozen-dried by the method described by Williams (1953a). There were no visible differences between particles which had been suspended in distilled water or in the appropriate isotonic buffer.

¹ Aided by a grant from the National Foundation for Infantile Paralysis.

RESULTS

The electron micrographs of the frozen-dried and the air-dried T-phages are shown in figures 1 to 12. The frozen-dried viruses are generally

show little or no evidence of a hexagonal shape, but the even-numbered ones show the distinct intimations of this kind of shape which have been seen previously.

Electron micrographs of the seven T-phages prepared by freeze-drying and by air-drying. Uranium shadowed. $\times 50,000$ for all figures.

Figures 1 to 6. T1 to T6, frozen-dried.

six-sided in projected contour; the odd-numbered ones sometimes appearing as almost regular hexagons, and the even-numbered ones having a hexagonal form with two parallel sides about 50 per cent longer than the other four sides. In the air-dried preparations the odd-numbered phages

A few detailed remarks will be given concerning the electron micrographs of the individual phage types.

The micrograph of the air-dried T1 phage (figure 8) is similar to those which have been obtained by others, and exhibits an essentially

spherical, but much flattened, head. The micrograph of the frozen-dried T1 particles (figure 1)

well, owing to the lightness of shadow, but they appear to be very slender and slightly curled.

Figure 7. T7, frozen-dried.

Figure 8. T1, air-dried.

Figure 9. T6, frozen-dried. The virus particle is seen standing on the end of its tail.

Figure 10. T4, air-dried.

Figure 11. T5, air-dried, deposited in 0.01 per cent gelatin.

Figure 12. T5, air-dried, fixed in 0.1 per cent formalin.

shows a preparation which has been shadowed very lightly to minimize distorting effects of the shadowing film. The shape of the head is distinctly hexagonal, with some evidence of an elongation of two sides. The tails do not show

The even-numbered phages are morphologically indistinguishable. A typical air-dried specimen is shown in figure 10. The head is seen to be a flattened form, approximately an ellipsoid, with a somewhat pointed end. As has been shown

by Herriott and Barlow (1952), the distinctness of form is enhanced considerably by formalin fixation. Their micrographs of fixed material appear quite similar to those shown here for the frozen-dried virus particles. The tails of the unfixed T4 phages, figure 10, evidence considerable unravelling into fine fibrillar structures, probably an artifact associated with surface-tension forces during drying.

Electron micrographs are shown of the three even-numbered phages as prepared by freeze-drying. As can be seen (figures 2, 4, 6) there is no observable morphological difference among the types. The head is seen as an elongated hexagon

made by the critical-point method (Anderson, 1952).

Only frozen-dried preparations of T3 and T7 are shown here (figures 3 and 7), since the air-dried forms are essentially spherical, and have been shown in a previous paper (Fraser and Williams, 1953). The frozen-dried forms are mostly hexagonal in contour with a short, stubby tail protruding from one point of the hexagon.

Two air-dried preparations of T5 are shown in figures 11 and 12. The former exhibits the appearance of the particles after drying from distilled water containing 0.01 per cent gelatin. The addition of the gelatin was found to be necessary

TABLE 1

Dimensions of the T-phages subjected to air-drying. The distortion of form and dimensions is demonstrated clearly by the low values of height-to-width ratios

(Dimensions in $m\mu$)

	T1	T2, T4, T6	T3	T7	T5
<i>Head</i>					
Previously reported*	50	80 x 65	45	50	100
Present determination	65	85 x 70	50	50	80
<i>Tail</i>					
Previously reported*	150 x 15	120 x 20	none	none	"distinct tail"
Present determination	150 x 10	110 x 15	doubtful	doubtful	180 x 10
Height-to-width ratio of head	0.4	0.6	0.6	0.6	0.4

* Information summarized by Delbrück (1950).

with a slightly constricted central region. The shape of the two ends is identical in those cases where the tail has been broken off. In figure 2, the particle at the far right shows this symmetry of form. (In this micrograph the central particle has above it a separate, broken-off tail, while the tail of the particle itself appears to be curled and relatively detached from the substrate film.) The tails of the even-numbered phages appear to be well integrated with a pronounced enlargement at the end. Figure 9 shows a T6 phage whose shadow indicates that it is standing on the end of its tail. This kind of orientation allows the cross-sectional shape of the object to be ascertained. This and other micrographs lead to the conclusion that the cross-sectional shape is six-sided and approximately hexagonal. The micrographs of the even-numbered phages shown here are in essential agreement with those obtained from preparations

to maintain attachment of head and tail upon drying. The head of the T5 phage is seen to be grossly flattened and spread out, while the ends of the tails appear to be frayed into three or four separate strands. The preparation shown in figure 12 was fixed in 0.1 per cent formalin for 30 minutes prior to spraying. The heads here are seen to be less flattened and show evidence of angular structure. The formalin apparently has reduced the degree of fraying of the tails as well as partially preserving the shape of the head.

