Bacteriophage quaternary structure studied with laser-light diffraction of periodic electron microscopic images

Donald Ralph DiBona
Iowa State University

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DiBONA, Donald Ralph, 1938-
BACTERIOPHAGE QUATERNARY STRUCTURE STUDIED
WITH LASER-LIGHT DIFFRACTION OF PERIODIC
ELECTRON MICROSCOPIC IMAGES.

Iowa State University of Science and Technology, Ph.D., 1966
Biophysics

University Microfilms, Inc., Ann Arbor, Michigan
BACTERIOPHAGE QUATERNARY STRUCTURE STUDIED
WITH LASER-LIGHT DIFFRACTION OF PERIODIC
ELECTRON MICROSCOPIC IMAGES

by

Donald Ralph DiBona

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Cell Biology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

Chairman Advisory Committee
Cell Biology Program

Signature was redacted for privacy.

Chairman of Major Department

Signature was redacted for privacy.

Dean of Graduate College

Iowa State University
Of Science and Technology
Ames, Iowa

1966
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INTRODUCTION

While our knowledge of the faculties of cells or even entire organisms is not comprehensive, a sizeable measure of evidence has indicated that biological functions are rooted in the most fundamental organizational strata. The disciplines of physics and chemistry have, in recent years, provided biology with techniques that permit the study of macromolecules both as organic chemicals and as minute particles that can be characterized with regard to size, shape, weight and electronic charge. Proteins, in particular, have been extensively described; amino acid sequences, the relative configurations of these constituents and the interactions of the molecule with its environment have been determined in many instances. Ordered aggregations of these macromolecules are readily detected with the electron microscope; however, while physical-chemical studies indicate that the size of most proteins is not beyond the resolution limit of that instrument, monomeric subunits are rarely observed as the individual components of fully-formed structures. The development and successful application of procedures for the solution of this problem have been the fundamental objectives in this study.

X-ray diffraction studies have suggested that proteinaceous structures are essentially crystalline; a fundamental
unit of construction is periodically repeated to generate the final, functional structure. Further indications are that only a very few crystal types are to be expected and diffraction studies on viruses other than bacteriophage have, on occasion, resulted in the proposition of models fairly accurately depicting the arrangement of protein monomers in the completed structure. Since most bacteriophage are structurally more complex than plant or animal viruses, they are not readily prepared for X-ray analysis, and electron microscopy has contributed virtually all that is known about their morphology.

The study of subunit structure with the electron microscope has been made possible by the advent of routine negative staining procedures (Brener and Horne, 1959), but these techniques, like those of X-ray analysis, have been most successful with plant and animal viruses. The quaternary structure or disposition of subunits in bacteriophage is not readily discernible because the surfaces of these particles are apparently too smooth to allow the development of strong contrast. In this study we will show that phage substructure can be studied with the electron microscope when the negative staining procedure is modified to emphasize particular morphological aspects of the sample and that the rules for quaternary conformations suggested by research on plant and animal viruses are more generally applicable.
Close scrutiny of electron micrographs of biological specimens reveals that more information is contained therein than is immediately evident. The degree of resolution present is frequently obscured by various aspects of the preparative procedures, principally the lack of complete and precise staining and the presence of random densities or "noise" in the background; with negatively stained material these complications are most prominent. Our approach to cancel this hindrance depends on the assumption that suspected structural periodicities are indeed recorded on high resolution electron micrographs and on the fact that the presence of such regularities can be capitalized upon in structure analysis.

Various image enhancement techniques make use of the regularity of the structure being studied; we have selected optical diffraction since it provides the least ambiguous results and can be used for the testing of proposed interpretations. The techniques of Klug and Berger (1964) have been modified by the use of laser-light as the diffracted radiation. Laser light, chosen for its high intensity and extreme monochromaticity, has permitted rapid analysis of a large number of specimens while our attempts with more conventional light sources required extremely long exposures making extensive sampling prohibitive.

In the process of establishing our procedures as routine and reproducible, samples other than bacteriophage were used.
These samples selected for simplicity of analysis included bacterial flagella, tobacco mosaic virus, ciliary microtubules and the shafts and tips of trichocysts. While successes were not apparent in all of these attempts, they served to demonstrate the efficacy of the approach and to suggest additional applications of laser-light diffraction in electron microscopic studies. These ancillary studies have not been discussed in detail, but illustrative results are presented.

Bacteriophage are known to be propagated by transfer of their nucleic acid component to a host organism and to have a proteinaceous coat that is a sturdy, protective package for the genetic material during the extracellular, inactive phase of the viral cycle. Exactly what fraction of the capacity for nucleic acid transfer resides in the phage protein is not known, but evidence, accumulated here, shows that a non-destructive, structural rearrangement accompanies the transfer process.

While the functions of the systems studied are vital to the interpretation of observations, it remains essential to attain first a non-ambiguous picture of the structures in question. In some instances the models proposed in this study might be ideally suited to the attributed functions, but microscopic evidence of function is nonetheless circumstantial. Proposed structures must then be evaluated on the
bases of minimum energy considerations and the plausibility of simple, self-assembly mechanisms. Theoretical discussion of bacteriophage quaternary structures will draw heavily upon the experimental evidence from this study and that presented by other authors.
The purpose of this review is to present a survey of selected, significant articles that are representative of the extensive literature on virus structure. Since much of our knowledge of quaternary structure comes from research on plant and animal viruses, article selection has included research on the structure of those pathogens as well as on bacteriophage. It has been necessary to sample both X-ray diffraction and electron microscopic studies; precise subunit structure determinations have only been possible when a multiplicity of techniques have been applied. Thus, after examining the development of preparative procedures for the electron microscopic investigation of viruses, we will look in depth at the most significant studies on virus quaternary structure and enumerate other findings pertinent to the research of this treatise.

Technical Developments

The technique of shadow-casting or the vacuum-deposition of a heavy metal at a known angular displacement from the direction of observation was the first to be used in the electron microscopy of viruses. The best results with bacterial viruses have been obtained by Anderson (1946), Hall (1950), Williams and Fraser (1953), Kellenberger and
Arber (1955), Williams and Fraser (1956), and recently Kellenberger et al. (1965). Many other studies have been conducted on shadow-cast preparations of phage, but these have contributed little information when compared to that obtained by other preparative techniques. Combined replication and shadowing have been somewhat more fruitful; however considerably more skill is required for routine application (Horne, 1961).

Three-dimensional structure is best observed with frozen-dried preparations that are subsequently shadowed. In this procedure as described by Anderson (1952, 1954) the specimen must, nevertheless, be exposed to a rather hazardous chemical treatment. Williams (1953) devised a method for simultaneous spray-deposition and freeze-drying of particles, but specimens still required shadowing before observation. The primary limitation of the shadowing technique is the size of the resulting metallic grain which is very limiting in any but the most experienced hands; consequently it has rarely given high resolution results (e.g. Hall and Slayter, 1959; Depue and Rice, 1965).

The positive staining of biological particles has been somewhat more useful for the observation of substructure, but very few good staining materials have been found. It was experimentation with positive staining procedures, however, that led to the serendipitous discovery of the most recent
major advance in the electron microscopic investigation of small particles. This technique, negative staining, had been used extensively for the light microscopy of bacteria (Fleming, 1941).

In electron microscopy Farrant (1954) was the first to note reverse-contrast effects on phosphotungstate-stained ferritin molecules. Hall (1955), while positively staining tobacco mosaic virus (TMV) particles with phosphotungstate (PTA), noted that in some regions of his specimen grids viruses were not stained but were surrounded by the heavy metal. In another study Huxley (1957) observed that the virus was penetrated by PTA so that the axial hole was observable. In practice the negative staining procedure involves the embedment of the sample in an electron-dense matrix of a heavy metal glass. The procedure might better be labelled "PTA-embedment", but we shall comply with the convention adopted by the majority of other workers in this field.

Routine use of negative staining was not considered feasible until the work of Horne and Brenner (1958) and Brenner and Horne (1959) who used the technique with unprecedented success for the analysis of structural components of the bacteriophage T2 (Brenner et al., 1959). Their technique involved suspending particles in a volatile buffer, 1% ammonium acetate or 2% ammonium bicarbonate, and mixing
the suspension with an equal volume of 1 to 2% PTA adjusted to pH 7.0 with N KOH. The sample solution was then sprayed onto carbon- or nitrocellulose-coated grids to assure that the specimen had dried before positive staining was able to take place. They implicated virus concentration as the controlling parameter in the regulation of surface tension in the drying droplets; too high a concentration of virus revealed very little in the way of contrast effects, whereas too low a concentration resulted in dense spots of PTA that clouded the appearance of the sample to be observed. Recommended virus concentrations were about $10^9$ particles/ml.; their work with protein components suggested an optimum concentration of .1 to 1.0 mg. of sample/ml.

The first major modification in the negative staining procedure was that of Huxley and Zubay (1960) who did not apply the sample to the grid by spray-deposition. They applied formaldehyde-fixed ribosomes with a dropper, pulled off excess liquid with filter paper, washed the grid with buffer in the same manner, and then added a drop of PTA which was similarly removed. A further innovation in this study was the use of grids with perforated films allowing examination of particles trapped over holes.

Many minor changes in technique have since evolved and met with varying degrees of success. Principal among these has been the use of the uranyl ion instead of PTA. Results
with this heavy metal have been limited since there is an enhanced possibility of accidently obtaining positive staining; this is particularly problematic when dealing with nucleic acid-containing structures. Excellent results have been obtained with uranyl acetate (Fernández-Morán, 1962) and with uranyl formate (Finch, 1964).

The most successful results with viruses will be cited in the review of their morphology where the potential of the negative staining procedure has been most fully realized. Some outstanding results with non-viral particles have been achieved with bacterial cell walls (Thornley and Horne, 1962), bacterial flagella (Kerridge et al., 1962; Lowy and Hanson, 1965), mitochondrial elementary particles (Fernández-Morán et al., 1964) and cells and their associated virus (Almeida and Howatson, 1963; Parsons, 1963).

The most critical analysis of negative staining comes from Finch and Klug (1965) in a study of rabbit pappiloma virus (RPV). Adding negatively stained TMV to the reverse side of prepared grids and doing stereomicrography, they were able to determine that the dominant contrast contribution to the image originated from the side of the particle nearest the carbon substrate and that in most cases there were contributions from both sides of the particle. It will be seen that this phenomenon has usually complicated the interpretation of results but, in at least one case, it was used
to great advantage (Kellenberger and Boy de la Tour, 1965).

The possibility of further improvement in negative staining results and small particle observation in general seems to be dependent on our understanding the nature of protein surfaces and their interactions with electron-dense materials. In the next few years it is probable that improved methods for the development of contrast will enable us to take better advantage of the resolving power of the electron microscope.

Methods for facilitating image interpretation

The problem of inadequate contrast is the principal limiting factor in the observation of proteinaceous substructure in the electron microscope. Ruska (1965), in a discussion of the current status of electron microscopy, illustrated the enhancement of contrast in under-focussed images but warned against their use since the corresponding alterations in observed structure are great. The negative staining procedure has aided in the alleviation of the contrast problem, but it too has its limitations; the only structures that can actually be observed have been surrounded by enough of the heavy metal to provide contrast, and in many cases the heavy metal is not applied precisely where it is desired. Bradley (1965) summarized the view of many microscopists who believe that under-focussing is a great asset in the
observation of subunit structure. We can not classify underfocussing as an image enhancing procedure since, without a knowledge of the in-focus image, the results of underfocussing are not predictable and thus measurements of shape and size are not meaningful.

Galton (1878) showed that the effects of "noise" or random density distribution in photographic images could be effectively cancelled by the superposition of several identical negatives in the printing process. Markham and his co-workers (Markham, 1963; Markham et al., 1963; 1964) have applied Galton's reasoning to their own approach to the study of negatively stained images. Rather than using identical negatives, they have developed techniques for the superposition of periodic elements of virus particles. With the use of a stroboscopic flash and a rotating image they have successfully selected for various rotational symmetries by adjusting the speed of the rotating image and the flash rate of the stroboscopic lamp so that the image was effectively illuminated at successive, precise fractions of a turn, the reciprocals of these fractions being integral. The resulting effect was thus photographed. For the linear analysis of specimens, a comparable approach was used; the sample is attached to a contact switch so that moving the pair along a stage in a direction parallel to the particle long axis periodically triggers a strobe lamp and exposes
the film positioned in a vertically mounted camera. Thus, in both cases, images of segments of the structure were superimposed. When done precisely over the repeat spacing of the sample, meaningful structures were enhanced and random densities were averaged out of the background. Trial and error was found to be the only effective means of determining the correct distances for traverse, and, while results were invariably striking, they are suspiciously artifactual as was noted by the authors. The successes of Markham's group with these methods will be noted as virus structure is reviewed.

Gachet and Thiery (1964) have applied these procedures for "harmonic analysis" or "integration" with less sophisticated, manually controlled, sampling procedures on a variety of macromolecules, cellular organelles and viruses. Linear and rotational integration techniques have been applied in numerous other circumstances, although most results were not convincing.

A more precise procedure for harmonic analysis has been borrowed from the field of X-ray crystallography. The optical diffractometer provides a complete Fourier analysis of specimens in the form of a Fraunhofer diffraction pattern. Such instruments have been built for testing models of structures that have undergone X-ray analysis (e.g. Taylor et al., 1951; Hughes and Taylor, 1953; Wyckoff et al., 1957;
Taylor and Thompson, 1957). The idea of using such an instrument for periodic analysis of electron micrographs was first realized by Klug and Berger (1964) who used an optical diffractometer in an investigation of negatively stained actin, catalase and TMV. Finch et al. (1964), in the companion paper on the use of this instrument, have provided an additional application of the technique; the results of that work will be discussed in conjunction with the structure of the polyheads of T4 phage.

Optical diffraction with laser light has been reported by Nowick and Mader (1965) who taped negatives of hard-sphere models to the telefoto lens of a camera, illuminated them with a helium-neon (HeNe) laser beam and thus simulated the diffraction patterns found in the analysis of alloy thin films. The phenomenon of optical diffraction and the special case of laser-light diffraction will be discussed in the section on "Interpretation of Optical Transforms".

Virus Substructure

Tobacco mosaic virus (TMV) and quaternary structure

The history of structure studies on TMV closely parallels the development of biological progress in the fields of X-ray diffraction and electron microscopy. This pathogenic agent was the first virus discovered (Beijerinck, 1899); the
first isolated, purified and crystallized (Stanley, 1935); and the first to show evidence of subunit construction by X-ray diffraction (Bernal and Fankuchen, 1941). Electron microscopy has recently provided a view of the subunit arrangement in the TMV particle (Finch, 1964). Most of the current thought on many aspects of virus structure and function come, in fact, from research on this most exhaustively studied of all viruses.

While Stanley (1935) thought that TMV consisted of a single protein and a bit of ribonucleic acid (RNA), by 1938 Bernal and his collaborators (Bernal et al., 1938) had X-ray evidence that it was rod-shaped and probably hollow, and by 1941 Bernal and Fankuchen (1941) had established that it and other viruses consisted of an orderly array of apparently asymmetric units. These initial findings have proven to be the fundamental background for subsequent analyses of virus morphology; subunit construction has been found to be the case wherever structure analysis of viruses has been fruitful. It remained for Harris and Knight (1952) using techniques for the end-group analysis of polypeptides to confirm that the proteinaceous component of TMV was composed of chemically identical subunits of relatively low molecular weight. Since then the protein has been completely characterized through the efforts of several investigators to the point where the exact amino acid sequence is known, thus permitting a precise
statement of subunit molecular weight (17,531).

Physical-chemical studies on the TMV particle enable us to interpret the kinds of protein-protein interactions involved in the intact conformation. Caspar (1963) reviewed the studies on stability and assembly of the virus and summarized the evidence that the great stability of TMV depends largely on the protein-RNA interactions. The reconstitution of disaggregated TMV protein with and without RNA lends credence to the supposition of an in vivo, self-assembly of subunits (Fraenkel-Conrat and Williams, 1955) and statistical considerations of the self-assembly of subunit arrays (Caspar, 1963) make this mechanism seem much more feasible than the construction of whole viral particles by direct incorporation of individual amino acids.¹ A general picture of assembly has been developed where, with genetic determination of amino acid sequence, the remaining structural parameters are determined subject only to environmental conditions. The formation of secondary structure, which is the relative three-dimensional disposition of adjacent amino acids (e.g. the α-helical conformation), is apparently spontaneous as is the formation of tertiary structure or the relative positioning of the polypeptide chains. Proteins at

¹See Campbell (1965) for a comprehensive review of the current thinking on protein synthesis.
the tertiary level of organization are referred to as sub-units, aggregations of which are termed quaternary structures. Kauzmann (1959) summarizes the kinds of non-covalent bonding that are responsible for tertiary structures and relates how these forces may be instrumental in the formation of quaternary structures.

Attempts at the *in vitro* reaggregation of quaternary structures have been unsuccessful with other than rod-shaped particles except in the case of some enzymes where very few subunits are involved (Reithel, 1963). Since the periodic spacing of subunits generally falls in the range of tens of Angstrom units (Å), the study of subunit construction by electron microscopy requires the use of delicate preparative procedures. X-ray analysis is possible to the level of a few Angstrom units, but sample preparation is not always possible; when it is achieved, the information on quaternary structure is found by examination of low-angle diffraction patterns. The information obtained about this level of organization derives its primary significance from the ensuing implications about the development of viruses and sub-cellular organelles.

The disaggregated TMV particle only returns to "active" conformation under strict environmental conditions, and the reconstituted virus is infective only when TMV-RNA has been incorporated into the rod structure (Fraenkel-Conrat and
Protein subunits, monomers, in the absence of RNA can be made to aggregate into exactly the same conformation that they possess in the infective unit, but frequently an alternative structure will be formed (Franklin and Com- moner, 1955).

A discussion of the dimensions and the precise positioning of protein monomers in the TMV particle will then serve to illustrate how these parameters might be found for other viruses. Williams and Steere (1951) measured the length of TMV with shadowing techniques for electron microscopy and arrived at a statistical mean value of 2980 Å. Hall (1958), taking maximum care in purification, titration and microscope calibration, was able to calculate percentage error in his measurements and presented a figure of 3020 Å (±50 Å). Light scattering measurements (Boedtker and Simmons, 1958) also agree with a length of about 3000 Å for TMV while hydrodynamic studies, because of necessary approximations to an ellipsoid of revolution, yielded slightly greater lengths. That the rod has an axial hole, observable in the electron microscope, was first noted by Huxley (1957) in one of the first successful applications of negative

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1Sinsheimer (1957) provides a review of the findings that implicate nucleic acid as the genetic material; Stent (1963) and Bocciarelli (1965) survey most of the available information on the infectious cycles of viruses.
staining.

Using the theory of diffractions from a helix (Cochran et al., 1952), X-ray diffraction results with oriented gels of TMV have yielded helix parameters for the particle; these findings are reviewed by Klug and Caspar (1960). In any one strain of TMV the X-ray patterns are consistent, and Watson (1954) explained the positions of the significant reflections by postulating a non-integral number of subunits per turn of the helix. The axial repeat distance, or spacing over which a pair of subunits are completely superposed on the long axis of the helix, was measured to be 69 Å (±0.5%). Watson's proposal was that 3n + 1 subunits existed along three turns of the helix which then had a pitch of 23 Å. More precisely, Franklin and Klug (1955) have determined that the number per three turns is 49.02 (±.02%). Franklin and Holmes (1958) working with mercury-substituted TMV supplied by Fraenkel-Conrat showed that chemical and structural subunits were identical and that there were 2130 such units per particle. The RNA in TMV is arranged in a helix comparable to and within the protein matrix (Caspar, 1963).

Thus it was possible to schematize the picture of this virus, and such has been done by the late Rosalind E. Franklin; this diagram is reproduced in a great many places (see Caspar, 1963). The TMV particle is, then, about 3000 Å long and 180 Å in diameter. The protein subunits are arranged in
a flat helix and form 130 turns of 16.33 per turn. The rod has an axial hole with a 20-Å diameter, has a protein shell continuously grooved at a radius of 40 Å to a subunit thickness of about 8 Å, has in this groove a single strand of RNA with about three nucleotides per protein subunit and is apparently serrated since the protein monomers are tapered at their exposed ends.

In the electron microscope negatively stained TMV appears to exist in helices of both hands, and frequently the handedness seems to change over the length of the particle (Finch, 1964). This phenomenon is not an artifact of preparation but one that, nonetheless, complicates interpretation. Recently Finch and Klug (1965), in a discussion of contrast topography in negative staining, cite this phenomenon as an example of image contrast arising from either side or both sides of the particle. The crystallographer is unable to determine helix handedness; in some cases this information can be obtained by electron microscopy. Using precision replication techniques and tracing the orientation of the specimen from the grid to the resulting micrograph, Depue and Rice (1965) have proved that the helix of F-actin is right-handed; their procedure is exacting and has not yet been successfully applied to viruses. Markham et al. (1964) with their linear integration procedure have shown evidence that the TMV helix is left-handed.
The alternative, non-infective, TMV-protein conformation that forms on reaggregation is an array of stacked-discs or, more appropriately, a zero-order helix. Microscopy of these preparations is generally more informative than is that of particles in infective conformation; Nixon and Woods (1960) and Hart (1961) have shown cross-striations in these protein shells while Markham et al. (1963), with harmonic analysis, demonstrated sixteen subunits in end-on views of short segments. An integral number per turn is, of course, required by the stacked-disc configuration.

The most illustrative electron microscopic demonstration of TMV structure is provided by Finch (1964) who presents the only reported micrographs showing subunit array. Finch, using uranyl formate negative staining shows the three dominant helices of the particle (n=1, n=16, n=17 by the terminology of Klug et al., 1958). The intersections of the 16 and 17 series are, by definition, the subunits of the particle, and these are readily seen; the primary series, represented by the 23-Å striation is frequently observed completely across the particle, and the 23-Å serrations along the long edges are similarly obvious. Stereomicrography revealed that, where the particle diameter had increased from 180 Å to 200 Å, the evacuated specimen had been partially flattened.
Other rod-shaped plant viruses

Rod-shaped, single-stranded RNA, plant viruses other than TMV have, in a few instances, been found to possess the same general body plan. They have not been investigated in exhaustive detail, and chemical corroboration of preliminary findings is lacking. In an electron microscopic investigation of barley stripe mosaic virus (BSMV), Harrison et al. (1965) provided micrographs of uranyl-formate negatively-stained particles that showed a pronounced cross-striation spaced at 25-27 Å. They confirmed the presence of an axial hole reported by Gibbs (Gibbs, et al., 1963) who had, however, suggested cross-striations at 23-Å intervals. Finch (1965), in a low-angle, X-ray diffraction study on oriented gels of BSMV, found a pitch of 26.1 Å (+0.2 Å) and submitted evidence that the number of subunits per five turns was nearly integral. In the same report, tobacco rattle virus (TRV) was comparably analyzed; its helix pitch was recorded as 25 Å (+_0.5 Å) and there was indication of 3n + 1 subunits for every three turns as in the case of TMV. Markham (unpublished results reported by Finch, 1965) has stroboscopically analyzed end-on views of short segments of TRV and observed a 25-fold symmetry. Negatively stained TRV has shown striations across the particle spaced 25 Å apart (Nixon and Harrison, 1959). Beet yellows virus (BYV) filaments (Horne, 1959) have a similar appearance with electron-dense striations
spaced at a distance of 25 to 30 Å.

Although much work has been done with other rod-shaped plant viruses, it has been less informative. However, the results illustrate the value of uranyl formate as a negative stain and the simplification of the microscopist's problem when material is amenable to X-ray analysis.

Spherical viruses

The spherical viruses have similarly shown subunit construction. None of these has been studied with the completeness of TMV, but recently Klug and his collaborators published an authoritative pair of papers on the subunit structure of turnip yellow mosaic virus (TYMV) (Klug et al., 1966; Finch and Klug, 1966).

In a discussion of spherical viruses it is helpful to utilize the terminology of Caspar, et al. (1962) which might be paraphrased as follows:

Virus: the particle or any of its parts in any phase of the infectious cycle.
Virion: the fully assembled, complete, infectious particle.
Envelope: the membrane (perhaps from the host organism) which encloses the virion. This term will not concern us as this structure is not found in bacteriophage.
Capsid: the proteinaceous surface layer, made up of an array of identical subunits. In particles with complex morphology, like bacteriophage, the capsid refers to the entire proteinaceous surface.
Structure units: smallest equivalent subunits of the capsid - the structural building blocks of the helix or closed shell which constitutes the capsid.
Capsomeres: regularly arranged, morphological arrays of structure units; those arrays observed as units in the electron microscope.
Nucleo-capsid: the combination of nucleic acid and capsid.

A topological analysis of capsid morphology has been presented by Caspar and Klug (1962). This geometric consideration of virus structure was based on an appraisal of the problem in close-packing identical, asymmetric units on nearly spherical surfaces or in helical arrays and deals with observations by both X-ray diffraction and electron microscopy. Tomato bushy stunt virus (TBSV), TYMV (Caspar, 1956) and poliovirus (Finch and Klug, 1959) had been examined by X-ray diffraction and found to possess cubic symmetry which was actually resolved to 5:3:2 or isosahedral symmetry. The icosahedron is a closed polyhedron made up of twenty, equilaterally-triangular facets. Rotated about a vertex, the structure is repeated five times per rotation or once for every seventy-two degrees of arc; there are twelve such axes of rotation. The centers of the twenty triangular facets act as three-fold axes of rotation; the structure is thus repeated every 120 degrees. The thirty mid-points of triangle sides are two-fold axes. Thus, X-ray patterns of gels of randomly oriented spherical viruses show three superimposed spot-patterns reflecting these three symmetries. While the symmetry of spherical viruses is icosahedral, the actual shape may more nearly approximate a sphere and, as this was
the case with those first examined, they were thusly named. Caspar and Klug (1962) showed, with the use of models, that this configuration is geometrically feasible if the units of the shell are assymetric, structurally equivalent and bonded together in nearly equivalent (quasi-equivalent) fashion throughout the structure. This arrangement places all of the units in very nearly identical environments, is in keeping with minimum energy considerations, and makes plausible the assembly of a spherical virus shell from a two-dimensional hexagonal array or P6 net (Pawley, 1952). Crick and Watson (1956) suggested that the packing of identical units on an approximate sphere required cubic symmetry and that the number of units allowed was 60 or some multiple of that figure. This allowed for tetrahedra and octahedra with 2:3 and 4:3:2 symmetries respectively as well as for the 5:3:2 symmetric icosahedron. Tetra- and octahedra are harder to visualize in keeping with maximum, hexagonal packing considerations, but we shall see that some investigators find increasing evidence for the presence of octahedral forms.

Helical forms are more easily understood by this kind of reasoning. A regular two-dimensional net or lattice of equivalent forms can readily be bent into a cylinder with coincidence of subunits possible in an endless variety of positions; all such units are thus in exactly equivalent, immediate environments except those at the top and bottom
of the helix. Thus it becomes reasonable to assume (Caspar and Klug, 1962) the formation of helices as mistakes in icosahedron or sphere assembly, an explanation particularly attractive for the polyhead mutant of phage T4 (Epstein et al., 1963).

The theory of Caspar and Klug (1962) yields the following description of spherical capsid morphology. Capsomers are made up of either five or six structure units, depending on whether or not these arrays are centered on the vertices of an icosahedrally symmetric surface. With five structure units per vertex capsomere and six per surface or side capsomere, a consideration of the inscribed angles of an icosahedron dictates that the bonding angles between structure units will vary less than five degrees; this situation constitutes quasi-equality. In cases where the traced icosahedron does not position any capsomeres exactly on vertices, skew classes are formed which then have a handedness - dextro or laevo. Two consequences of icosahedral arrangements are then apparent. First, the number of capsomeres is not necessarily sixty but, rather, is confined to those values that are sums of some or all of the numbers 12, 20, 30, 60 or 60n, where n is integral; Horne and Wildy (1963) present Goldberg's diagrammatic proof. Second, the number of structure units will always be sixty or a multiple of sixty.
The consequences of icosahedral symmetry are supported by consideration of some actual capsomere number evaluations. By X-ray diffraction Finch and Klug (1959) showed that poliovirus and TYMV have identical icosahedral symmetries; thirty-two capsomeres have been determined for TYMV by Huxley and Zubay (1960) and, independently, by Nixon and Gibbs (1960) on the basis of electron microscopy. Humar wart virus (Noyes, 1964), SV40 (Bernhard et al., 1962) and polyoma virus (Wildy et al., 1960) all appear to have 42 capsomeres, twelve pentagonal and thirty hexagonal in gross outline; these shells should then be expected to yield 240 structure units as opposed to 180 for poliovirus and TYMV. Rabbit papilloma virus (Finch and Klug, 1965) has 72 capsomeres or 420 structure units and adenovirus (Horne, 1959) with 262 capsomeres would be expected to possess 1560 structure units. Adenovirus appears to be the most strictly icosahedral of all the viruses thus far investigated since it has visibly triangular facets in negative-stained preparations, and six capsomeres can readily be counted along a triangular base. Horne (1959) presents a model with 262 units (capsomeres) as viewed along a two-fold axis of rotation; his model is strikingly similar to the negatively stained image of an adenovirus capsid.

The number of structure units is then for all examined cases a multiple of 60 in keeping with the prediction of
Crick and Watson (1956). Hosaka (1965) has proposed a method for counting capsomeres by analysis of the ratio of capsomere separation to maximum capsid diameter. In actual practice this method would require greater measuring accuracy than is presently available; furthermore, it does not account for the presence of skew classes or the size differential between five- and six-fold capsomeres. Horne and Wildy (1963) have proposed an equation for the determination of capsomere number, but this too has limited use since it necessitates being able to count the number of capsomeres along a triangular edge. Despite the controversy over methods for determining capsomere number, there is general agreement with regard to the symmetry of spherical viruses.

**Bacteriophages**

We have looked broadly at helical and spherical forms of plant and animal viruses but have not yet mentioned the morphology of bacteriophage, the principal subject of this study. Relatively little is actually known about the quaternary structure of these extensively studied, pathogenic agents; capsomere configurations are rarely observed, and phage shapes are seldom simple spheres or helices. The majority of phage thus far observed are more complex than plant or animal viruses and possess both helical and polyhedral components that have been respectively termed tails
and heads. The general features of phage structure have been amply reviewed by Stent (1963).

**T-even bacteriophage**

*E. coli* B. is host to a series of seven virulent bacteriophage referred to as T-phage and numbered T1, T2... T7; the even-numbered members of this series T2, T4 and T6) have been studied more often with the electron microscope than have any other bacteriophage. Using shadowing techniques, Anderson (1946) described the head portion of the T-even phage particle as a bipyramidal hexagonal prism measuring 900 - 1000 Å in length and 600 - 800 Å in width. The tail structure appeared much more narrow, about as long as the heads, and was not well described at that time. In the next decade many other investigators, notably Williams and Fraser (1953), Kellenberger and Arber (1955) and Williams and Fraser (1956) examined shadowed preparations of the T-even phages. Kellenberger and Arber (1955) were able to show that the tails of T2 actually penetrated the walls of the host in the act of injection. It remained for successful negative staining to reveal more precisely the fine structure of the T-even phage particle.

Brenner and his co-workers (Brenner, *et al.*, 1959) separated the structural components of T2 and characterized isolable fractions by electron microscopy, ultracentrifugation
and protein finger-printing techniques. Intact phage showed head profiles suggestive of the bipyramidal hexagonal prism described by Anderson (1946); particles that had lost their DNA (ghosts) were shown to possess a 20-30 Å thick head membrane. The tails possessed a rod-like segment with a length of 550-600 Å, a diameter of 75-80 Å and an axial hole 25 Å wide termed the core since it was surrounded by another proteinaceous component, the sheath. Brenner's group determined that the sheath of T2 contained about 200 repeated subunits of approximately 50,000 molecular weight and no detectable N-terminal amino acid. The sheathed core was shown to terminate in a plate-like structure that has been termed the base-plate and was apparently hexagonal in outline. Proteinaceous fibers were found to originate from each of the six apices of the base-plate and measured 1400-1600 Å long and 18-20 Å wide; these fibers were apparently bent or "kinked" at about half their length.

The T-even sheath structure had been previously shown to contract after chemical treatment with cadmium cyanide (Kozloff and Henderson, 1955) or with hydrogen peroxide (Kellenberger and Arber, 1955) and after alternate freezing and thawing (Williams and Fraser, 1956). With the enhanced appearance of sheaths in their negative stained preparation, Brenner et al. (1959) were able to make accurate measurements of the dimensional changes occurring in contraction. They
found that contracted sheaths had pulled away from the distal end of the core, shortened to a length of 350 Å and increased in diameter from 165 Å to 250 Å; the diameter of the axial hole increased from 75 Å to 120 Å. It was extremely difficult to measure the length of non-contracted sheaths as the structure was not well resolved near the base-plate, and sheaths could not be isolated in non-contracted form. Thus they assumed that non-contracted sheath was as long as isolated core (about 800 Å) and computed that the volume of sheath material was constant at $1.3 \times 10^7 \text{Å}^3$.

End-on views of contracted sheaths (Brenner et al., 1959) revealed 15 subunits. Having deduced 200 subunits for the sheath structure by molecular weight determinations, Brenner and his collaborators postulated that the contracted sheath was a helix with thirteen turns; the extended sheath was believed to have had 24 - 25 turns since that many striations were seen by negative staining. Ultracentrifugation of tail fibers (Brenner et al., 1959) suggested a molecular weight of 100,000 or less; because of their observed shape, this weight suggested that they were indeed bundles of single chain α-helices which, at lengths of 1300 Å, would have a molecular weight of about 100,000. Anderson (1960) showed the presence of a "collar" or plate-like structure at the proximal end of the T2 tail which was confirmed by Fernández-Morán (1962) who also elaborated on the attachment of fibers to the
base-plate.

The T-even phage is then a complex of DNA and a great many proteins; head, sheath, core, base-plate, fibers and collar are all structurally obvious. Hershey (1955) detected an internal protein that could account for 3% of the phage sulfur; Levine, et al. (1958) found a different internal protein that accounted for 4-6% of the total phage protein; they also uncovered the polyamines, putrescine and spermidine. Still another polypeptide was discovered by Hershey (1957) and, independently, by Ames, et al. (1958). Koch and Dreyer (1958) confirmed that a lysozyme was a structural part of the phage; this enzyme had been reported previously by Koch and Weidel (1956) and Barrington and Kozloff (1956). Brenner and Horne (1959) and Horne and Wildy (1963) reported slightly adjusted values for the measurements cited originally, but the morphological diagram is not seriously different.

With a general view of the anatomy of the T-even phage particle, it is worthwhile to examine in more depth the controversies that have arisen over the morphology of the head and sheath.

T-even heads

The head protein of T2 was isolated by Van Vunakis et al. (1958) with duonol treatment at alkaline pH. Sarabhai and collaborators (Sarabhai, et al., 1964) found that one
cistron coded for the majority of the polypeptides which they had isolated from the T4 head. In a study of conditional lethal mutants, Epstein et al. (1963) observed an elongated structure which they termed "polyhead"; since other phage structures had apparently been made without error, they concluded that a mistake in head-protein synthesis had accounted for this variant structure that was apparently polymerized head-protein. Finch et al. (1964) examined this structure by optical diffraction of micrographs (Klug and Berger, 1964) and analyzed it as a flattened helix with a nearly hexagonal array of subunits, 70 Å center to center, on the visible surface. Bradley (1965) interpreted the variable diameter of observed polyheads to be a variation in the number of subunits per turn of the helix. While this does not seem to be in keeping with general rules for quaternary structure formation, it may be that some tertiary conformations permit a range of subunit aggregations. Furthermore, no one has produced any evidence as to the extent of outside control that governs head assembly, and as yet it is an assumption that quaternary structures are spontaneously formed in vivo.

Bradley (1965) also offers an alternative to the bipyramidal hexagonal prism concept for normal head morphology. By inference from negatively stained profiles, he proposes that the head surface is a bipyramidal hexagonal
anti-prism where top and bottom facets are out of register as in the case of an icosahedron. Moody (1965) took a more concerted look at the requirements for a plausible subunit structure in his analysis of the T-even head. He brought out the point that certain crystals do form bipyramidal hexagonal prisms and anti-prisms, but that these are always solid crystals. With all the interior molecules in equivalent environments, a variability in surface environment is a trivial departure from a minimum energy situation; such a departure is not allowed for surface crystals like the head membrane of the T-even phage particle. Moody proposed four general structures which might be inferred from the appearance of the head in electron micrographs. He pointed out that pentagonal antiprisms (like icosahedra) can more easily account for the various hexagonal profiles than can hexagonal antiprisms. After considering the presence of occasional multiple-tailed forms, icosahedral heads and the P6 sheet, polyhead structure, he chose a bipyramidal, pentagonal antiprism as the most likely model and the one that best accounted for polyheads as extensions of the equatorial portions of normal heads. Moody called this head shape a prolate icosahedron which signifies that it violates icosahedral symmetry by possessing isosceles rather than equatorial triangles in the equatorial positions. A capsomere arrangement is not proposed in detail, but there is evidence that his model could accommodate the 1800 to 2000
molecules suggested by the data of Van Yunakis et al. (1958).

Cummings and Kozloff (1962) claimed that a short-head form (apparently icosahedral) was required for nucleic acid transfer and that the elongated forms must change to the short form before injection can take place. They demonstrated this by reversible prevention of head shortening with polyglucose sulfuric acid. In thin sections Cota-Robles and Coffman (1963) observed that T2 particles which had apparently injected their DNA still had elongated head profiles. They also suggested that very little of the phage tail penetrated the cell wall in contrast to the findings of Kellenberger and Arber (1955). Whether or not the head membrane of the T-even phage particle is instrumental in the injection process is still unresolved, and proposed models do not account for the possibility of a "contractile" head. It is, of course, possible that various strains of T2 are quite different in their mode of action, but no discussion of this has been found.

Bradley (1965) adsorbed anti-T4 head-protein antibodies to intact T4 heads in an attempt to mark individual capsomeres and stated that a center-to-center spacing of 70 Å was thus indicated. While this spacing is in keeping with that measured on polyheads, these results can not be reported as conclusive since the micrographs presented were low in contrast and did not permit accurate measurements. Further it
would be expected that anti-head antibodies would attach to individual subunits if steric conditions allowed; there is no reason to expect capsomeres to act as antigenic centers. If a steric fit on adjoining subunits were not allowed, the closest possible packing of antibodies, measured at about 50 Å in diameter, might govern their final distribution.

An ingenious analysis of the polyhead conformation was presented by Kellenberger's group in a pair of recent publications (Favre et al., 1965; Kellenberger and Boy de la Tour, 1965). The first displayed intracellular polyheads in thin sections and provided a careful genetic and immunological analysis of the factors governing the production of polyheads. In the second paper polyheads were regarded as nearly, completely flattened helices. This assumption was demonstrably valid, and evidence that the surface was a rolled P6 lattice allowed interpretation of the resulting images as characteristic moiré patterns. This approach is highly feasible since Finch et al. (1964) had shown with optical diffraction that phosphotungstate was contributing contrast to the image from both sides of the particle. When both sides of the flattened helix approximate hexagonal nets, the angular displacement of the two can be detected by comparison of resulting images with those obtained from model nets

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1A moiré effect is achieved when two identical gratings or lattices are superimposed slightly out of register.
displaced at known angles; the calculated, angular displacement should then equal exactly twice the pitch of the helix being considered. Using this procedure, Kellenberger and Boy de la Tour found that the pitch of the helix was variable depending on the number of subunits per turn. While various helices were found, the diameter was shown to be constant within any one polyhead structure. The capsomere diameter was established to be between 70 and 80 Å and was occasionally observed to have a 20 to 40 Å central hole. Studies on buoyant density and chemical stability suggested that polyhead protein was not necessarily identical to that of normal T4 heads.

The T-even sheath

The sheath of the T-even phage has been the subject of as much experimentation and conjecture as has the head structure. Its contractile ability makes it an attractive target for studies on subunit conformation and rearrangement. Originally very cautious statements were made regarding the contractile nature of this structure; the contraction was explained as a disappearance of the tail's distal segment with a subsequent diameter increase of the proximal segment (Kellenberger and Arber, 1955; Kozloff et al., 1957).

Kozloff and Lute (1959) first demonstrated that a potentially reversible contraction was actually taking place.
Micrographs of shadowed preparations showed extended, contracted and partially contracted sheaths. Contracted sheaths could be induced to relax by the addition of 0.05 M adenosine triphosphate (ATP), and it was shown that each T2 particle contained approximately 115 molecules of ATP and 20 molecules of deoxy-ATP (dATP) bound to the tail protein. These high energy compounds were hydrolyzed to the nucleoside diphosphates and inorganic phosphate as the sheath contracted.

Brenner et al. (1959), as discussed earlier, have shown the contracted and relaxed states in PTA negative staining. Significant analyses of the structural rearrangements in sheath contraction have been offered by Bradley (1963a) and Kellenberger and Boy de la Tour (1964). Bradley proposed that extended sheath had a pitch of 40 Å which reduced to 33 Å in the contracted state and that extended sheath had six subunits per turn of the primary helix; the latter proposition was also asserted by Fernández-Morán (1962). More obvious than actual subunits in their uranyl acetate preparations are the striations across the long axis of the sheath in its non-contracted or extended state.

In an examination of normal and polymerized sheaths of T4, Kellenberger and Boy de la Tour (1964) deduced that "polysheath" found in their lysates was, in fact, an extended polymer of sheath material in a helical arrangement comparable to that of contracted sheath. Their observations
on extended sheath confirm the findings of Brenner et al. (1959) and Bradley (1963a) that specify 24 to 25 striations spaced at about 40 Å in negatively stained images. Since they never observed free or isolated extended sheaths, they concluded that this "poised" structure was not independently stable and that it gained its conformational integrity only by interaction with the core. The stable structure, they proposed, is contracted sheath that was readily found intact and apart from the core. The polysheath appeared to have the same surface conformation as contracted sheath and was equally stable. Polysheath seemed to have an affinity for free base-plates which were, however, never found at both ends of the structure. The model they proposed for polysheath is at least three interwoven helical bands each of which has a 30° pitch and two or three rows of subunits; contracted sheaths were postulated to be merely two turns of polysheath material. Bradley (1963a) had proposed that, in contraction, six units of one ring interdigitated with six of the ring above, and Kellenberger and Boy de la Tour postulated the same kind of eventual result based on a structural change in individual subunits. An allostERIC effect was invoked to account for the change in exposed chemical groups, and the ATP found by Kozloff and Lute (1959) was postulated as an allostERIC effector.

Anderson and Stephens (1964) showed degraded phage T6
treated with alkaline pH and suggested that degraded sheath material was a single helical strand. Similar observations were made by Brenner et al. (1959), Brenner and Horne (1959) and Bradley (1965).

In light of the accumulated evidence there is reasonable proof that the T-even tail has contractile ability; yet none of the proposed hypotheses for structural rearrangements accompanying contraction account for all the data, and there is little evidence that sheath contraction precludes the release of phage DNA.

Other sheathed phage

Additional phage that have apparent contractile mechanisms have also been described, but even the most illustrative of these do not offer much in the way of new information. Most of the implications of these findings are based on the comprehensive study of the T-even phage and show that slightly different structures might be involved in similar functions.

Williams and Fraser (1953) surveyed all of the T-phages of E. coli and found no contractile structures in the odd members of the series; these observations have repeatedly been confirmed notably by Bradley (1963a). A lysogenic phage of Bacillus cereus shadowed by Kellenberger (1957) was the first phage other than the T-evens to show evidence of a sheathed tail, but no contractions were noted. The first
phage to be discovered (Twort, 1915) and named for him is a lytic agent of *Staphylococcus aureus*; Vieu and collaborators (Vieu *et al.*, 1964), in a negative staining study, suggested an octahedral head for phage Twort and described a sheath structure that displayed longitudinal striations on its surface. The sheath was demonstrated in both relaxed and contracted stages without pronounced changes in visible surface structure. Phage S3K of *S. aureus*, another lytic agent, has been shown with contracted sheaths by Seto *et al.* (1956). Feary *et al.* (1964) have examined phages 7v, 7m and 7s of *Pseudomonas aeruginosa*; phage 7v apparently has a contractile sheath without demonstrated substructure. *B. subtilis* phage SP8 has a sheath observed in both contracted and uncontracted states by Davison (1963).

Frampton and Brinkley (1965) in studying lysogeny in *E. coli* derivatives obtained lysates of primarily incomplete phage, but among the resulting collection of phage components were sheath-core combinations comparable to T-even structures. The intact phage that they described did not all have sheaths, but all had apparently icosahedral heads. Four bacteriophage of *Vibrio coli* have been investigated by Fletcher (1965), all of which have sheaths, base-plates and apparently icosahedral heads; tail fibers were suggested for all four. deKlerk and co-workers (deKlerk *et al.*, 1965) studied several *Lactobacillus* phage that showed head substructure, while it was
not possible to count capsomereres on these apparent icosahedra. Micrographs of contractile structures in several different phage were presented. The phage effective against \textit{L. fermenti} showed clear base-plates with attached spikes; the phage of \textit{L. casei} have been described as having octahedral heads and base-plates that remained attached to contracted sheaths and were pulled along the tail core by the process. Sheath substructure was indicated for some of these phage, but calculation of helical parameters was not possible.

In an examination of a pyocin of \textit{P. aeruginosa}, Ishii and co-workers (Ishii, \textit{et al.}, 1965) found that this bactericidal substance bore a striking resemblance to the isolated cores and sheaths from T2 phage (Brenner \textit{et al.}, 1959). Chemical treatment of the pyocin revealed that the core and sheath proteins were conformationally stable under very different conditions. The sheaths were observed in both contracted and extended states; extended sheath had 35 cross-striations by negative staining, and the contracted structure showed twelve cogs in end-view. Evidence for base-plates and tail fibers was also presented. In agreement with the hypothesis of Kellenberger and Boy de la Tour (1964), they found no extended sheaths apart from cores, but such observations could have been impaired since these structures measured about the same length, 1200 Å. Upon contraction the change in dimensions by the sheath was comparable to the
T-even situation; the length was reduced to 460 Å, and the diameter increased from 150 to 180 Å. There was, in one instance, the possibility that the intersection of three apparent sets of striations on an extended sheath represented subunit resolution. The origin of contrast was not obvious in this preparation, and thus it was difficult to state that the image represented a single surface.

Reference to sheath and core assemblies as contractile structures is merely in keeping with current terminology. Chemical evidence of contraction, to our knowledge, has not been obtained for other than the T-even phage. A few additional citations on phage contractile mechanisms will be included in the "Discussion" section since they apply to the research of this study; other references on the tail fibers and cofactor requirements for the T-evens can be traced through Kozloff and Lute (1965).

The phage of Staphylococcus aureus

The bacteriophage effective against S. aureus have been studied by several investigators, principally Farrant and Rountree (1953), Vieu et al. (1964), Bradley (1963b), Rosenblum and Tyrone (1964) and Lapchine and Enjalbert (1965). The most recent and authoritative work on this group (Lapchine and Enjalbert, 1965) separates these phage into four morphological categories. Twort phage, S3K, 119, 130, 131
and 200 appear to be similar; all are virulent and possess contractile mechanisms comparable to those described previously. The temperate phage examined were divided into three categories, and none of these viruses possessed apparent contractile mechanisms.

Two of these categories contained phage with apparently icosahedral heads, 500-580 Å in profile diameter. These phage were classified on the basis of tail length; phages 29, 42d, 55, 75, and 77 had lengths from 220 to 2400 Å for their tail segments, and phages 52, 52a, 53, 71, 80 and 187 had lengths from 1500 to 1750 Å. Phage 53 was the only member of either series with a striated tail. The fourth category included phages 3a, 3b, 3c, 6, 7, 42e, 47 and 54 which showed extremely elongated heads and 75 by 3000-Å tails. The heads in this group measured from 400 to 550 Å wide and from 780 to 920 Å long; the length-to-width ratio of the profiles remained fairly constant at slightly less than two to one. There was little evidence for cross-striations on these tails and meager suggestion of an axial hole in those that had ghosted. Evidence was presented, however, for a "plug" or tail attachment site inside the empty heads while its observation was not clear enough to merit a discussion of size and shape; structures of this type have occasionally been seen elsewhere (e.g. Moody, 1965). Bradley (1963b) has observed tail striations in a number of lysogenic
staphylococcal phage.

The elongated head shape of some of the staphylococcal phage have also been noted by Rosenblum and Tyrone (1964) who observed them in phages 42c and 52a, while 52a has an icosahedral head in the preparations of Lapchine and Enjalbert (1965). Kellenberger (1957) observed elongated heads in a lysogenic phage of B. cereus, and Schmidt and Stanier (1965) saw them in preparations of Caulobacter phage OCB13. No one has presented a model for the subunit construction of these heads which are far more assymetric than those of the T-even phage. Moody (1965) has provided a diagrammatic sketch of an icosahedron, elongated with two extra rows of equilateral triangles equatorially disposed; this sketch approximates the shape of some of those phage heads just mentioned.

Other non-sheathed phage

Bradley (1963b) made the most recent observations on the phages of the T-series of E. coli and reaffirmed that the odd members do not have sheathed tails. Phages T1 and T5 were shown to have tails measuring 1500 Å and 1700 Å long respectively, and each tail was about 100 Å in diameter. The heads of both were presumably icosahedral with T1 measuring 500 Å in profile diameter and T5, 650 Å. Forty cross-striations were counted in negatively stained T5 tails, and there was
the suggestion of an axial hole in the tails of ghosted particles. T3 and T7 were morphologically indistinguishable, having head-profile diameters of 470 Å and tails only 150 Å in length. Comparing negatively stained profiles with those of a wire model, Bradley proposed that the head of T3 was octahedral.

Phage lambda (λ), the most studied in the analysis of lysogenic mechanisms in E. coli, was described by Kellemberger (1962) to have no demonstrable sheath structure; the tail dimensions were 1490 Å by less than 100 Å. The head shape of this phage, too, is apparently icosahedral with no resolvable subunit structure. Phage P22 of Salmonella is described as being very much like phage T3 and T7 (Bertani, 1958). Brock et al. (1965) found no morphological substructure in the tails of streptococcal phage while the heads were nearly spherical.

Simple phage

The rod-shaped phages have not been examined in enough detail to reveal evidence of subunit construction, but Bradley (1964) has observed a suggestion of an axial hole in isolated cases; this phenomenon has not been adequately described. The spherical or minute phage have been observed in ever-increasing numbers as interest grows in their mode of replication. Phage φX174 of E. coli has been the most
exhaustively studied; Hall et al. (1959) observed icosahedral symmetry in the twelve-knobbed surface of this phage.

Nucleic acid transfer

The classic blender experiment of Hershey and Chase (1952) showed that in the process of infection DNA was actually transmitted to the host while virtually all of the structural protein remained outside and was no longer required in the infectious cycle. This work has been instrumental in the development of thought that implicated DNA as the genetic material. From the standpoint of phage function, it has been reinforced by several authors who have repeated the work with tailed phage other than T2 and even with the rod-shaped phage, M13 (Salivar et al., 1964).

The problem of mechanics involved in nucleic acid injection has been attacked by many investigators (Stent, 1963), but it has been, perhaps, the least fruitful of all the areas of phage research; a review of the structural evidence has not made a solution obvious. Horne and Wildy (1962) contend that, even if the core of the T-even phage penetrates the coli wall, there is no evidence to necessitate DNA transfer. The adsorption phenomenon has been more successfully investigated, and it is well established that the attachment of the T1 and T5 phage is a two-step process, the first step being reversible (Garen, 1954).
The most significant recent paper on DNA transfer is that of Lanni (1965) who presents striking evidence that the actual transfer of nucleic acid is a two-step process for phage T5 and that the second step is dependent on protein synthesis by the host for its initiation. Evidence is also submitted that this intercurrent protein synthesis is induced by the first-step-transfer fragment of DNA which amounts to about eight percent of the T5 DNA content. Phages isolated by blending after having transferred this 8% fragment have lost their infective capacity. The first-step-transfer has been shown to be temperature dependent and, perhaps, dependent on the action of a lysozyme.

No clear-cut structural evidence for this type of process has been found. Analysis of all the micrographs reviewed here indicates that structural rearrangements implicated in DNA transfer can be made to take place with or without DNA loss. Similarly, DNA loss is noted in all of the described conformations of phage capsids but, in these cases, there is no way to discern whether the DNA was originally present or if the capsid was assembled without it.

Considering the great number of phage that have been examined with electron microscopy, there is very little evidence regarding quaternary structure. The structural studies on bacteriophage other than the T-evens have been characterized by a lack of detail, and interpretation has
often been characterized by neglect of the rules of symmetry and subunit packing that have evolved from research on plant and animal viruses. These rules should probably apply to all viruses and subcellular components of comparable shape.
MATERIALS AND METHODS

Bacteriophage

Samples of the temperate phages 6, 29, 42b, 52a, 53, 77, 80, 81 and 83 of Staphylococcus aureus and phages T4 and T5 of Escherichia coli were provided by Dr. P. A. Pattee, Department of Bacteriology, Iowa State University, Ames; the staphylococcal phage used and others of the International Typing Series have been described by Pattee and Baldwin (1961). Bacteriophage M13, active on male strains of E. coli (Hofschneider, 1963), were obtained from Dr. W. O. Salivar, Department of Bacteriology, University of Wisconsin, Madison. Each of the samples received was adequate for the preparation of several electron microscope specimens, and a variety of preparative procedures were used on each of those listed.

For the study of environmental effects on the quaternary structure of a sheathless phage, the staphylococcal phage 80 (Ø-80) was selected and was needed in large quantities. Ø-80 could not be successfully propagated in broth cultures, and usable titers had to be accumulated by the soft-agar, overlay technique (Adams, 1959) with several hundred Petri plates. The harvesting of phage was carried out by washing each of the plate surfaces with 10 ml. of sterile P and D (phosphate + dextrose) broth (Pattee and Baldwin, 1961), homogenizing
the soft agar layer with repeated mouth pipetting and collecting the "crude lysate". Partial purification involved spinning out bacterial debris and soft agar in a Sorvall RC-2 centrifuge equipped with a GSA rotor operating at 7000 rpm (ave. 6280 g) for thirty minutes. Phage were pelleted from the resulting supernatant by centrifugation at 30,000 rpm (ave. 78,410 g) for two hours in a Spinco L-2 centrifuge equipped with a 30 rotor. The pellets which were cloudy after this treatment, were resuspended in 10^{-3} M tris buffer with 10^{-2} M MgCl₂, pH 7.2, and the centrifugation steps were repeated. Clear, glassy pellets were obtained after the second high-speed centrifugation, were resuspended in small volumes of the tris buffer and stored at 4°C. Fractions of this sample to be used for experimentation were further purified by a repetition of the centrifugation procedure. The other phage examined were concentrated for electron microscopy by one to two-hour centrifugation at 30,000 rpm and were maintained in the media in which they were received.