Figure 5 is a micrograph of frozen-dried T5 phages. Many of the heads are seen as fairly regular hexagons with the tails appearing for the most part to be joined at one corner of the hexagon. The tails show no evidence of fraying but do appear to be curled.

The results of measurements of the sizes of the heads and tails of the seven T-phages are shown in table 1. Magnifications of the electron micro-

graphs have been determined by the use of polystyrene latex spheres (diameter = 260 m μ) which were added to the suspensions prior to spraying. The ratio of height to width of the phage heads has been determined by measurement of the shadow lengths; the local shadow angle is known from measurement of the shadow lengths of the spherical polystyrene latex particles.

DISCUSSION

Entrainment during sublimation. A problem encountered in the freeze-drying of the T1 and T5 phages is the preservation of the attachment of head and tail. It is found that lightly centrifuged (11,000 and 9,000 rpm) phage particles, when air-dried, exhibit almost complete head-and-tail integrity. When the same preparation is frozen-dried (Williams, 1953a) and sublimed at -40°C , however, the electron micrographs show well preserved heads but an almost complete absence of tails. Not only are the tails separated from the heads, they are nowhere to be seen in the preparations sublimed at this temperature. But when the sublimation is carried out at -65°C , the tails are found to be intact in about 80 per cent of the virus particles. We believe that the rate of sublimation of ice is sufficiently great at -40°C and warmer to entrain the dry, tenuously attached tails and remove them from the subliming surface. It is interesting to note that no difficulty of similar nature is encountered in the sublimation of the ice from the even-numbered phages, equipped as they are with larger and presumably more firmly attached tails. It is believed that the curled tails of the T1 and T5 phages are an artifact introduced during sublimation. Finely stranded material dried in the absence of surface tension is subjected to considerable distortion of form by random thermal motions at room temperature. An object as fine as a T1 tail literally waves in the breezes of Brownian motion until portions of it strike a solid surface, such as the collodion film, and stick where they happen to hit. The result is a randomly curved form.

Possibility of artifacts. An examination of the electron micrographs of the air-dried phages, and of the height-to-width ratios in table 1, discloses that considerable distortion of form has occurred, presumably through forces of surface tension incident to drying out of the aqueous suspending medium. The frozen-dried virus particles, how-

ever, have height-to-width ratios of almost unity, and it is probably safe to conclude that only very small surface-tension forces affected the particles upon sublimation. There still remains the possibility that the sharply angular contours are an artifact induced by the freezing process, a possibility particularly to be suspected in view of the hexagonal structures known to exist in ice crystals. There is some evidence, however, to discount the notion that the presently observed hexagonal contours of the phage particles represent this kind of artifact. The even-numbered phages have been shown to have a generally hexagonal form when prepared by the critical point method, and T5 has been reported also as having an "almost crystalline appearance" (Anderson, 1952). The T2 phage has been shown by Herriott and Barlow (1952) to be sharply angular when photographed subsequent to formalin fixation. As our figure 5 demonstrates, T5 also has strong suggestions of angularity when prepared by this type of fixation. In view of these similarities of form resulting from fixation methods other than freezing, it is considered unlikely that the hexagonal contours observed for all the T-phages are artifacts due to freezing.

Three dimensional form. A major question arising from the observations reported here is the true *three dimensional* shape of the phage heads. The electron micrographs indicate that the heads of the odd-numbered phages are fairly regular hexagons when seen in plan but do not furnish good evidence about the three dimensional structure which gives rise to this two dimensional projection. At first glance it might seem that the shadowing process would reveal the three dimensional shape. Different facets of the polyhedron make different angles with the shadowing direction, and one might hope that the resulting variations in thickness of uranium upon the several facets would reveal (by variations in electron opacity) their spatial orientations and hence the form of the whole structure. Unfortunately, this is not so; the opacity of the body of the phage head is appreciable and probably masks severely the opacity variations which might otherwise depend upon facet orientation alone. In addition, there may be an effect equivalent to a creeping of the uranium film, previously discussed by Williams (1952). At present then we can form an idea of the three dimensional shape of the phage heads only from observations of the