Samples of Ø-80 were examined by negative staining after various lengths of treatment over a broad pH range (2.0 - 12.0); they were chemically treated in solutions of urea (.8 - 8.0 M), 10% Na-perchlorate, 10% H₂O₂ and modifications of the tris buffer described.

All the phage were examined both with and without
formaldehyde fixation; when used, this reagent was added to the suspending vehicle to a final concentration of 5 to 10% and specimens were allowed to fix for a minimum of twenty minutes.

Negative Staining

Considerable experimentation with various negative staining methods in the early phases of this study led to the development of procedures specifically appropriate for studying particular aspects of particle morphology.

Copper grids, 300 or 400-mesh Athene-type, were coated with formvar films, grey to black in interference color; grids were subsequently coated with carbon by vacuum evaporation. A range of carbon film thicknesses were used; heavier films are more hydrophobic and enhance the negative staining procedure while thinner ones, although less stable in the electron microscope, permit higher resolution work if treated very gently. Resolution did not appear seriously impaired by carbon layers that were noticeably grey and hydrophobic enough to permit routine negative staining. The use of chemically treated samples which were not in the best vehicle for specimen preparation necessitated the utilization of these heavier grids so that staining would be adequate for routine observation on the first attempt at microscopy. Formvar nets or perforated films, as used by Huxley and
Zubay (1960), were also tried, but particle dimensions were not constant from one sample to the next when specimens were trapped over holes; the use of these grids was discontinued.

**Phosphotungstate (PTA) methods**

Principal variations in the use of PTA involved pH, heavy-metal concentration and method of applying sample to the grids. A pH of 7.2 was found to give the most reproducible results and was used throughout the latter stages of the study. Stock solutions of PTA were made up at concentrations of 2% (w/v) in distilled, demineralized water and adjusted to pH 7.2 with N NaOH. PTA negative staining was carried out by the three basic procedures outlined below.

**Nebulizer methods**

Brenner and Horne (1959) suggested that spraying a mixture of equal volumes of sample and 2% PTA on carbon-coated films provided an amorphous dense background, thin enough to permit particle observation; further, they prescribed that the sample be suspended in a volatile buffer and that the wetted grid be dried quickly in vacuum to prevent PTA crystallization. In our hands, their technique was not universally applicable but was used both as reported and with many minor variations to outline particles, thus preserving their three-dimensional structure fairly well but
not delineating surface irregularities which for the bulk of the study were of primary concern.

Dropper addition of sample

Using the technique above with regard to PTA concentration and rapid evacuation of wetted grids, some samples were prepared by adding the PTA-particle mixture to the carbon-coated surface. This procedure resulted in heavy deposition of electron-dense regions where particle observation was possible only at the periphery of such areas. With lower PTA concentrations (0.5 to 1.0%), the technique was more serviceable since the matrix did not mask the particles to such an extent, but results were not readily reproduced. The addition of minute traces of bovine serum albumen (BSA) aided in spreading the PTA-glass over larger areas but added a graininess that confused interpretation of surface structure.

The most successful PTA procedure was a variation of the Huxley-Zubay (1960) method. Here the sample was deposited on the grid with a fine-bore pipette and allowed to stand for 10 to 60 seconds before it was withdrawn by touching the edge of the grid with bibulous paper. As the drop receded, phage particles were left behind on the grid surface. There was little suggestion of preferential particle alignment with this method. The sample droplet was not completely drained
but was only removed to the point where meniscus had vanished and grids appeared wet only by reflected light. Grids were then either washed by a repetition of the method outlined above with distilled water or buffer, or were immediately negatively stained. The washing step was only useful when the sample contained large amounts of non-volatile compounds. The staining was done in the same manner before grids had completely dried. PTA at a concentration of 2% was used when maximum contrast of particles and background was desired, and several consecutive drops of a ten-fold dilution of the stock were used to study surface structure.

**Uranyl formate negative staining**

The results of Finch (1964) with tobacco mosaic virus substructure indicated the efficacy of uranyl formate as the negative stain best suited to a study of quaternary structure. While several authors have reported the use of the uranyl ion, seldom have specimens been clearly negatively rather than positively stained. Finch (1964) and Leberman (1965) reported its use in concentrations of 1%, but we have found that successful results were most often obtained with very weak dilutions of 1% stock solutions. Staining was only possible when the pH (4.2) of stock solutions was unaltered and, for this reason, it was necessary to run controls for uranyl formate experiments with grids prepared by PTA.
procedures. The uranyl stain was used with all the methods described above for PTA, and again best results were with separate deposition of particle and heavy metal; optimum concentration was about 0.05%.

Freeze-dry negative staining

When water is very rapidly cooled to the range -150 to -200°C, it forms an ice with a crystal structure different than that usually encountered at the normal freezing point. The unique and useful aspect of this, much colder, ice, is that its density is comparable to that of water in the liquid state; thus we observe a liquid-to-solid transition with only minor changes in the water "structure". If a hydrated biological structure is quick-frozen to this very low temperature range and subsequently placed under high vacuum, the water is pulled off without apparent alteration of the disposition of structural proteins, nucleic acids and lipids, and with minimal rearrangement of the lower molecular weight moieties. The most convincing argument for this preservation of conformational integrity is that lyophilized preparations of bacteria, viruses and various subcellular particles regain their normal activities when rehydrated and returned to "active" temperatures.

Freeze-substitution techniques were not tried because the required vehicles are not good solvents for the heavy
metal salts. On the theory that the negative staining process, the actual apposition of heavy metal glass to the sides and internal surfaces of the specimen, can take place before the water of hydration of the specimen is lost, the following procedure has been used. Grids prepared in the fashion described above were affixed with sample, washed, and touched with a drop of 0.5% PTA, pH 7.2, which was quickly withdrawn with bibulous paper. The prepared sample was placed immediately into the electron microscope specimen-holder and the holder was plunged into a large volume (300 ml) of liquid nitrogen where it was held until the violent bubbling associated with the cooling ceased. The holder was then inserted in the microscope's specimen chamber and immediately pumped down. The transfer of specimen from liquid nitrogen to microscope was done as quickly as possible to prevent a high degree of frost formation and to assure that the specimen would be at least partially evacuated before its temperature had risen appreciably. The pumping was allowed to continue for thirty minutes to one hour before the specimen was examined, in order to allow the grid temperature to rise to a point where thermal stability could conceivably be gained during electron bombardment.

While the outlined procedure is rather direct, it must be pointed out that success with this technique was very limited; only one grid in 15 to 20 displayed both particle
preservation and good negative staining. This procedure was used to indicate the extent to which particles were flattened or distorted by non-freeze-dry methods.

**Electron Microscopy**

Specimen grids were examined with an RCA EMU - 3F electron microscope, equipped with a 5-mil condenser aperture and a 37 micron objective aperture and operated at an accelerating voltage of 100 KV. Instrumental magnification was always 29,900X as determined by periodic calibration with a crossed diffraction grating replica (Fullam, Inc., Schenectady) with 2160 lines/mm. Micrographs were recorded on Kodak Medium projection plates and enlargements were made on Kodak Kodabromide F4 and F5 and Agfa contrast-grade 6 papers. All measurements quoted were made on original plates as measured with a Nikon Profile-Projector (Model 6C, Nippon Kogaku, K. K., Japan).

**Optical Diffraction**

Extensive experimentation with photographic integration procedures for the analysis of periodicities in electron micrographs (Markham, 1963; Markham, *et al.*, 1964) led to the conclusion that these techniques were of limited value and frequently yielded ambiguous results. The demonstration by Klug and Berger (1964) of the possibility that very precise
harmonic analysis could be accomplished by optical diffraction suggested the applicability of this technique to the present study. An optical diffractometer was thus constructed after the basic design of Wyckoff et al. (1957); it utilized a Hg-arc (HBO 200) light source and lenses of four-meter focal length. Preliminary results indicated a satisfactory degree of precision, but the majority of our samples required exposures of several days; since extensive sampling was prohibitive, the use of this instrument was discontinued.

Attempts to circumvent the problem of long exposure time led to the development of procedures for optical diffraction in a standard light microscope. With proper condenser alignment and pin-hole light sources, the diffraction patterns (optical transforms) of highly ordered, high-contrast samples could be observed by positioning the specimen on the stage and viewing the back-focal plane of the objective with a telescopic eye-piece or by positioning the sample at the back-focal plane of the objective and observing the resulting pattern with a conventional ocular assembly. Gall (1965) has used the latter approach in a study of red blood cell microtubules. These procedures were discontinued because sample size was necessarily small and suitably intense, monochromatic light was lacking.
The use of laser emission was suggested by the extreme monochromaticity and intensity of such sources. A helium-neon (HeNe) gas laser (model TL-1, Bendix corporation, Cincinnati Division, Cincinnati) was loaned to us by Dr. P. H. Carr, Department of Physics, Iowa State University, Ames. This instrument has an optical power output of 0.5 mW from either end of the chassis; the emission from one end is a nearly parallel beam, 2 mm. in diameter, and that from the other is very slightly divergent. The laser light is spatially coherent and plane polarized; the wavelength is precisely 6328 Å.

Initial use of this source with the microscope assembly revealed the great sensitivity of laser light to any and all impurities in the optical systems. While this provides an excellent means for checking the quality and cleanliness of a lens system, it was virtually impossible to distinguish diffraction patterns from background for any but the most perfectly regular, high-contrast samples. The task was then to utilize a system with a minimum of optical surfaces, and an instrument more like a conventional optical diffractometer was thus designed.

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1 Observation of the laser beam as it is reflected by an apparently smooth, white paper card, reveals a bright red spot that appears to be "granular"; this phenomenon is due to combined diffraction and interference effects produced by the randomly oriented fibers in the card itself.
Using the diverging beam with the confocal mirrors adjusted to give a single, circular mode, the chassis was otherwise masked and positioned 2.86 meters from a front-surfaced mirror which then directed the laser emission down an optical train that included sample, focusing lens, reflection shield and camera in that order; all were mounted on a three-meter optical bench and supported by three-pronged lens holders on centerable mounts. The high intensity of laser emission makes critical alignment a routine procedure, and thus the instrument was realigned for each use. The general features of this diffractometer are diagrammed in Figure 1; lens specifications and exact dimensions are quoted there and in the corresponding legend.

In a conventional, optical diffractometer (Hughes and Taylor, 1953; Wyckoff, et al., 1957), an additional plano-convex lens is situated before the sample plane and at a distance from the source or pin-hole equal to its focal length; thus parallel light strikes the sample. While the beam is not parallel upon striking the sample in our assembly, we have found that a one-lens system provides clearer and more accurate pattern registration with laser-light. The use of a mirror is unfortunate but necessary with available facilities; sampling various areas on the mirror face located the "cleanest" surface.
Calibration

The use of parallel light striking the sample would enable the calculation of sample periodicities by measurement of spot separation in resulting patterns and geometric extrapolation to the corresponding Bragg angles since the focusing-lens determines only the position of pattern focus and pattern magnitude is dependent only on the wave length used and the specimen-to-film distance. With a non-parallel beam the instrument must be carefully calibrated for quantitative work. To this end 200-mesh electron microscope specimen grids were screened in the comparator, and those with the highest degree of order were selected for diffraction. The reciprocal magnification factor of the resulting optical transform allowed use of the diffractometer to determine the dimensional parameters of samples with otherwise unknown periodicities.

The section on "Interpretation of Optical Transforms" will provide details of the measurement of patterns, the rules governing the reciprocal relationship of sample and transform, and the general character of optical diffraction results.

Mask preparation

It was essential to use specimens with as high a degree of contrast as possible to obtain patterns with a minimum
"noise" level; in many cases suitable contrast was not present on original negatives. As we shall see in the following section, transform dimensions are not altered by changing from a positive to a negative image, and this conversion was used to provide images that permitted the smallest amount of undiffracted light. Selection rule considerations also enter into this choice. Highest contrast samples were obtained by a two-step photographic process: prints of electron microscope negatives were made on high-contrast paper (Agfa, contrast grade 6), and results were photo-copied on either Dupont Cronar film or Kodak Contrast projection plates. Loss of detail in this process can be minimized with care, and the resulting contrast and size of final images can be controlled to a high degree.

Silvered, lantern-slide, binding tape was used to frame the sample thus preventing light transmission or diffraction by the surround. Back reflections from the tape were unavoidable, but the absence of a collimating lens prevented the re-reflection of spurious light to the photographic film. The shiny-surfaced tape was used since it provided the cleanest edges, smallest thickness and greatest opacity to high intensity laser-light.

Optical path differences in the photographic emulsion necessitated the mounting of specimens in immersion oil between optically flat cover-slips; the necessity of this
procedure was first noted by Bragg and Stokes (1945) and has since been confirmed by many others notably Wyckoff et al. (1957) and Klug and Berger (1964).

Model patterns were constructed for demonstration of diffraction phenomena and testing structures suggested by micrograph analysis; for reasons of accuracy, these models were drawn on a large scale and subsequently photographically reduced to a usable size. A fully-framed, immersed photographic image represents and will be referred to as a "mask".

Photography

The photographic assembly consisted merely of a lens holder and a Tri-X film pack (Kodak). The film pack assembly slipped securely into the rigidly positioned holder and could conveniently be mounted or returned to a light-tight container in total darkness. Exposures were of the order of one minute and could be timed within two or three seconds by shuttering the beam with a black cardboard box that securely fit the exit end of the laser chassis; the relatively short exposure times made apparatus stability problems negligible. It was not necessary to build a light shield for the diffractometer since it was assembled in a window-less laboratory that could be totally darkened. Exposed films were processed for 12 minutes in Kodak Microdol X developer.
Diffraction patterns presented are contact prints of originals or slight enlargements. Only originals, however, were used in measuring the positions of reflections. Dimensions quoted on patterns relate to actual spacings in the specimens used and account for electron microscopic magnification and the alteration of that value in the construction of masks.
Figure 1. Line drawing of laser-light diffraction apparatus (not to scale). Total distance from exit point on laser chassis to mask is 376 cm.; mask to lens distance 6.0 cm., lens to film 163 cm. Position of the shield is not critical provided it is within 10 cm of the plano-convex focusing lens. In the alignment procedure, the first-surface mirror and the laser are mutually adjusted to direct light directly along the optical bench on which all components but the laser itself are mounted. Location of a clean surface on the mirror is done by observation of the focussed light source at the film-plane when there is no mask in place.
HeNe laser

shield
lens
mask

mirror

film
INTERPRETATION OF OPTICAL TRANSFORMS

Descriptions of simple diffraction phenomena are numerous; however those that deal with the problems of this study require a substantial understanding of the mathematical bases of crystallography. The microscopist can utilize the information obtained by optical diffraction procedures, provided he understands the basis of the diffraction process and is willing to investigate further the special cases encountered in the harmonic analysis of typical structures recorded by the electron microscope. The purpose of this section is to illustrate those diffraction phenomena essential to the interpretation of the optical transforms obtained here and also others derived from comparable studies. A clarification of the interpretation procedures that led to the conclusions of this research will provide more common ground for the discussion of results. The special case of laser-light diffraction permits the demonstration of large-aperture phenomena which are generally discussed only in diagrammatic or mathematical terms. It will be assumed that a knowledge of the wave-propagation of light and the situations that lead to constructive and destructive interference are understood.

The term diffraction (breaking-up) was first applied in 1661 by Grimaldi who observed that the edges of shadows from
point sources were not perfectly sharp. This phenomenon was not understood in Grimaldi's era since the foundations of geometrical optics had just been laid down by Isaac Newton who treated the propagation of light as a rectilinear transmission of corpuscles. Huygens' theory which could explain Grimaldi's observation was not accepted even after Thomas Young's interference experiments in 1803. The conclusive experiments of Fresnel with his bi-prism, and the invention and use of the diffraction grating by Fraunhofer finally made plausible the science of physical optics - the phenomena related to the wave nature of light, principally interference and diffraction. The equations of Maxwell and the eventual quantum mechanical treatment of light did not change the concept of diffraction, but the discovery of X-rays allowed it to be applied in a manner that has since constituted one of the most precise methods of experimental physics. The advances in X-ray diffraction, due largely to the findings of Laue and the Braggs, are reviewed by Lipson and Cochran (1953). The derivations of equations that demonstrate the dependence of the diffraction phenomenon on the wave length of radiation used are provided by the authors cited above and, in simpler form, by most textbooks in introductory optics.

The optical diffractometer described in the preceding section allows us to demonstrate the way in which
characteristic diffraction patterns are formed and some of the rules governing final intensity distribution. The samples used do not have 100% contrast, and there will be evidence of some non-predictable reflections on the transforms presented. Ordinarily optical diffractometry is done on samples made from holes punched in opaque card (Hughes and Taylor, 1953) with a pantographic punch. While that kind of instrument was not available to us, laser-light has offered comparable advantages.

The general features of all optical transforms are similar. Undiffracted or zero-order diffracted light will be focussed on the optical axis of the assembly at the point that will be referred to as the central spot of the pattern.\(^1\) Light that is diffracted from regularly arranged apertures forms families of beams. Each family consists of a parallel array of beams travelling from the apertures in a given direction; the pattern would then be focussed only at infinity were it not for the long focal-length (163 cm.) plano-convex lens which focusses each family at a particular spot on the film. Each of the resulting spots or reflections

\(^1\) In the photography of strong sources of light, film halation from this spot is virtually impossible to prevent; in X-ray diffraction a lead stop is routinely used to block the registration of the central spot but, with proper selection of sample contrast, the halation of the central spot has not seriously confused our results.
thus corresponds to a particular direction of light scattered by the sample or mask. An important consequence of the origin of reflections is that each is made up of individual contributions from each of the apertures; thus every spot on the pattern contains information about every aperture in the mask, and an irregularity in any of the apertures or scattering centers can significantly alter the sharpness of the transform.

Realizing that longer wave lengths of light are diffracted more or are scattered at broader angles, we can appreciate the need for monochromatic light. Figure 2 is the optical transform of a 200 mesh, electron microscope, specimen grid as registered in the back-focal plane of an objective lens in the microscope assembly previously described. A pin-hole source of white light from a tungsten bulb was used. Note that the diffractions are streaked away from the central spot. If the photograph were in color, the effect of spectral spreading would be more vivid, but the illustration has been labelled to describe the direction of increasing wave-length. A more important consequence of this illustration is that diffractions arise from the central spot; meaningful measurements must be made from the center of the pattern. These conclusions will be better appreciated when the same sample is diffracted by laser-light; in those transforms the characteristics of the pattern from a very
The lines of a diffraction grating are parallel and uniformly spaced. The diffraction pattern of such a grating is a linear array of spots which are spaced along a line perpendicular to the lines of the grating; the distance between spots on the pattern is reciprocally related to the perpendicular distance between the lines of the grating, i.e. gratings with 30,000 lines/inch give rise to patterns with spots spaced three times as far apart as those from a grating with 10,000 lines/inch. A spot pattern, then, not only corresponds to a direction of symmetry but also provides precise information about the translational separation of apertures in the sample. We have referred to the central spot of the pattern as the zero-order diffraction; by convention the spots are given consecutive, integral, positive order designations as they proceed to the right or above the central spot; reflections oppositely positioned are assigned the corresponding negative values.

The principle of the phase microscope serves to point out still another characteristic feature of optical diffraction results. Each element of the source is diffracted separately by the sample as is each wavelength which was noted in Figure 2. The sum of these reflections will provide an image of the light source with the zero-order reflections comprising the strongest image. In the phase microscope the
Abbe theory of image formation is fundamental to operation. The effective light source used is an annulus or ring that is focused on the back-focal plane of the objective lens. The diffraction pattern of a biological sample is seldom highly enough ordered to permit observation of its optical transform which is also registered in this field plane; the individual diffractions, containing the information required for image reconstruction by the ocular, take the shape of annuli. This shape has been chosen so that the zero-order reflection can be selectively retarded by a matching annulus positioned in this field plane without excessive overlap of the significant diffractions. Information about large spacings in the sample will be recorded close to the central annulus and overlap this image in many cases, but information about the very smallest separations will be displaced furthest from the center and thus eventually show the best phase contrast; however this may not be evident if such spacings approach the resolution limits of the microscope. The importance of large numerical aperture in the objective and the use of low-range wavelengths for gaining optimum resolution is similarly understood since these conditions permit more of the optical transform to be registered on the back-focal plane of the objective for subsequent image reconstruction. The ocular optically transforms the image on the back-focal plane of the objective, and image formation may be viewed
as a double-diffraction process as it was by Abbe. It is tempting to think of the possibility of reconstructing images from photographed optical transforms, but this cannot be done since information about the relative phases of the reflections is lost when the diffraction pattern is photographed. The complications of the phase problem are reviewed by any of the explanatory treatments of X-ray diffraction procedures in the literature.

A consideration of source-shape effects indicates that a pin-hole source is the obvious selection for precise measurement of transform dimensions. The shape of individual apertures in the sample is not as easily controlled, and the effects of various aperture sizes and shapes must then be considered. The effect to be demonstrated is that the shape and size of individual apertures governs the overall intensity distribution of the transform. Very small apertures yield very broad scattering envelopes; the pattern intensity from one spot to the next falls off gradually as their order assignation increases. Larger apertures give rise to transforms where the intensity decreases periodically from the center, the overall decrease being more rapid with larger aperture size. This phenomenon and its basis are referred to as selection rule considerations. Individual apertures and arrays of apertures will be considered in that order.

Figure 3 shows the optical transforms obtained when
various simple masks serve as individual apertures. In 3a, a circular mask or hole is the source of diffractions which take the shape of concentric rings. This pattern reflects the only symmetry present in the mask; the periodic placement of maxima and minima along a radius is a measure of the scattering envelope of intensity. Figures 3b, c, and d are the optical transforms of rectangular apertures and depict two orthogonal series of reflections arranged perpendicularly to the sides of the rectangle from which they arose. The shape of the individual reflections along the vertical axis (meridian) and those on the horizontal axis (equator) are lines rather than spots. This is true for all three of the transforms since all have the same kind of symmetry. The shape of the spots is also a function of the mask since each diffraction on the meridian will carry satellites, right and left, indicative of the equatorial spacing. In the cases pictured here, the equatorial separation of spots is not resolvable, and the satellite diffractions serve only to elongate spots horizontally. Since this aperture shape is optimum for framing most of the elongated structures used, it is well to understand that the kind of effect just observed will be present on the diffraction pattern of samples so framed. Frames will be necessary since it is essential neither to let too high a percentage of the incident beam pass the sample undiffracted nor to have the
diffraction pattern of the surrounding region registered on the pattern of primary interest. The most significant features of the rectangular aperture's transform are: 1) that the short side or width of the mask gives rise to a distribution of spots whose intensities fall off slowly as they progress along the equator in either direction from the center, and 2) that the longer diameter of the rectangle (a longer spacing) is represented in more closely spaced lines along the meridian; these spots diminish much more rapidly than those orthogonally displaced to them. The broad appearance of the meridional spots is similarly due to the persistence of intensity in the horizontal direction. If the rectangle is considered to be a pair of orthogonally arranged slits, we can visualize the effect of slit size on intensity distribution. The presence of the three pictures (Figures 3b, c and d) makes this more lucid.

The size of the scattering envelope becomes extremely important when considering a regular array of apertures. Arranging apertures in periodic fashion, effectively forming a lattice, is of great interest to this particular study since lattices are to be examined in detail. To reiterate, the overall intensity of the pattern will be a function of the size and shape of individual apertures, which we shall assume are identical, while their distribution will yield a pattern of characteristic symmetry dependent upon their
arrangement. Figures 4 and 5 demonstrate the two-dimensional case. The positive and negative images of a 200-mesh grid and their respective optical transforms show striking characteristic differences while the patterns have comparable symmetries. Note that in the first case (large apertures) the spot distribution is square but spot intensity does not diminish progressively as order increases along a row from the zero-order diffraction. Rather, although the decrease is progressive from first to second to third orders, the fourth-order diffractions show an increase over the third. The transform of the negative image (small apertures) shows identical symmetry, but here intensity decrease is progressive and continuous to the observable extremities of the pattern. Available film size limits the extent to which the pattern can be photographed, and it is assumed that if we were to sample further out we would begin to observe the imposed selection-rule effects imposed by this size aperture.

The fact that symmetries are identical for both positive and negative images is of practical value in the diffraction of electron micrographs. Most of the structures in which we are interested are formed of close-packed globular proteins which are large apertures compared to the spaces that separate them. Heavily negative-stained preparations alleviate this effect somewhat by adding electron density along the sides of the globules and even partially masking them, but the
Figure 2. Ten-fold enlargement of optical transform of a 200 mesh (200 lines/inch) electron microscope grid (see insert for shape and relative orientation of grid). The arrow heads in the upper right quadrant of the pattern describe the direction of increasing wave-length. This transform was registered in the back-focal plane of a compound microscope objective lens with white light as described in the text.
Figure 3. Demonstration of framing effects. Figure 3a is a laser-light transform of a circular aperture (insert shows reversed contrast enlargement of mask used). Circular or annular masks give rise to diffraction patterns of the type presented here. The spacing of the concentric rings is inversely proportional to the hole's diameter. Contact print (actual size) of transform. (This and all subsequent transforms shown are in reversed contrast and have been produced with the laser-light diffraction apparatus described in "Materials and Methods" and in Figure 1.) Figures 3b, 3c and 3d are transforms of various rectangular apertures (inserts are corresponding 4-fold enlargements of masks used). Equatorial and meridional density distributions and the rate of intensity decrease are clearly related to mask shape; narrow slits give rise to larger spot-to-spot spacings and broader scattering envelopes. This type of transform will be superimposed on the meaningful diffraction pattern of all samples that are framed with rectangular apertures. Transform enlargement: X 2.0
negative image of such preparations will almost always provide smaller aperture sizes. The effect of reversed contrast will be considered again when we take up the special case of helical arrays.

Figure 6 shows a longer exposure of the transform recorded in Figure 5 and demonstrates better the fact that observed spots are merely relative maxima of a complex but asymmetrical distribution of intensity. The information contained in this background is more subtle than the mere spot distribution on the pattern but is particularly useful as an indication of the precision of placement and size of the scattering centers or apertures. Since this precision is not very high in the case of negatively stained images, we shall find that this background becomes very asymmetrical and an annoyance. There is an indication that the spatial coherence of laser light makes these transforms more subject to this kind of pattern registration than they would be were the light multiphasic. The concentric circles in the center of the pattern undoubtedly arise from the annulus that surrounds the square array of the sample.

The positive image and transform of a 75 by 300-mesh grid is shown in Figure 7. The effects of aperture size and shape, the rule of reciprocal relationships, the effect of a circular frame and the combination of these factors are summarized in this illustration.
Figure 8 (a - d) shows the effects of further complication of the simple lattice transform by the addition of major discontinuities in the pattern. Close analysis of the two-grid situation, Figure 8b, reveals that the grids are diffracted independently; i.e. each gives rise to a pattern identical to that for a single grid, and no new spots are found; the two transforms are cleanly superimposed, and inter-lattice interaction is not indicated except in the central array that reflects the two annuli and their separation distance. The three-grid case is similar, no new spots being displayed. Figure 8d is the case for overlapping grids, and the inter-lattice interaction is represented by a family of new reflections. These spots are not very prominent since they do not arise from as extensive a periodic array as the apertures in the individual grids.

Observed Structures

The applications of this method to the interpretation of electron micrographs are numerous since periodicities are prevalent in biological structure. The true advantage of this technique is realized when periodicity is only suggested or, indeed, only suspected. If the image is high in contrast and does contain a regular distribution of apertures, optical diffractometry can accentuate the periodic aspects of the structure. As we have seen, the optical
Figure 4. Transform of 200 mesh electron microscope grid (insert is enlargement of mask used but printed in reverse contrast). Actual measurements on the 200 mesh grid revealed that there were 77.94 lines/cm. This spacing gave rise to reflections spaced at 7.133 mm. on the transform. For the sake of calibration, therefore, 1.00 mm. on a transform corresponded to 0.915 mm. on a mask (1.00 mm. on mask corresponds to 1.092 mm. on transform). Intensity variation along the major axes of the pattern is evidence of the shape of the scattering envelope from the individual open squares of the grid. Contact print of transform as recorded, two-second exposure.

Figure 5. Transform of a reverse-contrast 200 mesh grid image. Spot distribution is identical to that in Figure 4 but shape of the scattering envelope is broadened since effective apertures are now very fine lines rather than squares. Contact print of transform as recorded, two-second exposure.

Figure 6. Longer exposure of sample used for generation of Figure 5. The intensity of all spots is increased, and the complexity of the pattern arising from the annulus around the grid and its interaction with the grid lattice is readily observed. Contact print of transform as recorded, five-second exposure.

Figure 7. Transform from positive image of 75 x 300 mesh grid (insert) demonstrating the reciprocal relationship between lattice-spacing and reflection distribution and the different shape of the scattering envelope in the two directions. Contact print of transform as recorded, two-second exposure.
transform of an image represents the regular and irregular aspects of the image in very different fashion. The significant diffractions may be selected, analyzed and extrapolated back to the image itself; this procedure is the principal experimental method of this study. To apply it meaningfully, in this study or any other, it is necessary to have a sound appreciation for the structure of electron microscopic images and how they are related to the structures they represent.

The preparative procedures for electron microscopic examination of small particles have been repeatedly held accountable for the apparent distortions in examined samples; there is little disagreement about the presence of harmful effects in evacuation and electron bombardment of specimens, and the extent of these deleterious procedures is the subject of much conjecture. Proper application of optical diffraction can quantitatively estimate the degree of distortion induced in those structures where perfect regularity is predicted by both X-ray analysis and theoretical considerations of subunit structure. The negative-staining process has been discussed by most of the investigators who have utilized it; Finch and Klug (1965) (see "Literature Review") have successfully located the origin of contrast, and Klug and Berger (1964) have indicated that optical diffraction can differentiate the sides of the sample when contrast
originates from both faces. Particle flattening due to evacuation is the greatest hindrance to interpretation since its degree is very hard to estimate. Visual measurement of the variation in particle diameter is helpful because it provides a statement of the range over which particles are flattened. Optical diffraction should then be conducted on both seriously flattened and apparently erect particles of about the same contrast. A comparison of the resulting transforms and notation of the shift in spot distribution can compensate for the lack of non-ambiguous information that would be obtained if the particles were perfectly preserved.

Apart from considerations of particle flattening, images obtained from the electron microscope should be regarded as two-dimensional projections of three-dimensional structures. Aperture spacing along the optical axis is not easily simulated in diffraction as it is used here, and therefore the optical diffraction of a projected test structure is not identically analogous to the X-ray diffraction of a real structure.

Minor amounts of disorder in periodicities will be averaged out by the diffraction process since each reflection on the pattern arises from all of the apertures in the sample. An optical-averaging procedure for spacings is provided which is appreciably less subject to human error than careful measurement over a large number.
of repeats.

Transforms of Helical Arrays

X-ray analysis of helical structures has provided very accurate determinations of subunit configurations and has been possible whenever oriented gels or crystals of a sizeable sample can be obtained. Although the orientation of samples in such preparations is not perfect, the X-ray process is also an averaging procedure, and the size of the sample allows averaging over many thousands of particles. There is little, if any, disorder in individual particles. As stated above, optical diffraction allows harmonic analysis over at least a portion of the sample but constitutes an averaging over only tens of disorder loci which occur from one subunit to the next. The principal advantage of this technique as an averaging procedure results from the averaging of periodic direction as well as spacing. Many samples will reveal an apparently precise array of parallel lines or bands, but the condition of parallelism is not necessarily apparent from one line to the next. The line perpendicular to the parallel array may not fall on a discreet axis of the particle, and its degree of deviation may be impossible to measure. In these cases a knowledge of particle axes and an average value of band orientation can provide a very fundamental parameter of quaternary structure and suggest a
specific class of possible subunit arrays.

The presence of alternate configurations of aggregated TMV protein has been discussed. The stacked-disc and helical configurations are basically different in that no vertical (long axis) rise is expected for the line connecting adjacent subunits in the stacked-disc situation; for a helix such a rise must be present and to a degree that depends on the number of subunits per turn and the space allowed between gyres. Figure 9 (a - d) illustrates the difference in these two situations for a completely flattened particle. The sketches represent stacked-disc and helical particles negatively stained so the space between gyres is uniformly delineated. The frame about each model structure was laid as precisely as possible so that its axes might parallel those of the structure to be considered. In this way the axes of the particle will be marked on the transform by the diffractions from the frame, the short axis marker arising from the frame's long axis and vice-versa. This framing procedure was used throughout the course of image testing and greater dependance was placed on the equatorial marker since it originated from the sides of the frame that could most accurately be positioned. It is a perpendicular to this line that is considered the transform meridian. Figures 9b and d are different only in that the reflections from the "helix" are skewed with respect to the meridian. In both
cases the lines that represent reflections are elongated parallel to the equator, their shape being dependent on the frame transform. Structures that have been stained comparable to the models shown have been numerous in this study, and this straightforward kind of analysis has been particularly useful.

Whether the structure is a stacked-disc or a helix, the meridional separation of reflections will be inversely related to the vertical or axial separation of turns in the particle. The angular displacement of helix reflections will only be a measure of helical angles for the type of representation presented where the particle has been completely flattened and effective apertures are truly straight lines. Figure 10a shows the shape of a helix projection in the non-flattened particle.

This brief introduction to helix diffraction theory must be considered only that. To fully appreciate the value of helical transforms, it is helpful, if not necessary to invoke the terminology of Cochran et al. (1952) who have presented an equation for the distribution of intensity in a helix transform that accounts for the effective distribution of scattering centers when viewing the particle along a perpendicular to its long axis.

A continuous helix is comparable to a wire spring since it constantly rises as it turns about an imaginary,
cylindrical surface; a discontinuous helix positions units in space in a constant relationship to one another like the steps on a spiral staircase (a "helical" staircase would be a more appropriate name). The transforms of these two types of structures will reflect their differences although the considerations required for pattern interpretation will be similar. Figure 10 (a and b) shows a helical projection and its corresponding optical transform. If each of the reflections is considered in terms of classical rectangular coordinates (see Figure 10c), we can better understand what considerations have to be made for analysis. The projection of a helix shows that the axial separation of discontinuities is not altered from its value in the three-dimensional case; the radial separation in the three-dimensional helix is represented in the projection by spots that are spaced closer together as they approach the edges of the image. The rate of decrease in this horizontal disposition is very precise and is related to the curvature of a continuous helix that can be traced through the discontinuities. The equatorial or horizontal coordinate of a reflection thus represents a cylindrically averaged value of the radial distribution of discontinuities as they are disposed on a projection. Meridional displacements are also average values, but of axial distribution which is precisely reproduced by the projection. The consideration of equatorial displacements
Figure 8a. Enlarged images of the masks used for Figures 8b, c and d. (Masks are illustrated in the same contrast that was used.)

Figure 8b. Transform of two 200 mesh grids arranged as illustrated in Figure 8a part b. Comparison with Figure 5 demonstrates that no additional points are included on the pattern but that two patterns are cleanly superimposed. The complex pattern in the very center of the transform has arisen from the interaction of the annuli surrounding the individual screens.

Figure 8c. Transform of three 200 mesh grids arranged as illustrated in Figure 8a part c. As in Figure 8b, no additional points are found; pattern complexity arises only from the superposition of three individual diffraction patterns.

Figure 8d. Transform of two 200 mesh grids with a small area of overlap as illustrated in Figure 8a part d. Pattern complexity is greatly increased; the transform now consists of symmetry elements from each of the two grids plus the new elements contributed by the moiré image in the region of overlap.
is then relegated to a knowledge of the sequence of such reflections. Since we are concerned with partially flattened helices in many instances, it is advantageous that a solution to the transforms obtained is possible on the basis of meridional spot distribution. This analysis will provide some appreciation of the mathematical treatment of helix transforms.

The equation (Cochran et al., 1952) that can be applied to the solution of a helical transform is \( \mathcal{L} = \frac{C}{P} n + \frac{C}{P} m \) where \( C \) is the axial repeat period or the distance over which discontinuities are superimposed on the vertical axis of the helix; \( P \) is the rise/turn of the helix, the vertical and not the perpendicular separation of gyres; \( p \) is the vertical rise/discontinuity; \( n \) is the order of the Bessel function which, for our purposes, describes the effect of horizontal averaging; \( m \) is an integer, positive, negative or zero, and describes the diffraction order, and \( \mathcal{L} \) is the layer-line number of a particular reflection. The assignment of layer lines is thus a necessary prerequisite for the use of this expression and is accomplished by a geometric determination of the spacing required for a set of parallel horizontal rulings that will precisely intersect every reflection on the pattern. The numbering of layer lines proceeds in both directions from the transform equator which is numbered \( \mathcal{L} = 0 \). The diagram (Figure 10c) provides a graphical
statement of these considerations and the corresponding
legend provides the calculated values for the helix used.

The successful application of this equation can lead to
a knowledge of the subunit arrangement in a negatively-
stained, helical array if we are able to obtain an inter-
pretable transform. The \( n = 0 \) reflections, indicative of
rise/discontinuity, are the most difficult to obtain in
practice since meaningful diffraction by this aspect of the
lattice necessitates resolution of that separation. In the
case of TMV the rise/turn is known to be 23 Å; the rise per
subunit, only \( \frac{1}{16.33} \) this distance, is obviously not re-
solved by electron microscopy where with biological struc-
tures we could resolve separations in the range of 10 Å at
best. Nonetheless it is possible to resolve striations
between subunit arrays in directions other than that of the
primary helix (Finch, 196^). Such results can conceivably
give rise to \( n = 0 \) diffractions. Without a knowledge of \( p \)
it becomes necessary to test various values on the basis of
the recorded reflections; we can solve the equation for
cases where \( n \neq 0 \) if we are sure of the value of \( n \) for the
reflections found.

Figure 11 demonstrates the way in which helix handedness
can be determined by optical diffraction. A knowledge of
specimen orientation is essential, but this is easily
derived if care is taken to orient the sample in the electron
microscope so that the beam strikes the back side of the grid first and if the assumption holds that dominant contrast results from the side of the particle closest to the grid surface. The negative must be printed with the emulsion side away from the light source so that the viewed micrograph presents the helix of proper hand. If original negatives or copies of such prints are used as masks for optical diffraction, the sense of the helix will be demonstrated. This information cannot be derived from X-ray analysis and may be the feature of optical diffractometry that will make it a routine procedure, especially because of the simplicity of application.

Figure 12 represents the distortions to be expected in transform dimensions when the helix has been partially flattened. Axial displacements will not be changed if the helix compresses uniformly; if it flattens in irregular fashion, the pattern will be noticeably altered and may not be registered in recognizable fashion. The most obvious change in pattern conformation will always be a relative crowding of the Bessel functions toward the meridian. If proper sequence can still be assigned, this condition will not hamper interpretation.

The only way to compensate for our lack of complete knowledge of the degree of disorder and flattening in helical structures is to consider many samples, making accurate
measurements of their mean diameters and comparing these values to those predicted from a consideration of several transforms. Cross-checking of proposed structures is a relatively simple matter with this apparatus since a great many possibilities can be quickly tested.

Rotational Arrays

Occasionally, we will be fortunate enough to observe end-views of short segments of helical or stacked-disc structures. Such images characteristically suggest a division of the circumference into equal segments, and thus optical diffraction can resolve the number of such units in a complete rotation. When contrast is adjusted so that the sample is effectively a circular array of holes, we will obtain a transform that reflects this rotational symmetry, i.e. an array of six holes will give rise to a transform with 6-fold symmetry. Close to the central spot, the pattern will be merely concentric rings of as many spots as there were holes in the pattern. Moving out along a radius, the pattern will increase in complexity but maintain the symmetry described in the central region. Figure 13 displays three circular arrays and the corresponding transforms where this rule is obeyed.

Ideal masks cannot always be obtained, but nonetheless a reflection of symmetry will be produced if the mask can be
adjusted to yield fairly small apertures that are approximately identical. The most common image obtainable will be like Figure 14a; the corresponding transform is recorded in 14b, and this pattern obviously is not as clearly resolved as those from pin-hole apertures; the rules of symmetry are still obeyed.

An obvious application of this ability to resolve rotational symmetries is the case of spherical viruses or the heads of bacteriophage. Dealing with two-dimensional projections is a greater hindrance for this case than it was for helices since the structure represented on the electron micrograph is spherically rather than just cylindrically averaged, and we still have no accurate way of determining the extent of particle flattening. Examination of just the central portion of such images will, on some occasions, provide a measure of center-to-center subunit spacing. This fact can be utilized along with the assumption of icosahedral symmetry to predict how many such units can fit on the spherical surface. Hosaka (1965) has proposed this kind of an extrapolation to capsomere numbers, but offered no means for determining accurately the size of capsomeres.
Figure 9a. Enlargement of mask used to simulate the appearance of completely flattened stacked-disc arrays

Figure 9b. Transform resulting from mask of Figure 9a. Significant reflections are clearly on the meridian

Figure 9c. Enlargement of mask used to simulate the appearance of completely flattened helical arrays

Figure 9d. Transform resulting from mask of Figure 9c. Significant reflections are parallel to the equator but skewed away from the meridian. Vertical (meridional) separation of reflections is exactly that of the reflections in Figure 9b
Figure 10a. Photographic reproduction of mask used to generate Figure 10b. Image is a two-dimensional representation of a helix with 7.25 discontinuities (black spots) per turn. The significance of C (axial repeat), P (pitch) and p (rise/discontinuity) is indicated on the photograph. The partially drawn lines connecting discontinuities indicate two of the three dominant helices of the mask. The apparent distortion (helix bends away from the line of site at top and bottom) gives rise to the non-precise position of secondary reflections in Figure 10b.

Figure 10b. Transform from Figure 10a. Pattern is repeated about every primary reflection; reflections fall approximately on layer lines expected (see Figure 10c)

Figure 10c. Line drawing of expected point distribution on transform of Figure 10a for values of $n = \pm 1, \pm 2$ and $m = 0$. These four points (flattened circles) are oppositely distributed in Figure 10b where only one side of the helix is being viewed. Examination of the equation given indicates that very large values of $n$ ($\pm 7, \pm 8$) are necessary to position the reflections found on layer lines 1 and 3.
\[ f = 4n + 29m \]
Figure 11. Transforms of mask of helix with 7.25 discontinuities/turn as viewed from either side. Figure 11a is the image from the right-handed image (as viewed in Figure 10a) while Figure 11b is the left-handed transform. Handedness is therefore indicated by a skewing of the pattern clockwise for a left-handed helix and in the opposite fashion for a right-handed helix.

Figure 12a. Net (radial projection) of a helix with 8.0 discontinuities/turn illustrating exaggerated particle-flattening where no radial averaging contributes to the transform.

Figure 12b. Transform from Figure 12a illustrating the perfect rectangular point distribution obtained. Ninety degree-rotation of Figure 12a permits observation of the same symmetry found in the transform viewed directly. Values of m for the various rows of reflections are indicated at the bottom of the pattern.
Figure 13. Transforms of rings of six, five and thirteen holes (a, b, and c respectively). Inserts are contact prints of masks used which were made of holes punched in aluminum foil. Each pattern reveals the symmetry of the sample; the six-hole ring (Figure 13a) is the simplest since it has resulted in the registration of a hexagonal net of reflections. Figure 13b demonstrates both five- and ten-fold symmetry elements. The thirteen-hole ring (Figure 13c) shows thirteen-fold symmetry about the pattern center and, in the periphery, consists of many individual rings of thirteen reflections each.

Figure 14. Transform of thirteen-fold symmetric mask with random point distribution within each symmetry element (insert). Mask was constructed to simulate the random density distribution attained with negative staining but points were only included on the thirteen circles that make up the sample. The transform records the averaged distribution of points which is symmetrically meaningful. (The horizontal and vertical intensity spikes in the diffraction pattern arise from the rectangular mask used to frame the sample; that frame shape was chosen to avoid the superposition of other circular elements.)
OBSERVATIONS

The technical complications of this study have been numerous and have fallen into two categories: the attainment of well-resolved, negatively stained images and the development of optical diffraction procedures. The preceding sections have covered the evolution of those methods found to be most successful for our purposes. The comments of this section will be focused on the results obtained by electron microscopy and laser-light diffraction in that order.

Bacteriophage Structure by Negative Staining

Phage of Staphylococcus aureus

All of the staphylococcal phage examined here are temperate agents and have been shown previously to differ only slightly in structural features (Bradley, 1963b; Lapchine and Enjalbert, 1965). The most significant structural differences in this collection of viruses serve to separate them into two groups on the basis of head and tail morphology. Phages of each group have been examined, and large numbers of each strain have been measured with regard to overall dimensions; a single strain from each group has been selected for more extensive study. Results of the measurements taken on these phage are tabulated at the end.
Phage with icosahedral heads

The phages of this group are characterized by sheathless tails about 200 Å in length and heads that routinely show icosahedral profiles by negative staining. Lapchine and Enjalbert (1965) reported observations on eleven such phages and noted pronounced striations on the tails of one of these, phage 53 (Ø-53). The phage examined here, Ø's 29, 52a, 53, 77, 80 and 83, have all revealed striated tails, but only after the negative staining process was modified as described previously.

Figures 15 (a, b and c) are micrographs from PTA-stained samples of Ø-80. In these and all other micrographs of PTA-preparations, ghosted particles (ghosts) were identified by the apparent penetration of the heavy-metal salt into the heads of the particles. It is further assumed that whenever heads appear to be electron-transparent the agent's DNA is present and PTA has been unable to penetrate. The pronounced tail striations shown here were not as readily observed with formaldehyde-fixed samples.

The tails of ghosts appear to terminate proximally in an electron-transparent bar that measures approximately 30 by 120 Å. This structure is seen 80 to 100 Å inside the head profile, and its position has been observed to be constant from one preparation to another. The number of bands or
cross-axis striations on \( \phi-80 \) tails is not always readily counted, but statistical sampling has been possible because of the many preparations examined. Only those particles where non-ambiguous values could be obtained were measured. The mean number of bands on ghosts was 34 where two bands were assumed for the portion within the head; the mean number for intact particles was 36 but fewer of these could be counted since striations were usually obscured in the tail segment closest to the head.

The axial hole present in ghost tails was almost never observed in intact particles. Basal structures were difficult to characterize; they showed no discreet morphology, and their appearance was more or less diffuse from one preparation to the next. Figure 15a presents the clearest view obtained of the basal structure of \( \phi-80 \) where the arrow points to a star-shaped structure that could be a free base-plate; its hexagonal appearance resembles the basal structures observed for the T-even phage of \textit{E. coli} (Anderson and Stephens, 1964).

Unattached tails (Figure 15b) were present in all preparations examined and were found to vary widely in length except when base-plates were attached; in the latter instances mean values were 36 bands and a length of 1900 Å.

The electron-transparent lines on ghost-heads probably represent sides of the proteinaceous, triangular facets of
these apparently icosahedral surfaces. The fact that the observed view of ghost-heads is a projection makes it unlikely that the width (approximately 30 Å) of the limiting border is actually an indication of head protein thickness. The heads of non-ghosted particles reveal a granularity suggestive of capsomers, but not enough regularity can be observed to count capsomeres.

Spray-deposition of particles and PTA (Figures 16a and b) resulted in less displayed angularity of the Ø-80 head; the ghosts frequently showed circular profiles by this technique (Figure 16b). While the results achieved by this procedure are comparable to those with dropper addition of sample, reproducability was not routinely achieved.

Freeze-dry negative staining, as described, was attempted with Ø-80; the micrograph (Figure 17) shown represents the only success from among the thirty preparations made. This preparation was termed successful because at that point in the study, tail banding had never been observed as clearly and the grain size achieved was appreciably smaller than that of previous attempts. The TMV rod included for calibration purposes revealed a variability of diameter suggestive of particle flattening, which suggests that this preparation was not completely successful. Overall observations of freeze-dry negative staining revealed it to be difficult, if possible at all, to attain regularly.
When suspensions of Ø-80 in tris buffer were allowed to stand overnight at room temperature, negative staining revealed 90-100% ghosts (Figure 18). The use of .03% PTA at pH 7.2 necessitated considerable scanning to find samples adequately stained for the observation of substructure. The very thin PTA film that resulted was considered optimum for the observation of tail structures; axial holes and tail-banding were readily apparent. The tail-attachment-site and base-plates, however, were not well defined.

Figure 19 demonstrates the effect on Ø-80 of prolonged (6 hours) exposure to PTA. The resulting contrast effect is comparable to that obtained with dropper-addition of sample and PTA. While some ruptured ghost-heads were noted, the position of the proximal tail terminus was consistently about 100 Å inside the limiting membrane as in Figures 15 and 16 where PTA-particle interaction was limited to 10-20 seconds. The increased frequency of ghosts was similar to that observed when suspensions were left at room temperatures without exposure to PTA. Shorter treatments with PTA revealed no effect apart from the ghosting phenomenon, and longer treatments (up to 48 hours) did not provide an appreciable increase in the number of disrupted heads. Phage fixed with formaldehyde were more resistant to ghosting by both temperature and PTA treatments, but less graphic changes could not be observed.
The effects of altered pH are depicted in Figures 20 and 21. Alkaline pH values (Figure 20) apparently enhanced the affinity of base-plates for one another; this effect was found to be reversible. Alternate raising and lowering of pH in the range 7.2 to 9.5 resulted in the accumulation of increased numbers of ghosts. Acid pH (Figure 21) also was seen to cause ghosting and an increase in the "stickiness" of base-plates which, however, was not reversible in this instance.

The pH values selected for presentation of micrographs were chosen because they were reproducible extremes. Higher and lower hydrogen-ion concentrations produced non-reversible degradation that did not permit any structural observations. Negative staining at these values was also quite difficult since PTA crystallization was very rapid.

Treatment with perchlorate again produced extensive ghosting (Figure 22). While head membranes were apparently thickened in this preparation, perchlorate may merely prevent close apposition of PTA to head protein. Figure 23 shows Ø-80 particles after treatment for 30 min. with 0.8 M urea which reagent is commonly used for degrading protein structure in 4M to 8M concentrations; those concentrations successfully destroyed Ø-80's conformation with only 15 to 30-min. treatment at room temperature. No effects of urea were noted at 4°C. The particles in Figure 23 are from a sample that
showed the same appearance up to 12 hours; Ø-80 particles treated in this fashion were rarely observed in the center of PTA islets but were accumulated, rather, at the periphery with tails directed toward the droplet edge and frequently extending completely out of the PTA region. Tail banding and the angularity of heads were barely visible; the most pronounced effect was on the base-plates which appeared to be enlarged and resistant to PTA penetration.

A striking effect was produced by pelleting and resuspending Ø-80 in tris buffer without MgCl₂ or CaCl₂ (Figures 24 a and b). This procedure did not produce ghosting to a discernible extent but resulted in large numbers of heads disrupted most frequently at the site of tail attachment. The electron-transparent clot at the head end of the tail must certainly represent the DNA content of the phage and is apparently attached to the tail stem independently of the head protein. Uranyl formate-negative staining was not successful until the latter stages of this study and was used to only a limited extent with Ø-80 (Figure 25). Distinguishing ghosts from intact particles is difficult since the uranyl ion might be expected to stain the DNA of the head positively (Bradley, 1965). A less secure, but useful, criterion for ghosting is the presence or absence in the tail of an axial hole which was observed only on ghosted particles when PTA-negative staining was used. The validity
of this criterion is borne out by the fact that the shorter of the two tails, designated by arrows, has an axial hole and the other does not; the particles with tails showing an axial density also have less well-preserved head structures. The enhanced contrast of uranyl-formate preparations is better appreciated if it is understood that Figure 25 was originally printed on contrast grade 4 photographic paper; all previous figures were recorded on contrast grade 6.

Figures 26 (a - d) represents an overall view of our findings on Ø-29. The mean value of tail lengths on sixty, intact particles was 2100 Å, and that of 30 ghosts was identical; in both cases diameters were about 100 Å. Head dimensions were 660-680 Å, and a coarse granularity distinct from background was observed on intact heads somewhat more readily than it had been in Ø-80. Proximal tail termini in ghosts measured 30 to 40 Å by 140 Å and (Figure 26b) had a central vacancy that was continuous with the tail's axial hole and that was not as evident in this strain as it had been in Ø-80.

The base plates of Ø-29 were well-defined and showed a platform-like structure (Figure 26c) surrounding an electron-transparent mass, 240 to 270 Å in diameter and, on one occasion (insert Figure 26c), a polyhedral profile. The 80-Å bar apparently attached to one point of that mass by a slender rod (Figure 26c) was not otherwise observed. Note also the last somite-like segment of the tail which is just
above the top, transparent bar or plate of the basal structure and which has a paired appearance. The 270-Å distal breath of the basal structure is consistent with the view that isolated hexagonal arrays (Figure 26d and bottom center of 26a) are end-views of base plated that have a point-to-point diameter of 270 Å as well. The basal structure, then, appears to consist of a platform, 180 Å in diameter, with six 290-Å legs radiating at angles of approximately 30 degrees from the long axis of the tail; the platform surrounds a polyhedral mass, resistant to PTA penetration and positioned on a line continuous with the tail's long axis. Free base-plates, found in the collected lysate show a hollow, six-membered, ring structure with each of the members showing a circular cross section, 35-45 Å in diameter. The platform-like structure appears flattened in ghosted particles, and the circumscribed central mass is missing there as it is in free base-plates.

Phage 52a (Figures 27 and 28) has a similarly striated tail, measuring approximately 100 Å by 2000 Å. The head profiles in this strain are frequently flattened, particularly in ghosted particles. Surface granularity is observed on intact heads by uranyl-formate staining (insert Figure 28); tails show axial holes only when attached to ghosted particles, base-plates are poorly defined on whole particles, but hexagonal rings (Figure 27) are observed apart from
particles as in the case of 0-29. Many of the ring structures surround a central, electron-transparent mass, comparable in size to the members of the ring itself.