shapes of the shadows cast by the heads and from the geometrical consequences of the prevalence of the hexagonal two dimensional projections. It seems reasonable to assume that the phage heads would be most likely not to rest on one corner of their polyhedral form. The abundance of hexagonal contours implies then that whatever the three dimensional shape it must be such that when one facet of the polyhedral head is in contact with the collodion film the contour will appear hexagonal when observed along a line-of-sight perpendicular to that facet. The morphological consequence of the preponderance of hexagonal contours is that there are just three simple forms which the phage heads might have: an octahedron, a rhombic dodecahedron, and a hexagonal prism with bipyramidal ends. The first form is ruled out by the shapes of the shadows; an octahedron always casts a shadow having three linear edges, resembling the outline of wood-engravers' chisels, and further, an octahedral body will never be seen to overhang its shadow. The actual shadows observed are more complex in form, frequently exhibiting four or five linear edges, and the phage head generally overhangs the shadow in a direction perpendicular to the shadowing direction. The object at the top of figure 5, for example, casts a shadow having four linear edges, and the overhang at the top of the shadow is quite evident.

We are left then with a choice of two reasonable forms for the odd-numbered phages: the rhombic dodecahedron with twelve identical facets, and the hexagonal prism with bipyramidal ends, having eighteen facets. The former is a space filling form, while the latter is not. The three even-numbered phages could be of either form, also, since a rhombic dodecahedron distorted by a lengthening in a direction connecting two opposite, blunt corners will resemble the T2, T4, and T6 shape. But their shape is accounted for equally well by a hexagonal prism with bipyramidal ends in which the prism facets have been elongated. Work is now in progress which may establish the full three dimensional form of the seven T-phages.

Although the full meaning of the observed polyhedral form of the T-phages cannot be assessed at this time, we believe that this unique shape has significance with respect to the mechanics of growth and multiplication of the virus particles. Inasmuch as the T-phages represent

three distinct morphological types of virus, related originally only by their ability to grow on a common host, it is our prediction that all phages examined by methods which preserve three dimensional shape will reveal structures similar to those shown in this paper. Since the rhombic dodecahedron is a space filling form, and the hexagonal prism with bipyramidal ends is nearly so, it is attractive to speculate that the replication process might involve close packing at one of its stages.

Dimensions. An inspection of table 1 shows that the sizes obtained by us for the air-dried virus particles are in satisfactory accord with the previous determinations, except for the T1 and T5 phages.

TABLE 2
Dimensions of frozen-dried T-phages
(Dimensions in $m\mu$)

	T1	T2, T4, T6	T3, T7	T5
Head*.....	50	95 x 65	47	65
Tail.....	150 x 10	100 x 25	15 x 10	170 x 10
Height-to-width ratio of head.....	1.0	0.9	0.9	1.0

* Odd-numbered phages: distance between parallel sides. Even-numbered phages: maximum and minimum dimensions.

We believe that the dimensions given in table 2 for the frozen-dried phages are far more representative of the phages in aqueous suspension than are those listed in table 1. The greatest differences in the two tables occur for the even-numbered phages and for T5. The pointed ends of the former type are seen to be rounded off and retracted in the air-dried preparations, and this effect probably accounts for their decreased lengthwise dimension. The T5 phage is obviously flattened in the air-dried material, and it is not surprising to find that its head has much smaller dimensions when frozen-dried.

ACKNOWLEDGMENT

The authors wish to express their appreciation for the conscientious and excellent technical assistance of Miss Mary K. Brown, who was largely responsible for the preparation of the bacteriophage stocks.

SUMMARY

The morphology of the seven T-phages has been reexamined by electron microscopy of specimens prepared by normal air-drying from aqueous suspensions and by freeze-drying. The air-dried material shows evidence of considerable distortion of form, in accord with previous observations, and exhibits dimensions considered to be wholly unreliable. The heads of the frozen-dried phages frequently appear hexagonal in contour and have a height-to-width ratio near unity. Their dimensions listed in table 2 are believed to be the most reliable obtainable at present. Two types of three dimensional shape for the phage heads are considered to be likely: a rhombic dodecahedron, and a hexagonal prism with bipyramidal ends. No choice can be made at this time between these two likely forms.

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