Phage 53 (Figure 29) reveals dimensions and morphology almost identical to that for 0-80, except that unghosted heads are more angular. Figure 30 is a micrograph of a 0-77 particle, displaying an anatomy very like that of 0-29; no free ring structures were found in preparations of 0-53 or 0-77, and 0-77 has a basal nodule complete with an attached prominence like that of 0-29 (Figure 26c). A high magnification view of 0-83 (Figure 31) apparently reveals the subunit structure of the intact head. Maximum center-to-center spacing of the PTA-delineated masses on the surface is 65 Å. Capsomere counting was not possible since it is not clear which symmetry axis is coincident with our line of observation. This micrograph was printed to reveal the head-surface structure of 0-83, but other views suggest a morphology nearly identical to that of phages 53 and 80.

Phage with elongated heads and tails Phages 6, 42b and 81 were found to possess almost identical morphologies. These three strains were more amenable to uranyl formate negative staining than were the icosohedral-headed phage just discussed; use of two negative staining materials provided a more comprehensive view of the particles in question, and measurements were considered meaningful only when
Figure 15a. Ø-80 preparation showing general appearance of this strain by dropper-addition of PTA. Ghosts (G) are readily distinguished from intact particles (IP). PTA-treatment, dropper method. X 190,000

Figure 15b. Ø-80 intact particles and free tail (FT). Note that PTA delineation of tail-banding (B) is not perfectly regular (arrows) while the average center-to-center separation of bands is approximately 50 Å. Free tail (FT) with attached base-plate (BP) shows no clear axial penetration of heavy metal. OTA-treatment, dropper method. X 190,000

Figure 15c. Ø-80 ghosted particles showing axial holes (AH) of ghosted tails and bar-shaped proximal termini (PT). Base-plates (BP) are evident but their precise shape is not clear. PTA-treatment, dropper method. X 190,000
Figure 16a. Ø-80 preparation intact particles. PTA-treatment, spray-deposition. X 200,000

Figure 16b. Ø-80 preparation ghosted particle. Head membrane profile is nearly circular; axial hole of the tail is seen to extend completely from the proximal terminus through the basal structure. PTA-treatment, spray-deposition. X 200,000

Figure 17. Ø-80 preparation showing general characteristics of the phage by freeze-dry negative staining. PTA-treatment, as described for freeze-dry negative staining. X 200,000
Figure 18. Ø-80 overnight treatment in tris buffer at room temperature. Tail-banding and axial holes are readily observed. Note that all particles are ghosted, angularity of head profiles is lost and base-plates and proximal termini are not well contrasted. PTA-treatment; dropper method. X 230,000
Figure 19. Ø-80 preparation after 6 hour incubation in PTA (.03%). Many ghosted (G) and disrupted (DH) heads are observed. Intact particles (IP) show straighter tails than the rest of the sample. Proximal termini (PT) are located at about the same position within the head profiles of both disrupted and normally ghosted particles. PTA-treatment, dropper addition of particles and heavy-metal together. X 190,000
Figure 20. Ø-80 preparation after 30 min. incubation at pH 9.5. Most particles are ghosted; almost all are apposed to one another at their distal ends. PTA-treatment, dropper method. X 100,000

Figure 21. Ø-80 preparation after 30 min. incubation at pH 4.8. Virtually all particles found were ghosted. Staining is more complete than for alkaline treatment (Figure 20). PTA-treatment, dropper method. X 110,000

Figure 22. Ø-80 preparation after 30 min. incubation in 10% Na-perchlorate. Observed head profiles are thicker than those observed in the absence of perchlorate; very few intact particles were found. PTA-treatment, dropper method. X 120,000

Figure 23. Ø-80 preparation after 30 min. incubation in 0.8 M urea. Ghosting is not apparent, but head profiles have lost their angularity; basal structures (BP) are enlarged and directed toward the edge of the PTA-islet. PTA-treatment, dropper method. X 110,000
Figure 24a. Ø-80 preparation after suspension in tris buffer without MgCl₂ or CaCl₂. Particles do not display ghosting as in previous micrographs but many are disrupted (DP) at the proximal end. Head profiles are not continuous and, in most cases, are separated from the tail stem leaving a diffuse electron-transparent mass. PTA-treatment, dropper method. X 90,000

Figure 24b. Ø-80 preparation under same conditions as for Figure 24a. Proximal termini (PT) are observed in disrupted particles. Much fibrous material is found in the background (upper half of micrograph), and "clots" are parenthetically labelled DNA since this is likely to be their nature. PTA-treatment, dropper method. X 90,000
Figure 25. Ø-80 preparation in uranyl formate. Ghosts (G) are distinguished from intact particles (IP) primarily on the basis of the pronounced axial density distribution and distorted heads of the ghosted particles. Free tails (FT) also show an axial deposition of stain. Uranyl formate treatment, positive staining of DNA and negative staining of protein. X 175,000
Figure 26a. Ø-29 intact particle and free base-plate (BP) in end-view. PTA-treatment, dropper method. X 190,000

Figure 26b. Ø-29 ghosted particle showing position of proximal terminus (PT) and loose appearance of base-plate (BP). PTA-treatment, dropper method. X 190,000

Figure 26c. Ø-29 showing detail of base-plate conformation on intact particle. Insert (X 450,000) indicates the possibility of a nob-like (n) structure under the platform that outlines the basal structure. PTA-treatment, dropper method. X 190,000

Figure 26d. Free base-plates of Ø-29 in end-view. Engraved lines about the periphery of one such structure indicates that the structural repeat is very nearly every 60°. PTA-treatment, dropper method. X 190,000
Figure 27. Ø-52a preparation. Intact particles (IP) have characteristically flattened heads compared to the other icosahedral headed staphylococcal phage. Ghosts (G) show axial holes (AH) which are not observed in intact particles. Free base-plates (BP), apparently hexagonal as for Ø-29, were numerous in all fields of this sample. PTA-treatment, dropper method. X 150,000
Figure 28. Ø-52a preparation intact particle. Heavy-metal has been deposited in the axial region of the tail but predominantly in the proximal region. Insert shows two intact heads of Ø-52a printed to reveal that both positive and negative staining have evidently taken place. Uranyl formate-treatment. X 270,000

Figure 29. Ø-53 preparation of intact particle and ghosts. Morphology indicated is quite similar to that described for Ø80 (Figure 15). PTA-treatment, dropper method. X 190,000

Figure 30. Ø-77 preparation intact particle. Similarity to Ø-29 (Figure 26) is evident although the contours of the basal structure are not as pronounced. PTA-treatment, dropper method. X 190,000

Figure 31. Ø-83 preparation. Micrograph has been printed with very high contrast to reveal the sub-structure of the intact particle head surface. Globular transparencies are approximately 65-Å apart center-to-center. PTA-treatment, dropper method. X 450,000
dimensional consistency was noted. Particulars of these measurements, as well as those on the icosahedral-headed phage, are tabulated at the end of the section of "Observations."

Spray-deposition of PTA-stained, formaldehyde-fixed Ø-6 (Figure 32) was utilized since this procedure provided the straightest tails and facilitated measurement. Results with uranyl formate staining of formaldehyde-fixed material suggested that this combination of procedures was, unfortunately, incompatible. The elongated heads and tails of Ø-6 are immediately apparent in Figure 32. The site of tail attachment to the head is marked by the presence of an ovoid electron-transparency within the head profiles of ghosted particles; distal tail termini are not well-defined while the axial holes of ghosts and free tails apparently extend through these structures. The wavy head profiles (Figure 32, arrows) were rare, were observed only in formaldehyde-fixed preparations, and have not been found in the literature. Approximate head and tail dimensions are 1100 Å by 450 Å and 3500 Å x 90 Å respectively. (Measurements in this and all other examples of the staphylococcal phage were consistently 15-20% higher than those of Lapchine and Enjalbert (1965) who mentioned that their measurements were not made with a precisely calibrated microscope.) The tail attachment site within the head profile was also found in
free heads.

Ten-minute incubation in dilute peroxide at room temperature served to disrupt \( \Phi-6 \) structure. Dropper addition of PTA to unfixed material allowed the observation of tail banding and a bipolar, dark zone in the heads; the latter appearance has been previously reported (Lapchine and Enjalbert, 1965). Figure 33 contains a view of a disrupted head that has fractured away from the tail and left a small triangular region attached to the tail's proximal end.

The heads in this preparation appear only partially evacuated when compared to the ghosted particles of Figure 32. Basal structures are uniformly absent, and no positive evidence of free tail termini was found.

Phage \( \Phi-42b \) (Figure 34) presents the same kind of image as \( \Phi-6 \); base-plates are similarly diffuse in appearance when attached to tails, but the \( \Phi-42b \) suspensions were found to contain many free, hexagonal, ring structures as in the cases of \( \Phi-29 \) and \( \Phi-52A \). One such ring was noted for \( \Phi-6 \) in Figure 32. The beaded rings in preparations of elongated phage display a maximum point-to-point separation of 230 Å as opposed to 270 Å for the icosahedral-headed phage. Partially disrupted head membranes in phage \( \Phi-42b \) (free heads in Figure 34) suggest that the elongated head might be viewed as a pair of fused icosahedra.

Figures 35a, b and c show three aspects of \( \Phi-81 \) structure
as revealed by PTA negative staining. The wavy head membrane on free ghosted heads is reminiscent of, but not as pronounced as, the comparable images found for Ø-6 preparations (Figure 32). Again free heads contain the ovoid tail-attachment site; ghosted particles contain a bipolar dark zone, have an axial tail hole and more readily show cross-axis banding. Free, hexagonal ring structures are shown in Figure 35b but are not as clearly defined as previously. The free tails of Figure 35c were produced by peroxide treatment as used for Ø-6 (Figure 33); while two of the tails are of normal length, the third measures slightly over 4500 Å, and shows an axial hole over only part of its length.

Uranyl formate staining of Ø-6 (Figure 36) bears out further the assumption that ghosted particles can be identified by the presence of an axial tail hole, and that intact particles are positively stained with regard to the DNA-content of their heads. Substantiation of these assumptions is gained by the presence of a bipolar dark zone in particles that would thus be classified as ghosts. The positively stained heads reflect sharp angularity and slightly reduced dimensions; the clear zone delimiting the intact heads is approximately 20 Å wide, and in this and later figures there is a suggestion that this boundary is composed of spherical beads 20 to 25 Å in diameter. The faint suggestion of an axial deposition of uranyl formate on the tails of non-ghosted
particles could signify the hollow nature of intact phage tails and the smaller grain size of uranyl formate (Finch, 1964) that is able to permeate this space. The observation might also be made that the uranyl ion is positively staining an included strand of DNA, but this is still more presumptive.

Figure 37 exhibits a pair of very elongated, free tails found in samples of Ø-6; tails with such dimensions frequently were found but were never attached to heads. The results of uranyl formate staining in strains 6, 42b and 81 of the staphylococcal phage are summarized in Figure 38 and 39 where additional evidence of previous observations is presented.

The extensive measurements made on micrographs of the staphylococcal phage are summarized in Table 1. Of the parameters quoted, the enumeration of cross-axis, tail striations or bands is the most subject to error due to difficulties in observation.

Phage of Eschericia coli

Phages M13, T4 and T5 of E. Coli have been examined by the procedures described for the staphylococcal phage. T5 has been examined briefly here to provide corroboration for extending our observations on the staphylococcal, non-sheathed phage to a discussion of the structural evidence for nucleic acid injection mechanisms; T5 has been studied
Table 1. Measurement of staphylococcal phages

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aTop number indicates number of intact particles; bottom indicates number of ghosts.

bThe three values stated for this and other parameters indicate low, mean and high values and are recorded in that order.
Figure 32. Ø-6 preparation revealing elongated head profiles found for this strain. Proximal termini (PT) are observed as electron-transparent regions closely apposed to the head membrane in both free heads (FH) and ghosted particles (G). Axial holes are again present in ghosted (G) rather than intact particles (IP). Free tails (FT), base-plates (BP) and curiously wavy head profiles (W) are also indicated. PTA-treatment, formaldehyde fixation and spray deposition of sample. X 120,000
Figure 33. Ø-6 preparation after ten-minute incubation in 10% peroxide. Very few completely intact particles were noted, and the field contains principally free tails (FT), free heads (FH) and ghosts (G). Very short tail fragments were occasionally found (SFT) and rarely an apparently intact head without a tail (IH) was seen. The disrupted head (DH) suggests the stability of triangular segments of head membrane. PTA-treatment, dropper method. X 120,000
Figure 34. Ø-42b preparation showing base plates (BP) in end-view and free heads. Anatomical features of this phage are nearly identical to those found for Ø-6 (Figure 32) except that hexagonal ring structures (base-plates) were found in all Ø-42b preparations. PTA-treatment, formaldehyde fixation and spray-deposition. X 150,000
Figure 35a. Ø-81 preparation demonstrating intact particles (IP), the axial holes (AH) of ghosts (G) and proximal tail termini (PT) within the profiles of free and ghosted heads. PTA-treatment, formaldehyde fixation and spray-deposition. X 150,000

Figure 35b. Ø-81 preparation revealing presence of hexagonal ring structures (BP) as observed for Ø-42b. PTA-treatment, formaldehyde fixation and spray-deposition. X 150,000

Figure 35c. Ø-81 preparation after incubation for 30 min. in 10% H2O2. Banding and axial holes are in evidence for the free tails shown. PTA-treatment, dropper method. X 150,000
Figure 36. Ø-6 preparation in uranyl formate. Intact particles (IP) are identified by the pronounced accumulation of heavy-metal within their head profiles. Ghost- (G) and free tails (FT) show a strong axial deposition of density; the heads of ghosted particles are not strongly contrasted but show a bipolar dark zone. Base-plates (BP) are noted only as slight protuberances at the distal end of tails. Uranyl formate-treatment. X 150,000
Figure 37. Ø-6 preparations showing appearance of elongated polymers of tail material (FT) and that axial density of these structures is similar to that of ghosts (G) rather than that of intact particles (IP). Uranyl formate-treatment. X 150,000
Figure 38. Elongated staphylococcal phage. Figure 38a is a Ø-6 preparation showing combined positive and negative staining effects. Phage heads are centrally, heavily stained, are surrounded by a thin (20-A) line and are extremely angular. Figures 38b and 38c represent Ø-42b ghosts. Each specimen shows a bipolar dark region in the head, a proximal terminus and an axial tail hole. Heads are decidedly less angular than those of intact particles of Ø-6, Ø-42b or Ø-81 prepared in the same fashion. Uranyl formate-treatment. X 150,000
Figure 39. Ø-81 preparation showing characteristic differences in the structure of ghosts (G) and intact particles (IP). A free tail (FT) is noted which shows axial heavy-metal deposition over only half its length. Arrows delineate four elements of a perfect hexagon and point to resolved subunits of an intact particle base-plate. Uranyl formate-treatment. X 150,000
at great length with regard to the kinetics of DNA-transfer (Lanni, 1965). The sheath and head of T₄ are two of the most heavily examined structures in phage research and were used in this study to shed light on the controversies over their conformation. Phage M13, rod-shaped and containing single-stranded DNA, challenged the resolution limits of our methods, and the findings of Salivar et al. (1964) indicated structural evidence for a specialized kind of DNA injection.

**Coliphage T₅** Observation of T₅ substructure was not as readily achieved as it had been for the staphylococcal phage. Uranyl formate procedures were ineffective and served only to stain the nucleic acid of the T₅ head positively while not adding any contrast to the tail. PTA results (Figures 40a - d) are adequate to demonstrate the similarity of phage T₅ to the icosahedral-headed staphylococcal phage examined. In ghost tails the similarity anticipated is evidenced by the presence of axial holes and bands which were not readily demonstrated for the intact particle. The proximal tail terminus was again indicated about 100 Å inside the ghost head; basal termini were poorly defined.

**Coliphage M13** Measurements of phage M13 suggested a large range of lengths in our preparations although we have no structural indications as to which
particles are infective. The width of MI3 was found to have a mean value of 65 Å in PTA preparations (Figures 41 and 42) and 75 Å in uranyl formate-stained samples. This disparity is due to the fact that samples frequently were partially buried in PTA preparations and effectively measurable diameters were smaller. A frozen-dried, negative-stained sample of MI3 (Figure 41) shows lengths that vary over a large range even within this small field and a width of particle images that is constant. Observed granularity on the particle surface is not demonstrably different from that of the background. After several samples were examined, a single image (Figure 42a) suggestive of particle unraveling was recorded and showed an apparently continuous 10 to 20-Å wide, electron-transparent strand extending from the 65-Å wide segment. Figures 42b and c represent images of sonicated MI3 that were obtained by PTA methods. A longitudinal periodicity is not suggested, and no regions showing axial holes are indicated. Both of these micrographs were obtained with preparations sonicated in a watch-cleaning sonicator, a treatment repeatedly found successful for the partial disruption of MI3 particles. Uranyl formate negative staining (Figure 43) revealed a greater degree of surface structure than any of several PTA variations that were tried. Background granularity is a decided handicap in interpretation of this micrograph; a
comparable hindrance was encountered in nearly all uranyl formate-treated specimens. In a few localized places, measurement of striation spacing was attempted; the mean value of such measurements was 50 Å, but the degree of uncertainty is high for structure that is low in contrast and close to the combined resolution limitations of specimen, technique and microscope.

**Coliphage T₄**  Phage T₄ was examined primarily to obtain well-resolved images of sheaths in contracted and extended states and to apply optical diffraction in a derivation of helix parameters. Investigations by other authors indicated that routine PTA procedures would provide resolution of the cross-axis striation on extended sheaths of the T-even phage but that visualization of contracted sheath substructure was only rarely possible. In neither of the two states were observations of sheath subunits gained by PTA procedures, and uranyl acetate staining (Fernández-Morán, 1962; Kellenberger and Boy de la Tour, 1964) did not routinely yield non-ambiguous images. Success with uranyl formate procedures indicated that the use of this heavy-metal might be the answer to subunit structure resolution in the T₄ sheath. Initial observations with PTA-negative staining methods were made to gain familiarity with the overall morphology of the particle; these observations provoked interest in the controversy over T-even head
conformation and the sequence of events in nucleic acid injection.

The technique of Brenner and Horne (1959) was used on the peroxide-treated particles (Figure 4×). Observations of such samples were limited by background grain and low contrast; however, structure that was seen here was only seen with the spray-deposition technique. Many ghosted heads revealed a central pair of horizontal, crescent-shaped, electron-transparent lines like those described as "folds" by Cummings and Kozloff (1962) and thought to be indicative of a head-shortening necessary for nucleic acid injection. The collars of T4 particles are prominent in Figure 4×, but tail fibers and base-plate spikes are not well-defined.

Figures 45 - 50 were obtained by dropper addition of sample and PTA. A wide range of heavy-metal concentrations (0.01 - 2.0%) was used before satisfactory contrast effects were achieved; the optimum concentration was determined to be 0.4%, one drop of which resulted in the deposition of an extremely thin but nearly uniform and almost grainless film. This technique was useful for the observation of large numbers of particles and permitted identification of the state of contraction in almost all cases; base-plates, spikes tail fibers, sheath striations and indications of head geometry are all readily viewed (Figure 45). The T4 collar is seen less clearly and usually is a very faint
electron-transparent line between head and proximal end of sheath. The unstained appearance of the axial region in some tails is attributed to a lack of PTA penetration between the external surface of core and internal surface of sheath. No information has been derived about the size of this space or if, in fact, it exists. Base-plates are apparently continuous with the most distal turn of the sheath helix.

Figure 46 is a high magnification view of both untriggered and triggered particles. The sheath length is approximately 1100 Å, and the number of cross-axis striations is estimated to be 25. The length of sheaths has been found to vary between 900 and 1150 Å for extended or untriggered particles, and sheath diameters range from 200-230 Å. Maximum tail-fiber lengths were close to 1400 Å. Contracted sheath parameters had mean values of 500 Å for length and 270 Å for width. The substructure of contracted sheaths was rarely obvious in PTA preparations. Greater detail of sheath dimensions will be covered in the discussion of observations with laser-light diffraction.

With regard to the question of the sequence of events in nucleic acid injection and the controversy over whether or not sheath contraction is a necessary prerequisite to injection, a series of observations have been made. Ghosted particles were again identified by the penetration of PTA into the head membrane; nine examples of ghosted particles
are illustrated in Figure 47, and a series of non-ghosted particles is presented in Figure 48. At first glance five states of T4 conformation are noted. Particles are seen with extended sheaths both with (Figure 48a - e) and without (Figure 47h and i) DNA-filled heads. Non-ghosted particles and ghosts are viewed with base-plates attached to contracted sheaths (Figures 48k - o and 47a - g respectively); non-ghosted particles are also observed with contracted sheaths where base-plates are still attached to the tip of the core (Figures 48f - j). Caspar and Klug (1962) have mentioned that the T-even head defies the law of icosahedral symmetry because it is only formed around a DNA nucleus. If this claim is true, the particles in Figures 47h and 47i have released DNA without sheath contraction. Examining the profiles more closely reveals that the ratio of head length to width is quite variable and that the apparently icosahedral profile is not always present in ghosted particles while it is frequently seen in non-ghosted phage. Base-plates that are still apposed to sheaths after contraction reveal that the spikes have perhaps been repositioned in the plane of the proximally disposed base-plate segment. This spike arrangement is also suggested for particles without contracted sheaths but with icosahedral head-profiles (Figures 47i and 48e) and is not always the case, however, as evidenced by Figures 48d and j.
The phenomenon of partial ghosting is suggested by Figures 47d - f where PTA penetration has not occurred to the extent found in the other ghosts presented. The observation might be made that ghosting is only complete in particles with shortened heads, but Figure 49a disputes this claim. The great variety of head profiles causes difficulty in proposing a model and indicates that the head membrane is extremely flexible under the conditions of specimen preparation used.

Loosely wound, apparently continuous, helical structures were occasionally obtained in T4 preparations (Figure 49a - c). These structures were considered to be examples of "relaxed" or super-extended sheath material since they were found attached at one end to the proximal segment of cores (Figure 49, a and c) or free with attached base-plates (Figure 49b). The lengths of these structures was variable, and the diameters were in the vicinity of 200 Å; the strand width varied from 25-35 Å when it was observed as a single structure. Free base-plates, as described by Anderson and Stephens (1964), were also observed (Figure 49c).

Overnight exposure to hydrogen peroxide resulted in disassembly of T4 particles. PTA-treatment of such preparations (Figure 50) revealed separated heads (all ghosted), cores, contracted sheaths and fibers. Contracted sheaths were frequently observed in end-view as they stood erect on
the film; a pin-wheel appearance (Horne and Brenner, 1958) was noted, and as many as 15 units could be counted around the observed circumference. This view of contracted sheath permitted more accurate measurement of its dimensions; the axial hole diameters measured 110-120 Å, and the outside diameter of those that presented circular, cross-sectional views was 260 Å. Up to 14 striations were counted in side-views and were canted at variable angles with the sheath long axis.

Cores revealed an approximately 40-Å striation; 27 bands were frequently counted. Axial holes in the free cores were seen more clearly than in previous examination of intact particles and measured 15-25 Å in diameter. Fibers were 15-25 Å wide, variable in length with a maximum of 1600 Å, and showed a granularity which was not, however, clearly different than that of the background.

Uranyl-formate treatment of phage T4 was successful only in localized regions of specimen grids and provided very high contrast in those areas (Figures 51 and 52). Tail structures were more clearly visible than in PTA preparations except that base-plate spikes were rarely seen and contrast was not well developed along the whole length of tail fibers. Measured dimensions were comparable to those taken on PTA-treated samples, but head shape was never as elongated or as angular as before.
The particles in Figure 53 were peroxide-treated, and the many stages of contraction and DNA-loss that were described in Figures 47 and 48 were again found. Sheath substructure was resolvable for each of several stages of contraction observed; tail fibers were seen to lie along the sides of extended sheaths and to apparently be attached to collars. The details of sheath contraction and a further discussion of the observed stages will be included with the observations on the diffraction of T4 components.

Figure 54 shows the effects of overnight peroxide-treatment of T4 particles. Again phage disruption is apparent, but free, ghosted heads and tail fibers were not found. Isolated sheaths and cores were better defined in uranyl formate than in PTA; heads and tail fibers were apparently degraded by the formate procedure. The deeper penetration of uranyl formate was evidenced by the appearance of end-views of contracted sheath; the spokes of the pin-wheel (Figure 54) are contrasted closer to the center hole and suggest the degree of tilt of each sheath specimen by their flaring.

Some of the structural features of the examined phage have not been cited to this point because they will be better understood when compared with their optical transforms.
Figure 40a. Phage T5 intact particle. Note granularity of head surface and lack of clarity in the cross-axis tail striations. PTA-treatment, dropper method. X 310,000

Figure 40b. Phage T5 ghost showing proximal terminus (PT) within the head profile and a banded tail with an axial hole as was observed for the ghosted staphylococcal phage. PTA-treatment, dropper method. X 310,000

Figure 40c. Phage T5 ghosts with superimposed tails in distal region. Proximal tail termini are again in evidence. PTA-treatment, dropper method. X 310,000

Figure 40d. Aberrant phage T5 particle with elongated tail. Elongated tails were not found for particles with apparently full heads. PTA-treatment, dropper method. X 310,000
Figure 41. Phage M13 preparation demonstrating constancy of diameter (ca. 65 Å) and variability of length of examined rods. PTA-treatment, freeze-dry negative staining. X 180,000
Figure 42a. Phage MI3 particle partially disaggregated at one end. The nature of the filamentous thread extending from the top edge of the rod is questionable and could be DNA or protein since measurements in this range are not significant. PTA-treatment, dropper-method. X 270,000

Figure 42b. Phage MI3 preparation after ten-minute sonication. Particles are short and frayed, but surface structure is enhanced. PTA-treatment, dropper method. X 90,000

Figure 42c. Phage MI3 preparation after one-minute sonication. Particles are not seriously disrupted, and the phage surface is not contrasted as well as with longer sonication (Figure 42b). PTA-treatment, dropper-method. X 90,000
Figure 43. Preparation of phage M13 showing surface structure and occasional axial deposition of heavy-metal (arrows). Strand width is approximately 75 Å in this and similar preparations. Uranyl formate-treatment. X 360,000
Figure 44. Peroxide-treated phage T4 preparation showing intact particles (IP), ghosts (G), collars (CL), sheaths (S) cores (C) and apparent folds (F) on the heads of ghosted particles (as described by Cummings and Kozloff (1962)). Note that exposed cores (C) on ghosted particles show evidence of cross-axis striations. PTA-treatment, spray deposition. X 200,000
Figure 45. Phage T4 preparation demonstrating tail fibers (TF), base-plates (BP) and spikes (SP). Contracted sheaths (CS) are not well-delineated with stain but normal sheaths show a clear series of striations (ca. 40 Å spacing). Head profiles are elongated as opposed to the shapes observed in Figure 44. PTA-treatment, dropper method. X 190,000
Figure 46. High-magnification view of normal and trig­gered particles of phage T4. The intact particle (upper half of field) shows distinctive head contours, collar and tail apparatus including sheath, base-plate, spikes and fibers. The ghosted particle (lower half) reveals a shorter, broader head profile, a contracted sheath and a core with an irregular but generally axial penetration of stain (AH); the basal structure of the ghosted particle no longer shows a distal extension of the spikes as in the intact phage. PTA-treatment, dropper method. X 450,000
Figure 47. Nine views of individual particles of phage T4 exhibiting ghosting. Frames a through g show contracted sheaths but only a, b and c of these show strong PTA penetration within a shortened head profile. Frames h and i demonstrate PTA penetration of the head region but no apparent sheath contraction. Proximal termini are observed in several particles closely apposed to the inner surface of the head membrane. PTA-treatment, dropper method (except for frame h which is of spray-deposited material). X 190,000
Figure 48. Sixteen non-ghosted particles of phage T4. Frames a through e are included to demonstrate the variability in head profiles of apparently intact particles. Frames f through j are examples of particles whose sheaths have contracted without removal of the base-plate from the distal end of the tail. Frames k through o portray the appearance of particles with sheath-contraction, base-plate removal and no apparent ghosting (with the exception of the ghost in the upper left of frame o). PTA-treatment, dropper method. X 190,000
Figure 49a. Phage T4. Loosely wound, helical structure attached to proximal region of the core on a non-ghosted particle is probably a partially unravelled or super-extended sheath (SS). The core region above the attachment site of the helical structure shows some evidence of an axial hole (AH). The ghosted particle in the lower right of the field shows a proximal terminus (PT) within the head profile and is a further example of the type of particle shown in Figures 47h and i. PTA-treatment, dropper method. X 190,000

Figure 49b. Phage T4 preparation demonstrating a free super-extended sheath as evidenced by the attached structure at one end that is comparable to observed base-plates (BP). PTA-treatment, dropper method. X 190,000

Figure 49c. Additional example of the type of particle shown in Figure 49a where the loosely wound helix is attached at the normal site of the proximal sheath-core connection. PTA-treatment, dropper method. X 190,000

Figure 49d. Four views of isolated hexagonal ring structures with approximate base-plate dimensions and evidence of fiber attachment at vertices. PTA-treatment, dropper method (except lowest member of the series which is from a uranyl formate preparation). X 190,000
Figure 50. Phage T4 preparation after overnight treatment in 10% peroxide. Free head-membranes (HM), contracted sheaths (S) and cores (C) were found. Cores show a clear axial hole and some evidence of striation. Pinwheel-like structures (P) are quite probably end-views of contracted sheaths. PTA-treatment, dropper method. X 280,000
Figure 51. Phage T4 preparation. Intact particles show negative contrast but no evidence of positive staining. Head shape is less angular than in PTA preparations and sheath striations are very clearly marked. Tail-fibers are only contrasted for a short distance from the basal structure since the concentration of heavy-metal is very low (0.05%). Uranyl formate-treatment. X 115,000
Figure 52. High-magnification view of four intact particles from Figure 51. Details of sheath substructure are evident; in many areas four or five globular areas can be counted across the sheath between a single set of striations. A free core (C) with attached base-plate is also noted. Uranyl formate-treatment. X 210,000
Figure 53. Phage T4 preparation after 30 minute exposure to 10% peroxide. The aspects of structure noted for PTA preparations are again noted; primary differences are the clear demarcation of sheath striations in this preparation as opposed to PTA-treatment and the loss of angularity in the phage head as observed here. Figure 53d illustrates the position of tail-fibers (F) in the non-discharged particle. There is no indication of positive staining since only Figure 53c shows appreciable electron-density within the head profile and it could well be ghosted. Uranyl formate-treatment, X 220,000 (Figure 53a is X 190,000)
Figure 54. Phage T4 preparation after overnight incubation in 10% peroxide. Free heads and tail fibers are no longer detectable but sheath (S) and core (C) components of the tail apparatus are apparently resistant to peroxide exposure. Sheaths and cores show cross-axis striations as on intact particles, but all sheaths are contracted. The pinwheel- appearance of sheaths in end-view (P) is again noted, and these structures are more clearly contrasted than they were in PTA preparations. Uranyl formate- treatment. X 175,000
Laser-Light Diffraction

In order to report concisely our findings from laser-light diffraction studies of phage components, it will be necessary to temporarily digress from the strict recounting of observations so far followed. The examination of other fibrous systems is pertinent to the theme of this study only if it is utilized as a series of examples to illustrate results and the sources of anticipated artifact. For this reason an element of discussion must accompany the observations on flagella, trichocysts, TMV and ciliary microtubules; each will be assessed for its contribution to the study and for what it illustrates about the procedures utilized. When the cited examples have been thoroughly analyzed, the transforms of phage images can be straightforwardly presented, and their implications can be included in "Discussion" along with those conclusions drawn solely from electron microscopy.

Bacterial flagella

Flagellar structure has been investigated by negative staining by several authors, notably Kerridge et al. (1962) and Lowy and Hanson (1965). Both of these studies demonstrated that flagella are composed of helically arranged subunits, but negative staining accentuated the quaternary structure in such a way that a multiple-stranded helix was
suggested.

Elongated structures, like flagella, were found to be most readily stained with uranyl formate. Several flagella from contaminating bacteria were found in our preparations when crude phage lysates were checked; many of these appeared helical and were thought to be ideal test structures for development of optical diffraction procedures. Figure 55 is a collection of micrographs and the transforms that were obtained from them. The flagella were quite probably from three different sources and were not expected to yield identical patterns. A pattern meridian has been indicated on each transform to elucidate the equatorial distribution of observed reflections. What we have seen with regard to the handedness or screw-sense of helices indicates that each of these patterns is a combination of left- and right-handed contributions since contrast in the electron microscopic image is provided by both sides of the particle and viewing the back side of a helix through the front effectively changes its hand. As Klug and Berger (1964) have pointed out, if the helix hand were actually changing from right to left along the particle axis, the image would display a region of disorder at the site of the change; a region of this sort would give rise to reflections on the pattern meridian on the same layer line as the positive contributions from the right- and left-handed regions; none is found. Figures 55a
and b represent the simplest case of observed quaternary conformation where subunits are not clearly resolved but the particle appears to be a continuous helix. The pitch of a continuous helix is given by the layer-line spacing observed because, for this class of structures, the selection rule for reflection distribution reduced to \( \lambda = \frac{Cn}{F} \) (Cochran et al., 1952). The calculated pitch of this flagellum is then \( 48 \AA \), and the intensity distribution (R versus L) is indicative of dominant contrast from the right-handed component of the image which, according to Finch and Klug (1965), is arising from the side of the particle closest to the grid surface. The image has been printed and diffracted so that the proper screw-sense of the particle is preserved and is therefore determined to be a right-handed helix.

The herring-bone appearance of the flagellum in Figure 55c is again indicative of imaging from both sides of the particle; the corresponding transform (Figure 55d) reflects this phenomenon and similarly demonstrates right-handedness and a pitch of \( 48 \AA \). Figures 55e and f demonstrate typical results when the subunits of the structure are resolved; the indications of particle pitch and hand are recorded as before, but additional, less intense reflections are found closer to the equator which make pattern solution more complex. A strict limitation of optical diffraction procedures is thus exemplified. Not being able to resolve the
the rise per subunit in the particle makes it impossible to obtain reflections for \( n = 0 \), and, without this reference, a non-ambiguous assignment of order to the reflections obtained is similarly impossible. In the absence of the \( n = 0 \) layer-line spacing, the structure can be analyzed as a set of superimposed helices, and a logical structure solution might result if the degree of particle flattening could be determined.

**Trichocysts**

Trichocysts were obtained from the ciliate protozoan, *Paramecium aurelia*, by suspending individual organisms in drops of culture medium on carbon-coated grids; discharge was observed with the phase microscope as the drop dried and uranyl formate stain was applied as previously. Although intracellular debris was present in the grids examined, discharged trichocysts were easily identified by their striated shafts and golf-tee shaped tips.

Since particles were not surrounded by electron-dense zones, it was impossible to determine whether staining was positive or negative; in either case a regularly distributed electron-density resulted and served the purpose of the experiment. Diffraction of the images presented in Figure 56 was useful in pointing out the internal relationships between the periodicities present in *Paramecium* trichocysts.
The image and transform of the shaft (Figures 56a and b) each reveal a 550 Å period that is anticipated for this collagenous structure (Jakus et al., 1942). While other reflections are present along this direction, only those that are balanced with respect to the central spot can be meaningfully analyzed. Non-balanced spots cannot be explained in simple terms but probably arise as a result of particle-frame interactions.

Figure 56d is the transform of a length of trichocyst tip extending over 12 segments. The pronounced discontinuities are spaced 160 Å apart and are perpendicularly arranged with respect to the tip long axis. The individual bands between the discontinuities, therefore, do not represent turns of a continuous helix. Closer analysis of the tip segments reveals that they, too, have a defined substructure and afford an opportunity to test the diffraction procedure on an array of regular structures (a convoluted lattice). Figure 56f is the transform resulting from laser-light diffraction of the three segments inscribed in Figure 56e. The pattern is complex but indication of the 36 Å pitch is very clear. The equatorial splitting of the spots indicates the lateral separations of the three segments and provides an internal standard in two ways; segment periodicities can be calculated with reference to the well-established, longitudinal spacing, and only diffractions
that are equatorially split can have arisen from the apparently identical helices. Non-split reflections can be attributed to the frame, background noise or to one of the three helices that possesses a characteristic spacing not observed for the other two. Reference to Figure 56d reveals that the 36 Å pitch was indicated in that pattern as well but that its reflections were spread by the frame diffractions. Figures 56d and f provide a demonstration, therefore, of the additional lattice convolution due to the frame that surrounds the sample; the use of a nearly square frame (Figure 56f) resulted in nearly circular spots.

**Tobacco mosaic virus (TMV)**

The well-characterized structure of TMV makes it a logical selection for a test object. Furthermore, the use of this virus by Klug and Berger (1964) allowed a basis for evaluation of our results. TMV had been utilized for calibration purposes prior to the development of diffraction procedures, and many micrographs of PTA-treated virus were therefore available. Attempts to diffract these only illustrated the presence of the axial hole which was well-resolved in the micrographs themselves. Uranyl formate staining yielded better-defined particles (Figure 57a) than PTA-treatment had, but the transforms obtained (Figure 57b) were not comparable to those obtained by Klug and Berger.
While the micrographs used did not present the resolution of those published, the 23-Å repeat had been resolved and was reflected in the corresponding transforms.

PTA-preparation, while not affording high resolution did permit observation of large numbers of particles, and occasionally a proteinaceous rod with the stacked-disc conformation was found (Figure 57c). The transform of this structure is implicitly simple and confirms that the averaged density distribution in the corresponding electron microscopic image represented a grating of lines perpendicular to the particle long axis and spaced 47 Å apart. This spacing is roughly twice that expected for the stacked-disc conformation, but it is known (Markham, 1963) that the discs of the rod are paired and that the breadth of vacancies alternates along the long axis; PTA is only providing contrast at the sites of the broader inter-disc separation.

Since initial attempts to evaluate the laser-light diffraction system had been inconclusive for TMV due to the resolution differential between our micrographs and those used by Klug and Berger (1964), it seemed logical to test our system with their samples. Photo-reproduction of negatively stained TMV images from journal-print was not satisfactory, but their presentations of a catalase sample and a model TMV structure were readily photo-copied without appreciable loss of resolution. Figure 58a is a transform
made from a copy of their Plate 1a and displays more orders of diffraction than their Plate 1b while showing less central-spot halation. The transform of the TMV model (Figure 58b) is, however, less favorable with regard to the ratio of signal to halation because this transform was made from a photographic sample which, although very high in contrast, did not provide the absolute contrast of Klug’s mask made of holes punched in an opaque card.

A mask (Figure 58c) mimicking both sides of a TMV helix was constructed, yielded the transform shown in Figure 58d and served to illustrate that the reflections indicative of primitive helix pitch do not fall exactly on the pattern meridian.

**Ciliary microtubules**

Cilia have been of great interest to studies of contraction since their unique behavior is responsible for a large fraction of all biological movement (Fawcett, 1961). The microtubular components of this structure have recently attracted much attention as they are suspected to be the seat of contractility. Ciliary microtubules were obtained from J. L. Olpin, Department of Biochemistry and Biophysics, Iowa State University, Ames, and have been examined by both PTA

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1 Ciliary microtubules were a byproduct of KCl treatment of a rumen protozoan for isolation of the infraciliary protein.
and uranyl formate procedures.

Figures 59a and b illustrate the appearance of these fibrous doublets in PTA preparations and are presented for general information only since optical diffraction was inconclusive. Each component of the doublets is probably made up of several (possibly 10) sub-filaments, some of which are, perhaps, represented by the fibrous material found in the background. Uranyl formate treatment (Figures 60a - c), on the other hand, illustrated the beaded appearance of these sub-filaments on the tubule itself and gave rise to optical transform (like Figure 60d) that indicated a primary helix pitch of 35 Å. The frame diffractions interfere with the pattern on the equator so that the radial spacings are not clearly depicted. The complicated background of the pattern is disturbing but is representative of patterns obtained from samples where regular structure and randomized background were equally high in contrast.

The ciliary specimens provide a classic example of the various ways to view a helical arrangement. While there is indication from the transform that the tubule quaternary structure can be described by a single helix, microscopic evidence implicates the presence of individually stable strands of material, many of which would be required to satisfy the tubule's dimensions. The salient observation here is that the diffraction pattern of a microscopic
image is derived not from the biology of the structure but only from a pattern of transparencies and opacities.

**Phage ML3**

Observations on phage ML3 by negative staining techniques did not directly reveal a helicity although such a quaternary structure would be anticipated from theoretical considerations. Diffraction of the best resolved images of ML3 was seldom possible since particles were rarely straight for segments of more than a few hundred Ångstrom units. The particles illustrated with the corresponding transforms in Figure 61 were among the most suitable samples found.

Freeze-dry negative staining with PTA (Figure 61a) allowed resolution of helix pitch and hand as demonstrated by two sets of off-meridian reflections indicating axial separations of 52 and 85 Å (Figure 61b). The clarity of the pattern and the absence of background is attributed to the lack of random stain distribution when freeze-dry negative staining was used. Although a high degree of ordered structure is not noticed in the micrograph, it is reasonable to assume that the great majority of electron densities are meaningfully situated on the turns of the ML3 helix. The particle is left-handed if contrast contributions are predominantly from the side of the particle closest to the grid surface; this may not be the case for frozen-dried,
negatively stained samples since only the top side of such particles may be accessible to the heavy-metal. The inner pair of reflections confuses the question of contrast origin unless it indicates a second periodic spacing due to a secondary helix-series of opposite hand. The non-integral ratio of the observed spacings can be taken to mean that the number of turns per axial repeat is not a small number. In the absence of a value for the zero-order Bessel function (n = 0) which would fall on the pattern meridian, the helix selection rule cannot be solved non-ambiguously.

ML3 particles prepared by uranyl formate procedures (Figure 61c) displayed evidence of subunit structure and gave rise to optical transforms of the type presented (Figure 61d). The pitch of 52 Å is again indicated for the ML3 primary helix, but the separation of the inner pair of reflections has been increased to suggest a 75 Å spacing.

In summary, three helix series of ML3 have been recorded. Left-handed series of 52 Å and 75 Å and a right-handed series of 85 Å pitch are indicated.

Non-sheathed phage tails

Microscopic observations suggested that the tails of the examined phage of S. aureus would provide very simple diffraction patterns indicative of a stacked-disc structure and that the conformational change that accompanied ghosting
would be reflected only by a slight decrease in pitch.
Figures 62 - 65 indicate that the conformational change is
more complex than anticipated.

Frames lettered b (Figures 62 - 65) are transforms of
non-ghosted, particle tails; each of these displays the
strongest intensity on those layer-lines corresponding to
a mask periodicity close to that measured directly from
micrographs as band separation. The ghosted particle-tails
(Figures 62 - 65, frame d) yield greater meridional spacing
denoting a measureable decrease in pitch and show additional
off-meridian reflections closer to the equator. The change
in helix parameters is most graphic in Figure 64, the series
on $\phi$-80. The pitch decrease indicated is 9% (from 55 Å to
50 Å), and the reflections added in the ghosted tail trans­
form are distinct from background and represent spacings
of higher order for series B and C. The significance of
this change in the transform is not immediately clear, but
it might be proposed that the higher order helix series are
resolved in ghosted particles. Only one (series B) of the
pairs of reflections demonstrates the axial splitting of the
pattern on the mask, but the equatorial separation of these
spots indicates a spacing of over 150 Å.

The $\phi$-6 patterns (Figures 65b and d) demonstrate a
slightly different phenomenon; pitch decrease is also about
9% (from 47 Å to 43 Å), but the ghost transform displays
pitch reflections almost exactly on the meridian whereas
the intact-particle transform suggests the presence of a
non-zero-order helix. If the particle was uniformly dis­
torted upon drying, an array of stacked-discs might be
skewed out of the radial direction making its transform
appear helical. While some disorder is undoubtedly present,
we have generally assumed that it was random, and that the
transform would serve to "iron out" the angular irregulari­
ties.

The frozen-dried specimen and its transforms (Figures
66a and b) are low in contrast; here, where particle flatten­
ing should be minimal, the equatorial spreading of the
pitch-reflections is most strongly noted.

The sheath of T^4

Optical diffraction of PTA-treated, extended, T^4 sheaths
yielded a pitch determination of 42 Å. Patterns were simple,
placing the pitch reflections off-meridian and thus indi­
cating a true helicity. The transforms obtained from uranyl
formate-treated specimens (Figures 67 and 68) were complex
and yielded information about more than the turn-to-turn
period of the sheath helix.

The gross features of patterns obtained were consistent
with the structural evidence of contraction that was pre­
viously noted. Pattern complexity for extended sheaths
was reduced to three significant pairs of reflections (Figure 68). The distribution of these reflections in sheaths showing greatest extension (Figure 68b) suggested their assignment to layer lines 5, 12, and 17. Slightly contracted sheaths, as evidenced by the decreased ratio of length to width, yielded transforms that positioned reflections on layer lines 2, 5, and 7 (Figure 68d). Totally contracted sheaths maintained the 42 \(\AA\) pitch indications but had lost the other series (Figure 68f).

Correlating these findings with microscopic images points out that the nearly radial striations are maintained while their angular disposition is diverted toward the long axis. PTA-treated, contracted sheaths (Figure 67) revealed a second set of striations, more nearly axial but never with enough contrast or repeats to permit detailed optical analysis. Nevertheless, the observations of the helix series and the end-view of contracted sheaths will be instrumental in the proposition of a model structure.

Indications of subunits are present in the sheath images but are not resolved well enough to allow registration of the \(n = 0\) reflection that would indicate the axial rise per subunit. Therefore, a non-ambiguous selection rule for the sheath helix cannot be assigned, but the observation of reflection movement during contraction will permit a qualitative description of the subunit rearrangement.
Diffraction of cores was complicated by lack of striation contrast in PTA-preparations and the absence of straight segments with uranyl formate treatment. The two successful attempts at core analysis (Figures 69b and d) revealed only that the core had a 42 Å repeat period and, probably, a stacked-disc structure.

**Phage head substructure**

The granular appearance of phage heads in most preparations was similar to the grain of the background and, therefore, was not considered indicative of subunit structure. In isolated instances (Figure 31), a capsomere-like appearance was noted. The size of the units observed was about 50 Å, and it seemed reasonable that these represented structure units rather than whole capsomeres. A precise knowledge of the subunit-to-subunit spacing, when used with a knowledge of capsid diameter, would permit calculation of the number of subunits per phage from purely geometric considerations. A non-ambiguous solution on this basis, however, is dependent on a knowledge of subunit size with virtually no percentage error.

Regions of phage heads that lie parallel to the grid surface should present a view of the hexagonal arrangement of subunits or capsomeres and an array that would yield a diffraction pattern expressing the symmetries present. Models
of phage heads were built simulating the appearance of ideally negatively stained, icosahedral shells\(^1\) (Figure 70). The inscribed areas on these photographs were diffracted and showed (Figure 71b) that the six-fold symmetry of the projection was prevalent when sample areas had been chosen from the flattest parts of phage-heads.

Figure 71d is an optical transform of the ring of six globules seen on the head of phage 83 (Figure 71c). Six fold symmetry is present but not as obviously as desired because of contrast limitations. The determination of sample spacing in a hexagonal array is done by treating spot positions as representative of perpendicular separations between rows of spots in the mask. In this fashion the packing parameters of the surface can be determined. Surrounding the sample with a hexagonal frame would guarantee a hexagonal spot-distribution but frame-contrast is so much greater than that of the sample that the sample's transform would be overwhelmed. (This problem is comparable to that encountered with diffraction of cilia where the longitudinal striations were not clearly indicated on the pattern since

\(^1\)Model structures were made by folding photographs of an out-of-focus hexagonal array of pennies. It is interesting to note that hexagonal arrays of circles become arrays of hexagons when out of focus; this suggests caution in the assignment of shape to subunits in close-packed array.
they were coincident with frame diffractions.) The circular character of the transform recorded (Figure 71d) is due to the frame used; indications of hexagonality are present but not considered quantitatively meaningful.\footnote{Klug and Berger (1964) mentioned that they had begun experimentation with optical diffraction of circular arrays but that there were complications. While they did not elaborate on the difficulties, we would assume that pattern interpretation with low-contrast samples was a primary hindrance for them as well.}

Transforms of the T4 head (Figure 72b) reveal no regular, contrasted structure. The presented transform shows many orders of the frame diffractions with no indications of any significant reflections from the sample. Negative results of this sort are also informative when the system used to test for irregularities has otherwise given positive results in a consistent fashion. The subunit model of an elongated virus head (Figure 72c) is presented here for comparison with strict icosahedra; the reasons for its proposition will be covered in "Discussion".
Figure 55a. Micrograph of flagellum used to generate pattern of Figure 55b. PTA-treatment, dropper method. X 285,000

Figure 55b. Transform obtained from image of Figure 55a with a mask magnification of X 30,000. Meridians have been inscribed in this figure and in Figures 55d and 55f to indicate equatorial disposition of significant reflections. R and L (right and left) indicate the handedness indicated by the diffractions (arrows); it is clear that the most intense reflections are from the right-handed image components. Transform magnification X 2.0

Figure 55c. Micrograph of flagellum used to generate pattern of Figure 55c. Only the top fourth of this image was straight enough for masking. PTA-treatment, dropper method. X 330,000

Figure 55d. Transform from top region of micrograph in Figure 55c which was vertically oriented. Right-handed image components have again given rise to the strongest pair of helix-reflections (arrows). Mask magnification X 30,000, transform magnification X 2.0

Figure 55e. Micrograph of flagellum used to generate pattern of Figure 55c. (A section of tail from 0-80° is present at top of image.) Uranyl formate-treatment. X 260,000

Figure 55f. Transform from lower 75% of image in Figure 55c. Arrows indicate the right-handed contributions to the pattern which indicate a 48 Å spacing as in Figures 55b and 55d. Left-handed contributions (pair across the meridian and slightly closer to the equator than those indicated by arrows) suggest a slightly greater pitch and probably arise from a higher order helix series. Mask magnification X 60,000, transform magnification X 4.0
Figure 56. Diffraction of trichocyst components. Figures 56a and 56b are the image and transform of uranyl formate-stained trichocyst shaft. Arrows in Figure 56b point to reflections routinely obtained with images of trichocyst shafts; the skewed position of the reflections is due to non-rectilinear adjustment of the frame used. Meridional displacement of diffraction indicates a 550 Å spacing. (Micrograph shown at X 48,300, transform registered with mask at X 15,000 and magnified X 7.25). Figures 56c and d are the image and transform of a uranyl formate-stained trichocyst tip. Image shown at X 220,000. Transform registered with mask at X 15,000 and magnified X 2.7; mask was aligned with arrow vertical (from Figure 56c) and framed as indicated. Figure 56e (Uranyl formate-treatment, X 520,000) is a micrograph of three segments of the trichocyst tip shown in Figure 56c. Figure 56f is a transform from Figure 56e. Right- and left-handed components are indicated (R and L) and a 36 Å pitch is indicated. Transform registered at mask magnification X 30,000 and magnified X 2.0
Figure 57a. Images of uranyl formate-stained TMV. X 270,000

Figure 57b. Transform from image furthest to right in Figure 57a. Arrows indicate reflections of 23 Å pitch of specimen helix. Mask used X 30,000. Transform enlargement X 2.0

Figure 57c. PTA-prepared TMV showing a particle with stacked-disc conformation (inscribed by rectangle used as frame for Figure 57d. X 86,000

Figure 57d. Transform of mask inscribed in Figure 57c. Stacked-discs with 47 Å separation are indicated by disposition of the very pronounced reflections. Mask used X 30,000. Transform enlargement X 4.0
Figure 58. Diffraction of idealized samples. Figures 58a and 58b are transforms obtained from photocopied masks of Klug and Berger (1964) (their Plates la and lc respectively). A mask of holes representing projection of both sides of a TMV-helix is shown in Figure 58c while Figure 58d is the transform obtained from mask of Figure 58c and shows doubling about the meridian of the pattern from one side of the helix (Figure 58b). Arrow heads point out the layer-lines on which fall the reflections from the three dominant particle helices.
Figure 59. Two views of PTA-prepared ciliary microtubules as isolated by salt treatment. The doublet nature of these fibers is clearly indicated; the unlabelled arrow (Figure 59a) shows a point of disruption of the pair where one of the components has apparently been degraded. The possibility of a quaternary structure composed of many filamentous strands is indicated by the presence in the background of long thin transparent strands (F). X 130,000
Figure 60a. Individual microtubule from ciliary preparation demonstrating lateral projections (arrows) along the shaft. Uranyl formate-treatment. X 140,000

Figure 60b. Three ciliary doublets illustrating a surface granularity common to most preparations of this material similarly prepared. Uranyl formate-treatment. X 185,000

Figure 60c. Image of mask used to generate pattern of Figure 60d. Section of a single component from a ciliary doublet. Uranyl formate-treatment. X 280,000

Figure 60d. Transform obtained from mask of Figure 60c. Arrows point out balanced reflections which indicate right-handedness and a primitive helix-pitch of 35 Å. Mask used X 30,000, transform enlargement X 2.0
Figure 6la. Image of frozen-dried, negative stained M13 particle straight segment of which was tightly framed and used as mask for Figure 6lb. PTA-treatment, freeze-dry negative staining. X 90,000

Figure 6lb. Transform from straight segment of image in Figure 6la. Arrows indicate significant reflections. Primitive helix pitch of 52 Å and left-handedness are indicated (P). Secondary reflections suggest a helix of opposite hand and a pitch of 85 Å. Mask used X 30,000, transform enlargement X 2.0

Figure 6lc. Image of phage M13 as framed for diffraction. Uranyl formate-treatment. X 650,000

Figure 6ld. Transform of image in Figure 6lc. Primitive helix-pitch is again 52 Å (P), but unlabelled arrows denote balanced reflections indicating an additional left-handed helix with a pitch of 75 Å. Mask used X 30,000, transform enlargement X 4.0
Figure 62a. Image of Ø-77 tail (intact) used for registration of pattern in Figure 62b. PTA-treatment, dropper method. X 190,000

Figure 62b. Transform of image in Figure 62a suggesting stacked-disc structure and 55 Å pitch. Mask used X 30,000, transform enlargement X 4.0

Figure 62c. Image of Ø-77 tail (ghost) used for registration of pattern in Figure 62d. PTA-treatment, dropper method. X 190,000

Figure 62d. Transform of image in Figure 62c with balanced reflections indicating a pitch of 50 Å. Mask used X 30,000, transform enlargement X 4.0

Figure 63a. Image of Ø-81 tail (intact) used for registration of pattern in Figure 63b. Uranyl formate-treatment, X 190,000

Figure 63b. Transform of image in Figure 63a demonstrating stacked-disc structure and a pitch of 47 Å. (The transforms of this figure and Figure 63d were among those made with a wide range of mask magnifications in order to test the quantitative nature of the reciprocal relationship between mask and pattern.) Mask used X 28,000, transform enlargement X 4.0

Figure 63c. Image of Ø-81 tail (ghost) used for registration of pattern in Figure 63d. Uranyl formate-treatment, X 190,000

Figure 63d. Transform of image in Figure 63c with strong reflections at meridional positions (arrows) indicative of 43 Å pitch at this magnification of mask and pattern; pattern complexity is unexplained since comparable masks rarely gave rise to such a distribution of intensities. Mask used X 26,000, transform enlargement X 4.0
Figure 64a. Image of Ø-80 tail (intact) used for registration of pattern in Figure 64b. PTA-treatment, dropper method. X 190,000

Figure 64b. Transform of image in Figure 64a. Clear indication of 55 Å pitch and stacked-disc nature of the specimen is provided. Mask used X 30,000, transform enlargement X 4.0

Figure 64c. Image of Ø-80 tail (ghost) used for registration of pattern in Figure 64d. PTA-treatment, dropper method. X 190,000

Figure 64d. Transform of image in Figure 64b. The A series of reflections indicate a 50 Å pitch. See text (page 206) for details of pattern. Mask used X 30,000, transform enlargement X 4.0

Figure 65a. Image of Ø-6 tail (intact) used for registration of pattern in Figure 65b. Uranyl formate-treatment. X 230,000

Figure 65b. Transform of image in Figure 65a. Balanced reflections indicate a stacked-disc structure with a 147 Å pitch. Mask used X 30,000, transform enlargement X 4.0

Figure 65c. Image of Ø-6 tail (ghost) used for registration of pattern in Figure 65d. Uranyl formate-treatment. X 190,000

Figure 65d. Transform of image in Figure 65c. Reflections are still intense but considerable disorder in equatorial region is evident. Mask used X 30,000, transform enlargement X 4.0
Figure 66a. Image of Ø-80 ghosted tail used for diffraction. PTA-treatment, freeze-dry negative staining. X 490,000

Figure 66b. Transform of image in Figure 66a. Arrows indicate reflections that describe a pitch of 50 Å. The intensity of reflections is not balanced about the meridian and suggests right-handedness if only slightly. Mask used X 30,000; transform enlargement X 4.0
Figure 67. Sheaths and corresponding transforms. Figures 67a through 67h are uranyl formate-treated sheaths of T4 and their corresponding transforms. All samples indicate a primitive helix pitch of 42 Å (note strongest reflections close to the meridian. Micrographs shown X 215,000, masks used X 30,000, transforms enlarged X 2.0. Figures 67i through 67l are contracted sheaths of T4 (PTA-preparations) and their corresponding transforms which show again a pitch of 42 Å but in which reflections are further from the meridional axis of the transform. Figure 67i magnification X 210,000, Figure 67k magnification X 480,000. Masks used X 30,000, transforms enlarged X 2.0.
Figure 68a. Images of uranyl formate-treated sheaths of T4 showing three stages of contraction. Micrographs are presented with approximately the same degree of contrast that was used for registration of patterns (Figures 68b, 68c and 68d respectively). X 575,000

Figure 68b. Transform of image in Figure 68a (image b) revealing three sets of reflections (arrows) which are balanced about the equator and which might be assigned to layer-lines 5, 12 and 17. Mask used X 30,000, transform enlargement X 4.0

Figure 68c. Transform of image in Figure 68a (image c) again revealing three sets of balanced reflections but with a suggest layer-line assignment of 2, 5 and 7. Mask used X 30,000, transform enlargement X 4.0

Figure 68d. Transform of image in Figure 68a (fully contracted sheath, image d) demonstrating the persistence of the furthest spread diffractions but lack of registration of the others indicated in Figures 68b and 68c. In all transforms the primitive helix-pitch suggested is 42 Å. Mask used X 30,000, transform enlargement X 4.0
Figure 69a. Uranyl formate-treated core of phage T4 as separated from the particle by overnight peroxide treatment. Area inscribed in rectangle was used for generation of the transform in Figure 69b. X 475,000

Figure 69b. Transform of image inscribed in Figure 69a. 42 Å pitch is indicated, but reflections are very weak. Mask used X 30,000, transform enlargement X 4.0

Figure 69c. PTA-treated core of phage T4 as isolated by peroxide treatment and framed as used for diffraction mask. X 340,000

Figure 69d. Transform generated by diffraction of mask pictured in Figure 69c. Strongest pair of balanced meridional reflections again suggest a pitch of 42 Å. Mask used X 30,000, transform enlargement X 4.0
Figure 70. Models of various size "spherical" or icosahedral capsids constructed according to 5:3:2 symmetry. Each model is built from the same size capsomere; capsomere numbers of each are in parentheses beside the images. Images of this type are made with uneven illumination of model structures to simulate the variability of contrast in negative staining of viruses.
Figure 71a. Model of icosahedral capsid with circled region inscribing a reverse-contrast image of the mask used for Figure 71b

Figure 71b. Transform of mask in Figure 71a. Six-fold symmetry is readily apparent but measurement of capsomere spacing is not possible even with an idealized mask

Figure 71c. Ø-83 head from Figure 31 with circled region inscribing a reverse-contrast image of the mask used for Figure 71d

Figure 71d. Transform of mask in Figure 71c. Six-fold symmetry is suggested; arrows indicate directions of greatest density toward the center of the pattern but do not fall precisely on axes of a hexagon
Figure 72a. Micrograph of phage T4 with rectangle inscribing region of head used to generate the transform of Figure 72b

Figure 72b. Transform from Figure 72a. Only frame symmetry (rectangular) is evident indicating that no true regularity exists in the granularity observed in the microscopic image.

Figure 72c. Micrograph of PTA-stained T4 head and model proposed for conformation of the dried down specimen. The model structure has 280 capsomeres, but this number is not necessarily proposed for T4; a large range of capsomere numbers can be fitted to this proposed shape. Note similarity of contours.
Figure 73a. Transform of slightly bent spring (insert) demonstrating the unevenness of pattern registration when a helical mask is not perfectly straight.

Figure 73b. Transform of a true sine wave with a central line (insert). This mask is an idealized drawing of a true helix with an axial hole; axial hole has contributed to the complex distribution of intensity on the equator.

Figure 73c. Transform of a mask (insert) that allowed transmission of light through the teeth of one side of a bolt. This demonstration points out that a cleanly shadowed image of a helix could give rise to characteristic diffractions on the basis of the contoured edge. Transform extends into four quadrants because both top and bottom of gyres are seen by the illuminating beam.

Figure 73d. Transform registered by illuminating both sides of the bolt used in Figure 73c. This is a simple lattice convolution; each side of the bolt-image generates a continuous helix pattern but the separation of the two sides effects a splitting along the selected layer lines.
DISCUSSION

The data of a structural study are self-explanatory in many respects, but dimensional inconsistencies from one study to another are common in the literature on virus structure. Therefore, in order to discuss the significance of observed structures, an effort has been made to acquire independently all of the required dimensional data within this report. Quantitative statements will only be emphasized as precise indications of relative changes in conformation.

Non-Sheathed Phage

Microscopy of the lysogenic staphylococcal phage has provided consistent results; differences in the individual strains have been observed only as variations in the relative dimensions of common structures. It is generally assumed that the DNA-transfer function of the phage capsid is also common to all strains and that this transfer is from the head-reservoir through the tail. The degree and mechanism of capsid involvement in DNA-transfer is not well understood; it follows that a thorough understanding of phage quaternary structure is essential to a discussion of the process. In order to discuss phage structure in terms of function, we must first take a general view of what is actually involved.

The passage of a very long, 20-Å wide molecule of DNA
through a 2000-Å long channel of approximately the same diameter is mechanistically unsound without evidence for a powerful driving force. To account for the required force, Horne and Wildy (1962) and other authors have proposed that the great length of DNA is packed into a phage head under considerable pressure and that the mere availability of an opening in the head surface will effect rapid extrusion of the viral genome. This proposition is implicitly simple, but there is no evidence that packaging under pressure can take place in a bacterial cell. The problem of neutralizing the high negative charge on an elongated DNA molecule is partially resolved by the inclusion of polyamines and basic proteins; there is no evidence to suggest how these positively-charged components are neutralized to allow subsequent DNA unfolding for the transfer process.

A contractile tail structure does not, in itself, explain the forced linear passage of DNA but is routinely offered as the only solution to the problem. Similarly the question of DNA-transfer by phage not possessing contractile structures is seldom discussed. Both the suggested existence of episomes without proteinaceous capsids and the demonstrated ability of free phage DNA to infect the appropriate host successfully (Adams, 1959) become fundamental to this discussion since these phenomena suggest that the host can accept nucleic acid without active assistance from the
proteinaceous structure of a phage particle.

Therefore, we must consider the possibility of a passive and/or an active capsid role in the transfer of DNA to the bacterium. With these considerations, we can discuss each of the phage structures observed with regard to its potential role in the process. Conclusions about observed structural changes associated with ghosting may lead to propositions of transfer mechanisms, but these must be appreciated as theoretical and based on the assumption that particles which we have called ghosts are structurally identical to phage that have injected their DNA into a bacterium.

**Basal structures**

Six-fold symmetry was observed for all of the basal structures that permitted observation. Thornely and Horne (1962) observed this symmetry in the bacterial cell walls that they examined and reviewed similar findings from other studies.\(^1\) Steric fit between phage tail and host surface is thus accounted for; the receptor and base-plate need not

\(^1\)Hexagonal packing requirements and consequently six-fold symmetry are expected to be applicable for structures much larger than virus capsids. Caspar and Klug (1962) discuss this and cite the unpublished comments of R. Buckminster Fuller who has even applied these principles to architecture (the geodesic dome).
possess identically sized faces since concentric alignment of hexagonal rings will place all of the subunits of the base-plate in equivalent environments.

The proposition of a six-fold symmetric base-plate is disputed by other authors. Takeya and Amako (1965) claim a pentagonal ring structure for the base-plate of a Mycobacteriophage, and Bradley (1963a) suggests the same symmetry for T5; they substantiate their claims by proposing that five-fold symmetric base-plates are required since they are attached to the tail which is necessarily five-fold symmetric because of its attachment to an icosahedral vertex. In opposition to their presumption, Moody (1965) pointed out that six-fold and five-fold structures can accommodate one another. Evidence submitted here that phage tails end proximally in a specialized structure not necessarily attached directly to the head membrane also alleviates the symmetry requirements; further, the ring structures observed in the staphylococcal lysates are definitely six-membered. Correlation of the dimensions of observed hexagonal rings and side-views of base-plates attached to tails constitutes additional evidence that the hexagonal rings are isolated basal structures in end-view. The conclusions cited above for T5 and the Mycobacteriophage were somewhat dubious. If a hexagon is not lying exactly perpendicular to the line of sight, it can present a nearly pentagonal appearance;
the converse is not true. It is safe to conclude that observation of a six-membered ring is more positive indication of real structure than observation of a five-membered ring.

With regard to the function of basal structures, less definite conclusions can be drawn. Base plates were present in both intact and ghosted particles and cannot, therefore, be implicated as "stoppers" or "plugs" for the channel in the phage tail; the round structure seen under the basal platform of an intact particle (Figure 26c) is more suggestive, however. While a knob of this description was seldom observed with such clarity, it was noted that under less favorable conditions the base-plates of ghosts were routinely more sharply defined than those of intact particles.

There is, then, strong evidence indicating that basal structures are six-membered in keeping with the symmetry anticipated for the receptor site, that a structural modification of base-plates accompanies ghosting and that a centrally located mass within the basal structure is the most likely component for a plugging function.

**Tail shafts**

The measured tail lengths of assembled phage were highly consistent; although tails are variable in appearance from ghosts to intact particles, they are uniform within each of
these states. Evidence has been presented for the existence of unattached, variable-length polymers of tail subunits in all preparations. Why, then, do the tails of assembled phage exist only in prescribed lengths?

The possibilities for control of this consistency should be examined before a geometric explanation can be proposed for the change in conformation from one state to the other. The only generalization that can be made about helical symmetry is the statement that every helix is symmetrical about its longitudinal axis. A helical array of subunits may thus be described crystallographically as a space group as opposed to isometric shells which are point groups; significantly, therefore, helices are not self-terminating. Infinite addition of subunits is possible without alteration of symmetrical parameters or the constancy of subunit bonding sites.

Length-limited helices are found for many other biological structures, but only for viruses have they been well understood. The RNA of TMV is responsible for the 3000Å length of the infective unit; in its absence the length distribution of TMV rods is indeterminate. Bacterial flagellin forms flagellum-like helices in vitro (Abram and Koffler, 1964; Asakura et al., 1964) with a range of lengths, but flagella attached to bacteria are helices of almost constant, species-prescribed dimensions. Subunit polymerization with
no internal or external limitation is expected to result in a Poisson distribution of aggregate lengths (Oosawa and Kasai, 1962). The space-group nature of helices is, therefore, a hindrance to a simple, self-assembly mechanism which results in an aggregate of finite size. Consequently, additional considerations exist, and a regulator of helix length must be invoked; a nucleic acid strand whose genetically determined length is present in a bacteriophage might logically be postulated as a length regulator.

Lanni's (1965) work with phage T5 proves that DNA is transferred from that phage to its host in at least two fragments, but whether or not the fragments are separate within the undischarged phage particles is not indicated. If, in fact, the DNA lengths are maintained separately, Lanni's findings could be extended to suggest a precise length of DNA (the first-step-transfer by her terminology) in the tail of T5. The assignment of that location is particularly attractive because it physically positions this genetic fragment for injection before the bulk of the DNA.

Our findings support this hypothesis by showing a variation of tail structure from normal to ghosted particles; ghosts display axial tail holes approximately 20 Å in diameter. Nonetheless, a 20-Å axial hole ambiguously implicates the loss of DNA; an axial hole is logically assumed for every helical array since geometric construction
requires no subunit connections across the longitudinal axis. Such holes are not necessarily of resolvable diameter and, even when they are, may not be permeated with sufficient heavy-metal to provide contrast in the electron microscope. Therefore, it is reasonable to assume that intact and ghost tails may have axial holes of comparable diameter, but intact tails do not permit heavy-metal penetration.

Axial location of DNA in the tail helix would constitute a unique protein-nucleic acid relationship. If the tail of ø-80, for example, contained an axial DNA strand it would represent the only such case known for a viral structure since a tight co-crystallization of nucleic acid and protein is generally understood as the packaging solution for rod-shaped viruses. Nixon and Harrison (1959) demonstrated a strong similarity in the specific locations of RNA within the protein matrices of TMV and TRV. TRV was shown to have a comparable pitch (23 \AA) and protein-to-RNA ratio as TMV; yet TRV measures only 2000 \AA in length as compared to 3000 \AA for TMV. Caspar and Klub (1962) suggested that the RNA of TRV could be located at 3/2 the radius as in the TMV particle and cite the unpublished X-ray results of Finch and Klug. Rod assembly is thus conventionally regarded as a co-crystallization with the inference that a natural preference exists for specific kinds of overall packaging of nucleic acid and protein.
The first-step-transfer fragment of T5 was found to comprise 8% of the total phage DNA (Lanni, 1965). From the data reviewed by Stent (1963), a DNA molecule of this molecular weight \((7.2 \times 10^6)\) would have a length of approximately \(2.78\mu\) or more than sixteen times the length of the T5 tail \((1.700\mu)\). It is safe to conclude that a molecule of this length cannot be accommodated by the T5 tail in a "conventional" type of nucleic acid protein co-crystallization.\(^1\) The type of co-crystal found for TMV and TRV does not afford release of nucleic acid without disruption of the protein helix, and our evidence shows ghosting is non-destructive to the phage capsid.

Thus our discussion about tail structure in bacteriophage has led to two conclusions. First, the constancy of tail length suggests a regulated assembly system as for infective TMV. Second, a co-crystallization of nucleic acid and protein is the logical mechanism for length limitation, but known packaging methods (e.g. for TMV) do not seem applicable.

A reasonable alternate hypothesis can explain the observations. A fraction of the viral genome extends from

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\(^1\)If the DNA molecule were 19 - 20 Å wide, it would require 88 contiguous turns of a helix at a radius of 50 Å to package the structure, this does not seem even remotely probable.
the phage head for a distance equivalent to the measured tail length, and tail protein subunits are positioned about this structural axis. Several lines of circumstantial evidence support this theory. First, unattached phage tails found in the examined lysates are variable in length; all of those that are not of intact-phage length reveal axial holes and a banding pattern indistinguishable from tails on complete, ghosted particles. The absence of DNA in both (as proposed) might explain the conformational similarities between ghosted and free tails.

Second, the findings of Klug et al. (1966) and Finch and Klug (1966) on TYMV structure prove the presence of a specific quaternary conformation for RNA within an icosahedron. Extension of this information to DNA-bacteriophage heads is reasonable in light of the evidence for pre-formed DNA cores in phage maturation (Kellenberger, 1961); a stable ball of DNA could have a comparatively short, uncoiled segment that would direct tail protein polymerization. The bulk of the nucleic acid on the basis of its conformation, could provide a very different immediate physical environment and, therefore, foster the crystallization of an isometric shell from a chemically unique strain of protein subunits (head protein). If a specificity of DNA folding in the head-core is present to a high degree, a distinct length, if any, would be expected to extend, and a minor
degree of uncertainty in this specificity would then account for the slight variations found in tail lengths for complete capsids.

Third, in icosahedral-headed ghosts, tails apparently terminate about 100 Å inside the head. This phenomenon cannot be examined, unfortunately, in intact phage because heavy-metal penetration is lacking; a modified band of possible tail material is observed at a site closer to, but yet inside, the head membrane of elongated phage (Ø-6, Ø-42b and Ø-81) as well. The location of this proximal terminus suggests that non-sheathed phage tails arise from a point within the head-membrane and that the maintenance of head and tail attachment is not wholly due to an interaction between the two kinds of protein.

The most striking supporting evidence for this theory comes from the reduced-cation-concentration treatment on Ø-80 that resulted in a separation of the head membrane from the tail stem, leaving the tail attached to an electron-transparent mass which quite probably represents the phage DNA. (The lack of good negative staining in these preparations might be attributed to the absence of cations; this is speculative, but, in any case, the absence of axial holes in phage treated in this manner cannot be considered as evidence for tail-contained DNA.)

There is, then, some support for the hypothesized
presence of DNA in the non-sheathed phage tail. Constancy of length, the comparative appearance of intact, ghosted and free tails and the evidence for a strong attachment between DNA and tail implicate DNA as the regulator of tail-helix length. The corollary to this theorem is: tail protein subunits exist in different helical aggregations depending on the presence or absence of DNA and can convert from one stage to the other upon DNA loss. (No evidence exists for reversibility.)

**Shaft quaternary structure**

The principal contribution of laser-light diffraction studies on the non-sheathed tails has been the verification of a change in tail structure from intact to ghosted phage. The changes reflected are more involved than a decrease in pitch, length or number of bands, because analysis of transforms demonstrates a precise subunit rearrangement possibly common to all phage examined.

When the strongest reflections fall very close to the meridian, definite uncertainty exists as to whether the structure is a stacked-disc or a non-zero order helix. This lack of precision is due to possible errors in frame adjustment and/or sampling over a necessarily limited area. A column of stacked-discs need not have each of the circular components positioned exactly radially and, therefore, can
present a non-zero order helix on its optical transform. The band thickness on staphylococcal phage tails measured very nearly the same as the spaces between bands and made more than one order of diffraction hard to obtain. More significantly, a non-zero order helix with inter-band spaces of this relative size would be expected to show a steep pitch by positioning reflections well off the meridian, e.g., as in the case of flagella or trichocyst-tip segments. It is probable, therefore, that these phage tails are stacked-disc arrays and that the inter-disc connections are loose enough to permit heavy-metal penetration and a detectable amount of radial disarray. Similarly, if the assumptions with regard to DNA in the tail are correct, it would be logical to assume that the heaviest concentration of basic amino acids is in the shaft's center where their net positive charge would best serve a neutralizing function on the linearly arranged phosphates in the DNA-backbone.

Correct assignment of layer-lines is essential to selection-rule solution for a helix transform. In the solution for the transform of a Ø-80 ghost tail, for example, the principal reflections (A) must be assumed to indicate the solution for \( \ell \) when \( n = 1 \) and \( m = 0 \). This is partially verified by the Bragg spacing indicated for the A series which is shown to reflect helix pitch. If the assumed \( n \) and \( m \) values are correct and the helix repeats axially every
turn, \(\frac{c}{p} = 1\), and the primary reflections would be on the first positive and negative layer lines. The presence of series B and C closer to the equator disputes this but affords an opportunity to derive the correct layer-line index. If the meridional spacings of the A, B and C reflections are discreet multiples of a common factor, this factor can be used as the layer-line spacing, and the multiplicity factor represents the index number (\(l\)). There is no natural requirement that \(\frac{c}{p}\) and \(\frac{c}{p}\) be small numbers; they must be integral by definition, however. A degree of uncertainty arises since measured values are not likely to be small, integral multiples of a common spacing, but the roughness of our approximation will be measurable and can indicate the degree of uncertainty in the final solution. The ambiguity in this kind of a solution becomes evident here; without \(n = 0\) values, we cannot be sure whether postulated \(l\) values are correct on an absolute basis.

This approach makes the transform of Figure 64d partially soluble. One such solution places series A, B and C on layer lines 24, 13 and 12 respectively. Reflections B are suspect because of their split nature which does not reflect a frame spacing. A proposed solution uses only A and C and stipulates that the helix has a 50 Å pitch and repeats every two turns. The number of subunits per turn cannot be derived from the transform; however, if they are
spherical, 5.5 and 6.5 are the likely choices. These values are from tail diameter and pitch measurements with consideration of the tail as a non-zero-order helix. A stacked disc array, more likely on the basis of microscopic results, must have an integral number of subunits per turn. The halving of the pattern must then be explained as due to a staggering of the disc subunits from one turn to the next; six subunits for each disc would satisfy the microscopic picture and the potential requirement for six-fold symmetry.

Therefore, a physical description of the non-sheathed phage tail structure and its conformational change upon ghosting can be attempted. The subunits of the intact-particle tail are arranged in a stacked-disc configuration, six per disc. The discs are arranged loosely enough so that heavy-metal penetration is possible between turns, but the subunits of an individual disc are packed too closely to permit the development of contrast. Ghosting is accompanied by the twisting of the discs relative to one another so that the subunits are more nearly hexagonally arranged in the net representation, and an approximation to axial repeat occurs every other disc. The proposed interdigitation of subunits in the twisting of discs would be very slight but could account for an overall decrease in tail length as well as a decrease in pitch.
Sheathed Phage

Tail function

Kellenberger (1961) studied intracellular phage maturation and concluded that the T-even head membrane is built around a pre-formed DNA-core. The acceptance of this sequence removes the necessity to explain the elongated head conformation either as a self-assembled system or in compliance with icosahedral symmetry. The question of T4 head geometry will be discussed, but, for the sake of discourse on tail function, we need only consider whether or not it is assembled as Kellenberger has suggested.

If the T4 head cannot be assembled apart from a pre-formed core or template, all of the ghosted particles seen by electron microscopy must have been intact at one time and subsequently have lost their DNA. Such an assumption simplifies the analysis of the chain of events in DNA-transfer. Evidence that conformational changes in non-sheathed phage tails accompany rather than demand DNA-transfer has been accumulated here, and the possibility of a passive capsid function therefore, should be considered for phage with contractile mechanisms as well.

Observations (summarized in Figures 47 and 48) indicate that sheath contraction and DNA-loss are not necessarily interdependent. The presence of various combinations of
DNA-loss, sheath-contraction and base-plate removal from the core's distal end implicate the last of these as the key to the sequence. Ghosts are not found with contracted sheaths except when base-plates apparently have been pulled proximally along the core but are noted without contracted sheaths in a few instances. Therefore base-plate removal may facilitate but not necessitate DNA-passage, and DNA will be passed, less frequently perhaps, even without base-plate removal. The status of head-shortening or contraction as a prerequisite (Cummings and Kozloff, 1962) is at least as indefinite since it is also observed in combination with contracted and uncontracted sheaths, ghosts and intact particles and in the presence or absence of base-plates at the core's distal terminus.

A great many different linkage sites are apparent in the T4 tail, and an examination of these may clarify the problem. Sheath protein is probably linked differently at the top of the core than along the shaft since the proximal linkage appears to be more stable. (This linkage may be related to the presence of collar protein at that site although evidence is lacking.) The linkages between sheath and core along the shaft are evidently essential for the structural integrity of extended sheath. Kellenberger and Boy de la Tour (1964) arrived at this conclusion when they found no evidence of free, extended sheaths in their
preparations. The distal ends of the sheaths are apposed to base-plates in charged particles, and peroxide-induced sheath contractions can occur with or without elevation of base-plates; the distal linkage is perhaps more sensitive to oxidation than is the proximal sheath-core connection. Core and base-plate are also connected; prolonged exposure to peroxide separates them. The strongest linkage is the proximal connection between core and sheath. This bond is preserved under conditions where all the others mentioned are severed and, therefore, can provide a suitable anchor for the contraction process.

The extended sheath on the intact T4 particle reveals subunit structure, and optical diffraction studies enable us to propose a specific arrangement of subunits. Even so, we can discuss the sheath's stability without the use of a precise model. Negative staining with PTA demonstrated a series of cross-axis striations but no subunit structure and suggested that the separation of helical turns is greater than that of subunits along the primary helix. The presence of super-extended sheaths, apparently composed of continuous, single strands of sheath material, further implicates the greater stability of subunit-subunit bonds across the long axis. Super-extended sheaths, apart from cores, have a fairly constant pitch, a remarkable condition in view of the adverse effects of specimen preparation, evacuation and
electron bombardment on a loosely assembled structure of such proportions. It is thus safe to conclude that super-extended sheath is more stable than normal extended sheath which has not been observed in the free state.

The findings of Barrington and Kozloff (1956) and the micrographs of Kellenberger and Arber (1955) have largely dominated the thinking on DNA-transfer which centers about a "hypodermic syringe" analogy. We have seen little evidence for the interdependence of sheath-contraction and DNA-transfer, but the syringe analogy may be somewhat appropriate with regard to core injection. Since the tail fibers of T4 have the capacity for very strong attachment to the coli wall, the base-plate with its six spikes probably determines a precise, steric attachment to a receptor site (Kellenberger et al., 1965). If this attachment is secure, contraction of the sheath takes place with maintenance of the linkage at its proximal end with the core and distally with the base-plate; the core will be forced against and, perhaps, through the cell wall. The action of a lysozyme may assist core-penetration by digesting a region of the receptor locus. The absence of free, extended sheaths in any micrographs yet observed indicates that a contracted sheath is the more stable structure and that the pursuit of a minimum energy conformation is potentially the driving force for sheath-contraction.
The need for core penetration is questionable according to Cota-Robles and Coffman (1963). Stickler et al. (1965) provide an interesting observation on a bacteriophage of \textit{B. subtilis}; \textit{\textPhi}3610 displays a sheath in both contracted and extended states. Surprisingly, the sheath contraction is directed distally. (The strains isolated by Stickler and his co-workers were induced and have not been carefully examined with regard to their infectious cycles, but the phage described above was shown to be infectious.) In this situation, core injection is not mechanistically feasible as a consequence of sheath contraction. Therefore, core injection might not be a requirement for DNA-transfer.

The core-head attachment site is suggestive of findings for \textit{\textPhi}6 and the elongated staphylococcal phage. An internal electron-transparency is noted in ghost heads and is positioned at the vertex of tail attachment. Multiple-tailed \textit{T4} phage have been observed by Smith and Trousdale (1965), but the only micrograph of a ghost in their presentation did not reveal tail-attachment sites. They have not yet reported further findings on these particles. Moody (1965) discussed multiple tails in terms of the common vertices of the \textit{T4} head and noted the occurrence of an internal "nob" or "plug" (in his terminology) at the attachment site of each tail. Structurally, the core of \textit{T4} so closely resembles the conformation of non-sheathed tail shafts that its function may
also be similar and not dependent on sheath presence.

Womack and Barricelli (1965) have confirmed that only one strand of the T₄ DNA passes genetic information and have verified that the T₄ DNA is a single macromolecule 47 to 50μ in length. With a single molecule, a two-step-transfer process seems unlikely, but the indications of a stacked-disc structure for the T₄ core suggest a similarity to the non-sheathed phage tails just discussed. The phenomenon of an axial hole and a cross-axis series of striations are noted; unattached and ghosted cores apparently have the same structures by negative staining, and the most significant feature is their band-spacing which matches the pitch of extended sheath (42 Å). Kellenberger and Boy de la Tour (1964) proposed identical symmetries for extended sheath and core, but they did not show the core striations clearly. They suggested a required common symmetry for maintenance of the extended sheath conformation since it was never found apart from core. Our data corroborates these proposals, but we would draw less definite and somewhat different conclusions.

Whether or not extended sheath is dependent on the core surface for stability, the symmetries would be expected to match to a high degree to permit a constancy of interaction between the two. Other factors suggest that the interaction does not provide the sum total of extended sheath stability, namely the strong, proximal sheath-core attachment site and
the sheath-base-plate affinity. Contracted sheaths have here been found both with and without ghosting, but the change in core appearance was only found for ghosted particles. Thus, a set of conditions exist wherein the sheath changes its conformation without a corresponding change in the core and during which either sheath-base-plate or core-base-plate linkages are separated. These breaks occur with about equal frequencies in peroxide-induced contractions and suggest that the distal connections are as important as the sheath-core interactions along the shaft for extended sheath stability. This hypothesis cannot be proposed with a great deal of conviction for the contraction process in the infectious cycle since the precise localization of ATPase activity is lacking, but the same phenomena are observed (with very low frequencies) in the absence of peroxide. (It is essential at this point to restate that any conclusions about observed ghost particles rest on the assumption that their structure is identical to those particles which have lost DNA in vivo.)

Examination of the T4 particle has obviated a contradiction in the currently accepted theory of DNA-transfer by this agent. Either the non-icosahedral head is not made on a pre-formed core or sheath contraction is not a requirement for DNA passage. This conclusion rests on tenuous evidence but on the same kind of evidence that gave rise to
the theory it disputes. A safer statement can be made with regard to the behavior of the T4 sheath. Contracted and super-extended sheaths are apparently independently stable structures; normal, extended sheath is not as stable and depends on the distally-disposed tail attachments for stability (as well as on the sheath-core relationship along the shaft).

Sheath quaternary structure

The proponents of sheath-models have been hesitant to extrapolate to situations in which the sheath helix does not repeat once per turn (Bradley, 1963a; Kellenberger and Boy de la Tour, 1964). From a geometric point of view, helices with a one-turn-repeat are no more likely to be formed than any other class. Maximum hexagonal packing considerations are most strongly violated by a one-turn-repeat helix since this provides local environments where subunits are in a pseudorectangular array, i.e. each subunit makes closest contact with only four others. The cylindrical surface, about which a helix may be described, does not appreciably change the energetic status of a local environment; apart from chemical considerations, a pseudohexagonal array is expected for the helix net (unrolled helix) and for the intact helix as well.

Kellenberger and Boy de la Tour (1964) have proposed a
three-stranded helix for the sheath of T₄ (see "Literature Review"). This model is supported by the construction of an analogous situation with a three-stranded, polyvinyl helix embedded in an opaque fluid. The picture presented, while somewhat convincing, is not a perfect duplication of sheath images in electron micrographs. Their proposition further includes the possibility of the loss of one or two of these strands in protein-degrading environments, a degradation which implies in each strand an internal structural integrity surpassing that of the fully assembled sheath.

An apparent confusion over terminology should be clarified at this point. The description of a helix as multistranded is merely one way to characterize the helical parameters. The TMV helix, for example, can be imagined as a single-stranded, sixteen-stranded or seventeen-stranded helix since all of these series are simultaneously present. A helix, again, is an array of regularly spaced units on the surface of an imaginary cylinder. A single, continuous line may be drawn through all of the points on any helix if it passes from one point to the closest of the adjacent points displaced least with regard to the longitudinal axis; this continuous line describes the primary or primitive helix. Multi-strandedness is achieved by drawing a line through neighboring points but by selecting for a greater, regular, vertical displacement than in the primary helix situation;
more than one such line will be required to include all of the points. Examination of this mode of point selection will reveal that the number of such lines required is a measure of the number of subunits per turn. Two different series of multi-stranded helices can always be described, one of each hand. This explanation of multi-strandedness is the basis for a helix terminology (Klug et al., 1958) where a helix series is described on the basis of the number of continuous lines required to include all of the discontinuities. Thus the TMV rod is described as having three series: \( n = 1, n = 16, n = 17 \).

A true multiple-helix situation is one in which more than one molecular chain is actually present in the complete structure. In this situation the optical transform will be the same as for a single molecular chain containing the same three-dimensional distribution of points; thus a single optical diffraction pattern, even perfectly registered, cannot determine the number of molecular chains in the structure. The existence of a single chain in super-extended sheath, continuous when subunits are unresolved, is taken as a strong bit of evidence for the existence of
a single chain\(^1\) in normal-extended and in contracted sheath.

(Super-extended sheaths were dismissed by Kellenberger and Boy de la Tour (1964) as partially degraded remnants of "polysheath", an elongated polymer of sheath subunits in the contracted state. Brenner et al. (1959) and Anderson and Stephens (1964) had also noted this structure, but, in each case, the images did not reveal both sides of the helix.)

As in the case of conversion from a stacked-disc to a non-zero order helix, the change from a multiple- to a single-stranded helix (or vice-versa) involves a complex pattern of rupture and reformation of subunit-subunit bonds. It seems reasonable to postulate a tentative model for sheath quaternary structure on the basis of the information collected from microscopy and harmonic analysis only if: a) the data are satisfied within the limits of their accuracy, b) the findings of other authors (reviewed previously) are considered and c) the resulting model does not involve complexities that defy the substantiated

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\(^1\)Reference to a "single chain" or "single-stranded helix" from this point on, should be understood to mean that the class of bonds that link subunits in the direction of the primary helix are the bonds maintained throughout the quaternary rearrangements of the whole structure. Subunit-subunit interactions in every direction along the surface must be presumed (Tanford, 1964) but these may be more easily distorted or broken.
generalizations about subunit conformations from research on comparable systems.

The transforms of T4 sheaths strongly implicate the presence of a single-stranded helix; this is indicated by the constancy of pitch (42 Å) during contraction. The secondary reflections which apparently shift during the contraction process suggest an adjustment of the higher order helix series as the primary helix is radially expanded (thus increasing the number of subunits/turn). The subunit-subunit bonds are then distorted only slightly in the primary helix and the intermediate turn-turn interactions are too weak to arrest the contraction process once it has started (as evidenced by the low frequency of observed partial contractions). The variability in layer-line indexing is due to the necessary assignment of integral values so that an axial repeat period might be calculated. If n = 1 and m = 0, then \( \hat{l} = \frac{c}{p} \) or the number of turns per axial repeat. This value would assist in characterization of helix parameters but is not very meaningful since very slight changes in sheath conformation obviously cause large variations in axial repeat.\(^1\) Therefore, the basic contribution of the transforms

\(^1\)For example: a rearrangement that shifted the number of subunits/turn from 6.5 to 6.6 would change the axial repeat from 2 turns (13 subunits) to 5 turns (33 subunits); less discreet changes would more radically change the axial repeat period.
is indication of quaternary conformation behaving like a single chain of subunits adjusting the helix radius to accommodate a change in overall length. Although this appears to be very straightforward, it is in strong contradiction to Bradley (1963a) who proposed an interdigitation of subunits, and Kellenberger and Boy de la Tour (1964) who proposed that extended sheath subunits are attached firmly to the core and individually change their conformations to allow interdigitation that results in a rigidly stable, "multi-stranded," contracted state.

A total number of subunits has been proposed only by Brenner et al. (1959) who postulate 200 on the basis of centrifugation of whole sheath and subunits. Bradley (1963a), Fernández-Morán (1962) and Kellenberger and Boy de la Tour (1964) indirectly suggest \( 1^{4/4} \text{ to } 180 \). The number of turns of extended sheath is certainly between \( 2^{4} \) and 25 (not necessarily integral); for contracted sheaths, the number is close to 12 and harder to observe, but end-views strongly suggest \( 15 \pm 1 \) subunits/turn.

A proposed model should account stoichiometrically for the 135 high energy phosphate molecules found by Kozloff and Lute (1959). This number is not easily fitted to the data since at least 4 subunits are frequently seen on one side of a turn-surface in extended sheath. (Assignment of ATP and dATP locations to fit a precise number of core
subunits does not simplify the problem since a reasonable solution should provide equivalent environments for adjacent sheath subunits.)

The following solution is therefore proposed. The number of subunits/turn is non-integral and between 7 and 8 in extended sheath (satisfying the approximate halving of turn-number in contraction with an approximate doubling of subunits/turn to give 14 to 16); the number is close to 7.5 to most nearly satisfy hexagonal packing considerations, and the total number of subunits/sheath is then between 175 and 200. With 195 subunits, the data of Brenner et al. (1959) are 97.5% satisfied and, if 15 of these have no ATP or dATP requirement (since they occur at the top or bottom of the helix), 180 subunits remain to compensate for the 135 high energy molecules with a ratio of 4:3. This ratio does not suggest a discreet position for ATP and dATP in the sheath structure, and the proposed model is only one of many similar ways to explain the data.¹

These proposals should be clearly separated from strict conclusions about T₄ sheath quaternary structure. The principal conclusions are: a) sheath stability is due

¹Solutions that yield a 1:1 subunit/(ATP and dATP) ratio are numerous but all involve either an unreasonably small number of subunits or an unlikely fraction of the total number being exempted from ATP or dATP requirement.
primarily to a single stable chain of subunits bound in a fashion that is not radically altered as the sheath contracts, extends or super-extends, b) the turn-turn bonds of sheath helix are strongest in the contracted state, c) there are at least seven subunits/turn in the extended helix, and d) any detailed sheath model is necessarily conjectural at this point because of the conflicting data about the total number of subunits.

Rod-Shaped Phage

Studies on M13 have illustrated that the virus is probably helical and that freeze-dry negative staining provides nearly "noiseless" results when used successfully. Three helix series have been demonstrated. An axial hole is suggested by some of the microscopic observations on M13 but is apparently very small (10 to 15 Å in diameter).

There are no indications that M13 resembles the tail components of any more complex bacteriophage. The single-stranded DNA (Salivar et al., 1964) of this virus rod may have a pronounced effect on the structure in terms of a DNA-protein co-crystallization since the rod shows a steeper helix pitch than any other of the fibrous phage components analyzed. Tzagoloff and Pratt (1964) determined that only DNA was transferred to the host by this phage in the process of infection. We have found no evidence for a basal
structure or any modifications suggesting rod-polarity; nevertheless, if the M13 subunit is composed of L-amino acids arranged in a helical array as the data suggest, the rod must have a polarity, and only a separation of charge, for example, may be required to allow M13-host attachment. Liberation of DNA could involve breakdown of the rod structure; Tzagalooff and Pratt did only a radioactivity check to determine the continued presence of protein in the environment (after the fashion of Hershey and Chase (1952)).

Model proposition is not justified for M13, but the evidence from this study points to a non-zero order helix for subunit arrangement and an overall structural combination of protein and single-stranded nucleic acid more comparable to plant virus rods than to non-sheathed phage tails. An axial hole is probably present, but the location of DNA in the rod cannot yet be postulated.

**Phage Heads**

**Icosahedra**

Caspar and Klug (1962) have provided a convincing hypothesis of structure in spherical viruses. Icosahedral symmetry is expected to prevail in bacteriophage heads as well. Davison (1963) and deKlerk et al. (1965) have demonstrated a hexagonal packing of subunits and distinctly
icosahedral profiles for two different strains of phage. We have reviewed most of the pertinent findings on phage head morphology and, in the large majority of cases, icosahedral shape as well as symmetry was shown to prevail.

Octahedral heads are observed on rare occasions (Bradley and Kay, 1960), but these results should be viewed with skepticism. Observation of an icosahedron along its two-fold axis can logically be confused with that of an octahedron, and in the cases where octahedra have been claimed, most of the particle was evidently buried in phosphotungstate further confusing the picture. Crick and Watson (1956) had stipulated that an octahedron would satisfy cubic symmetry; such an arrangement would, nevertheless, place subunits in markedly different environments depending on whether they were positioned on a vertex, a side, or an edge. Octahedral 4:3:2 symmetry has not been observed for any viral capsids by X-ray analysis.

The staphylococcal phage described as having icosahedral heads are, in all likelihood, correctly described. In this study subunit structure was almost never observed while other parts of the particle provided well-resolved images. Our own observations lead us to the conclusion that capsomeres, as defined by Caspar et al. (1962), do not exist for any of the phage heads thus far examined. Capsomeres may only become visible when spherical viruses are dried for
electron microscopy. The bunching of discreet groups of five or six would be expected if they were icosahedrally distributed on a sphere. Phage heads are generally more angular than animal virus particles, and icosahedral shape in the hydrated state is proposed. Whether an isometric capsid is icosahedral in shape or just in symmetry, maximum stability is provided by the closest possible approximation to equivalent environments for each of the structure units. Therefore, it would seem logical that capsomeres are not indicative of anything real about the structure but are properly defined as clusters of structure units observable in the electron microscope.

In the final analysis no evidence has been put forth to dispute that the hexagonal profiles observed in negative stained preparations of bacteriophage are indicative of icosahedra. Structure units are generally too closely apposed at the surface to allow heavy-metal penetration. The prominent electron transparent lines observed on ghost heads probably represent the interfaces between triangular facets and the perseverance of these lines in collapsed heads suggests that the head conformation is icosahedrally shaped as well as 5:3:2 symmetrical.
Elongated heads

Moody's (1965) model for the head shape seems the most acceptable of those proposed, but issue might be taken with some of its specific characteristics. An important consideration in the proposal of a structural model is satisfaction of the general rules for comparable structures. A bipyramidal, hexagonal prism (Anderson, 1946; Bradley, 1965) does not meet this requirement. Moody reasoned that local environments should be quasi-equivalent even in structures not strictly icosahedral and tailored his model to approximate this situation.

While there is evidence for icosahedral-shaped heads, there is little reason to emphasize the importance of the individual, equilaterally triangular facets of the surface. These facets are stable structures, presumably because of their orientation adjacent to one another in the completed icosahedron. In this regard Moody's model is lacking. He has extended the central row of ten triangles to isosceles triangles and successfully achieved the measured proportions of the T4 head in a bipyramidal pentagonal anti-prism. The problem is to design both isosceles and equilateral triangles with one common side and identical subunits packed in nearly identical environments in each; there are a limited number of ways to achieve this.

If the unfolded capsid (theoretically speaking) is a P6
or hexagonal net, the subunits are in strictly equivalent environments. Folding the net in a regular fashion can account for an icosahedron as Caspar and Klug (1962) have shown, and if the lines of the folds are traced on the unfolded net, an array of adjacent triangles results (Figure 74a). The vertices of the triangles need not be positioned exactly on subunits, but the three vertices must be in comparable environments. Figure 74b diagrams a possible solution to the extension of the equatorial region as Moody has suggested but with the requirement that all triangular vertices are at comparable positions in the net. Two aspects of this type of solution are clear. First, other solutions are possible, but the isosceles triangle must have a height greater than that of the equilateral triangle by an even number of rows of subunits; second, two different varieties of subunit array will be present along the sides of the differently shaped triangles. Therefore, the resulting elongated (or prolate) icosahedron will be necessarily quantized with respect to its length-to-width ratio, but proportions can be attained by selecting a precise subunit-subunit spacing in the net and positioning triangular vertices appropriately. That the subunits on sides are in

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1 This ratio will be limited to discreet values depending on the number of extra subunit rows incorporated in the isosceles height.
different environments upon folding is the principle objection to Moody's model. Even if the head membrane is assembled about a DNA core, ghosted membranes are stable and should comply with energetic considerations.

An obvious source of faulty reasoning about the shape of elongated phage heads is the unwarranted assumption that head profiles from electron microscopy must represent the kind of folding present in vivo. The naturally occurring conformation of the T4 head, for example, might consist of rigidly defined triangular facets at top and bottom but approximate a true helix in its equatorial region. (A helical equatorial band is usually assumed by those who would justify a helix for the polyhead mutant.) In the state of evacuation observed with the electron microscope, the T4 head undoubtedly has collapsed to some extent. A model might then be proposed for the way in which the head collapses in this situation; this mode of distortion is not subject to the same energy considerations as is in vivo folding.

The model presented previously (Figure 72c) and shown again in Figure 74c is a simulation of the dried or evacuated state of a T4 head and has the unique feature of 20 identical facets in the unfolded state (Figure 74d). The indicated folding pattern involves the formation of equilaterally triangular facets at top and bottom and in the central region
of the body. It has been assumed that the triangular facets at top and bottom might exist in the hydrated state and represent a stable configuration for subunits anywhere in the head structure. The driving force for formation of this model is, therefore, an attempt to fold into the conformation where the maximum number of triangular facets are formed (20). The region connecting equatorial and terminal facets is pictured as being flat but in a more regular fashion than is expected for the head in a negatively stained preparation. The principal justification for this proposed conformation is its being the only model representing the kind of images observed as feasible distortions of an energetically sound structure in the hydrated state.

(A strong qualification should accompany any comments about $\Phi^4$ head structure. The observed geometry is quite variable with the alteration of sample preparation. If all of our observations had been with uranyl formate, for example, the head shape would have readily been dismissed as a slightly distorted icosahedron.)

The elongated heads of $\Phi-6$, $\Phi-42b$ and $\Phi-81$ have dimensions that coincide well with Moody's alternative proposition, that a phage head can be elongated by addition of extra rows of equilateral triangles. Micrographs of $\Phi-6$ heads stained with uranyl formate show a sharp angularity and a ratio of length-to-width which is close to that expected for an
icosahedron with two extra rows of equatorially disposed triangular facets. Broken heads of $\varnothing$-6 suggest a specificity of fracture sites to support such a model and emphasize the possibility of stability for individual triangular facets.

A model has been proposed for the shape of the T4 head in negatively stained preparations; it incorporates 5:2 symmetry and the formation of 20 equilaterally triangular facets. The $\varnothing$-6 head, representative of the elongated staphylococcal phage, is presumed to be 5:2 symmetrical and can be an icosahedron with two extra rows of triangular facets. The general conclusions of this theoretical analysis of head morphology indicate that non-icosahedral phage heads are more subject to distortion in preparation for electron microscopy than icosahedra and that a suitable model should account for this.

**Laser-Light Diffraction**

The current status of laser-light diffraction is difficult to estimate; its greatest usefulness at this point is, perhaps, an elimination of much of the human error in image interpretation. Theoretically, all of the information registered on film as an optical transform is meaningful, but much of what is observed is not easily understood. Optical diffraction theory supposes the complete coherence of incident illumination which is not totally achieved with
Figure 74a. Diagram of an unfolded icosahedron laid on a P6 net. The structure can be imagined as being constructed of five "ribs" (of four triangles each) which are assembled like staves in folding the structure.

Figure 74b. Diagram of a single rib from T4 as viewed by Moody (1965). Moody's proposal is for extension of the middle pair (equatorial) of triangles to achieve elongation of the icosahedron. The diagram shows several stages of elongation (multiple triangles included within one of the equatorial facets) and points out that only the equilateral facet positions subunits (spots) exactly on an edge as for terminal triangles.

Figure 74c. Model of T4 as proposed for the dried-down condition observed in negatively stained preparations. It is actually assumed that the equatorial region in the hydrated state is truly helical.

Figure 74d. Diagram of the model proposed (Figure 74c) in the unfolded state. Cross-hatched area delimits a single facet, twenty of which comprise the whole structure.
conventional light sources (Taylor and Lipson, 1964). The analytical capacity of coherent light, combined with the presence of large amounts of asymmetry in the masks of electron microscopic images, is thus determined to be responsible for the observed "hash".

One of the advantages derived from this technique has been the necessary production of better resolved, higher contrast micrographs which the technique demands. In this regard the successful application of uranyl formate staining has been essential. Among the direct advantages of the method is the ability to determine quickly if an observed distribution of density is truly indicative of regular structure; such demonstration was provided in the analysis of T4 heads where resolvable regularity was indeed absent.

The diffraction studies on M13 typify the character of results with phage components and with the other structures examined. When subunit structure is suggested on an electron micrograph, quaternary conformation is not necessarily being resolved. To completely describe the quaternary structure of a proteinaceous helix, for example, the axial separation of subunits must be precisely determined. We have illustrated how a continuous helix follows the selection rule for $l = \frac{C}{P} n$ where $\frac{C}{P}$ can only equal one. A discontinuous helix with the discontinuities completely resolved may be regarded as a convolution in which the component lattices are a
continuous helix and a parallel series of planes that lie perpendicular to the axis of the continuous helix and are spaced in accordance with the axial rise per discontinuity (p). In the projection of this convolution, the helix is a sine wave of appropriate pitch, and the planes are lines at right angles to the long axis. The intersection of the individual lattice transforms give rise to reflections characteristic of a discontinuous helix, and the strong meridional reflection is indicative of the many repeats in the lattice of parallel lines. The correct indexing of layer lines then characterizes the helix parameters as we have previously discussed. When \( n = 0 \) and \( m = 1 \) the layer line number is the number of discontinuities per axial repeat period; when \( n = 1 \) and \( m = 0 \), \( \ell = \frac{c}{p} \) or the number of turns of the helix per axial repeat. The ratio of these \( \ell \) values expresses the number of discontinuities per turn the value of which cannot be determined, therefore, without layer line indexing; only an ambiguous layer-line index can be assigned without the \( n = 0 \) reflections.

Electron micrographs with inadequate resolution of the axial separation of subunits in the helices studied reflect a different convolution in their optical transforms. The resulting images represent projections of a continuous helix of heavy-metal penetration, and occasionally a second or a third is also present. Corresponding transforms reflect
the convolution provided by the superimposed helices and yield continuous helix patterns for each. The existence of all three fundamental helix series technically defines the subunit of the structure but does not circumvent the problem completely in practice because layer lines cannot be correctly indexed.

Better resolution is the only true remedy for this difficulty, but diffractometer improvements could provide more lucid results of the type described in this study. Principal among these are the acquisition of the best possible, long focal-length lens and higher contrast masks. For use in the solution of X-ray diffraction problems, the diffractometer used here is, perhaps, equivalent to that used by Taylor and Lipson (1964), but proper evaluation would entail the use of identical masks.

Harmonic analysis of electron micrographs has been demonstrated to be feasible with the apparatus described. Like many other analytical tools, it faithfully records information when used properly but demands a degree of experience on the part of the investigator who would interpret the data. The value of a laser-light diffractometer as a teaching device has not been specifically discussed, but it obviously has great possibilities in this regard. As a research procedure, optical diffraction with any light source can aid in micrograph analysis and make possible the
interpretation of partially obscured regular structure if the regularity is truly resolved. Optical diffraction with laser-light, used in conjunction with high-resolution electron microscopy, provides an efficient means for precise structure analysis, in the range of tens of ångstroms units where X-ray diffraction is least useful; it is highly recommended as a routine procedure in the general area of electron microscopy.
SUMMARY

1. Modifications in conventional negative staining of bacteriophage for electron microscopy have been developed and shown to enhance observation of biological quaternary structure. Variations included a process for developing negative contrast on frozen-dried samples and the use of low concentrations of uranyl formate for optimum contrast and minimum grain size.

2. An instrument for the generation of optical transforms from periodic electron microscopic images has been constructed, tested on various fibrous systems and applied to an analysis of bacteriophage structure. This optical diffractometer is a one-lens system using HeNe laser-light (6328 Å) as the diffracted radiation.

3. Lysogenic bacteriophage of Staphylococcus aureus (strains 6, 29, 42b, 52a, 53, 77, 80, 81 and 83) have been examined in detail. All have demonstrated non-sheathed, cross-striated tails by negative staining; evidence from laser-light diffraction studies indicated that a quaternary rearrangement from one stacked-disc conformation to another accompanies the process of ghosting. The presence of axial holes in ghost tails suggests that either subunits have retracted from the center or a previously contained length of DNA has been lost. The latter of these possibilities is
proposed, and a structural role is attributed to the DNA of bacteriophage with regard to maintenance of head and tail attachment, limitation of tail length and the quaternary arrangement of tail subunits. A change in the hexagonal base-plate that allows DNA-transfer to take place is indicated, but other structural alterations are better explained as consequences of DNA loss.

4. Examination of the T4 phage of Escherichia coli has suggested a contradiction in the currently accepted theory of its structure and function; either the non-icosahedral head is not made on a preformed core or sheath contraction is not a requirement for DNA-passage. Although sheath contraction may be necessary for successful infection by T4, it is concluded that the significant function of this action is penetration of the host surface.

5. Observation of single-stranded, super-extended sheaths and laser-light diffraction of both extended and contracted sheaths suggests that sheath stability is primarily dependent on a single class of subunit-subunit bonds in the direction of the primary helix and that these are maintained throughout the processes of contraction and super-extension. Sheath and core each reveal a \(42^0\) pitch, but the core is a stacked-disc array while the sheath is a true, non-zero order helix.

6. Examination of the rod-shaped, single-stranded, DNA-coliphage M13 revealed a helical substructure, an axial hole
and no specialized terminal structures. M13 proved to be the best example to demonstrate the potential value of optical-diffraction of electron micrographs since substructure was only interpretable after harmonic analysis.

7. The head shape of the staphylococcal phage was found to be icosahedral except for $\varnothing-6$, $\varnothing-42b$ and $\varnothing-81$ where the geometry is explicable in terms of an icosahedron elongated with two extra rows of equilateral triangles in the equatorial position. T4 head geometry was found to be dependent on the preparative procedure used; a model with 20 identical, pseudo-triangular facets has been proposed for the structure usually observed with the electron microscope.

8. Laser-light diffraction has been demonstrated as a valuable technique for the clarification of electron microscopic results; the procedures used in this study can be very useful in comparable structure analyses whenever high contrast images can be produced.


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ACKNOWLEDGMENTS

The author is grateful to Dr. L. E. Roth for patience and understanding in supervising this research and to Dr. and Mrs. Roth for editorial help in preparation of the manuscript. Sincere thanks are extended to Drs. M. A. Rougvie and P. A. Pattee for many helpful discussions and much constructive criticism; their assistance as consultants was essential.

Acknowledgment is due the author’s wife, Randi, who has been invaluable in the maintenance of his morale and perseverance throughout the course of graduate school and the prolonged course of this study.

This research was supported by National Institute of Child Health and Human Development Grant # HD 1260 awarded to Dr. L. E. Roth.