A New Electron Microscope Imaging Method for Enhancing Detail in Thin Biological Specimens

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(Z. Naturforsch. **29 a**, 158–163 [1974]; received 28 March 1973)

An electron microscope imaging method is described which makes use of an electrostatic device, analogous in function to the absorbing phase plate of light microscope, to produce strong contrast in biological specimens. This device is situated at the back focal plane of the objective lens in place of a normal objective aperture. The images created provide an especially realistic representation of the specimen structure and contain information about it that would not necessarily be able to be detected by the conventional bright field method of observation.

Introduction

A major disadvantage of the bright field imaging method, when applied to thin biological specimens, is that it does not provide good contrast. It is true that this disadvantage can, to a large extent, be circumvented by 'outlining' these specimens in a film of heavy metal (negative) stain. However in this case it is the stain rather than the specimen that is imaged, so that the usefulness of the method becomes limited by such factors as accuracy with which the stain follows the contours of the specimen and the 'noise' which is created by structural irregularities within the stain.

A number of alternative imaging methods have been suggested which might be expected to improve on this situation (see, for example, the early paper by Boersch [1]), but the only high resolution method that has so far been applied successfully appears to be that involving an electrostatic phase shifting device [2, 3]. With this method, the negative stain is retained (it seems to play an important role in preserving the specimen morphology during irradiation) and the above difficulties are surmounted by creating images in which it is the specimen itself – and not the stain – that provides the strong contrast.

The electrostatic phase shifting device (also called an electrostatic phase plate because of the of analogy of its optical characteristics with those of the absorbing phase plate of light microscopy [4]) consists simply of a thin foil aperture and a fine, poorly conducting, thread spanning its diameter (see Figure 1*). It is placed symmetrically in the back focal plane of the objective lens so that the central portion of the thread intercepts a portion of the primary (unscattered) electron beam and becomes positively charged. The magnitude and distribution of charge at the centre of the thread are able to be adjusted by making changes in the illumination conditions, and with correct adjustment the electric field originating from the centre of the thread, together with spherical aberration, produces a quarter of a wavelength shortening of the optical path of the scattered wave which is uniform over a wide range of spatial frequencies to a limit close to $1/5\ \text{Å}^{-1}$. These are almost ideal phase contrast transfer conditions and hence this method of observation (providing the primary beam is not weakened too far) produces exceptionally realistic, high resolution, images.

The method, in addition, enables the biological material itself to be displayed in strong contrast. This is a consequence partly of the action of the thread in preventing a large (adjustable) portion of the unscattered electrons from contributing to the image and partly of the type of phase contrast, which is the opposite to that employed in conventional imaging.

This paper outlines the basic properties and describes the practical aspects and applications of this new imaging method.

Contrast Characteristics

There are two types of contrast to consider in high resolution imaging of thin biological materials; one varises from interference between the scattered and unscattered waves at the image plane, and the other as a result of a difference between the proportion of scattered and unscattered electrons

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* Figs. 1, 2, 4 and 5 on page 160 a, b.

leaving the object and contributing to the image (i. e. from apparent absorption effects). Traditionally, the former mechanism is known as phase contrast and the latter as amplitude contrast.

The action of the phase plate in intercepting (or, in effect, absorbing) various fractions of the primary beam permits a large variation in the magnitude of both these types of contrast. However, the efficiencies with which they are transferred to the image is essentially an invariable property, determined by the degree of spherical aberration of the objective lens and by the nature of the phase plate's electric field. This property can be demonstrated by showing the phase plate image and corresponding optical diffraction pattern of a thin carbon film, and an example of such a pair for optimum conditions (that is, when the image is exactly in focus and the phase plate is optimally charged) is given in Figure 2.

It can be deduced that amplitude contrast has only made a minor contribution to the overall contrast in the image in Fig. 2 from the fact that there is a negligible difference in intensity levels between the carbon film and the space within the hole that it contains. (Any difference in intensity levels between such gross features as these could only be due to amplitude contrast since the spatial frequencies involved – except at the edges of the hole – are too low for the phase contrast contribution to become significant.) The optical diffraction pattern of the carbon film image must therefore relate to the conditions of phase contrast transfer in precisely the same way as do the optical diffraction patterns of bright field images of thin carbon films when very little amplitude contrast is present [5].

Now this optical diffraction pattern shows a large region of approximately uniform high intensity extending over most spatial frequencies (apart from those lying in a very narrow band in a direction equivalent to that of the thread in the diffraction plane of the microscope) up to about $1/5\,\text{Å}^{-1}$. It therefore demonstrates unequivocally that the phase plate produces efficient, uniform, phase contrast transfer over a very wide range of spatial frequencies – and moreover, a range which is especially useful in studying biological specimens.

The optical diffraction pattern is essentially the square of the phase contrast transfer function and may be compared with the optimum phase contrast transfer function for the phase plate as was deduced originally by independent electron deflection experiments [2], Figure 3. Both results predict good phase contrast transfer over approximately the same spatial frequency ranges.

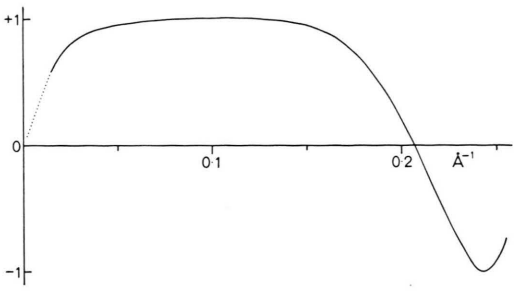


Fig. 3. The phase contrast transfer function (sine of the phase shift plotted against spatial frequency) for the in focus image when the phase plate is optimally charged. The function is very nearly rotationally symmetric over the full-lined part of the curve (cf. inset to Figure 2).

The phase contrast transfer function in Fig. 3 gives additional information however: parts of this curve which are positive coincide with conditions of bright phase contrast (that is tending to make thicker, more strongly scattering objects appear brighter). The broad plateau extending over the useful spatial frequency range is positive and the electrostatic phase plate is therefore primarily a bright phase contrast device. To appreciate the importance of bright, rather than the normally employed dark, phase contrast in enhancing detail in biological material it is now necessary to discuss briefly the amplitude contrast behaviour.

Amplitude contrast can become significant in the regions of Fig. 3 where the efficiency of phase contrast transfer begins to deteriorate; that is (neglecting the very high spatial frequencies) with spatial frequencies of less than $\sim 1/8\,\text{Å}^{-1}$, and especially with those less than $\sim 1/50\,\text{Å}^{-1}$. How important it actually becomes depends both on whether it is the carbonaceous material or the stain that is being considered and on how much of the primary beam is, in effect, absorbed. Its behaviour is therefore very unlike that of phase contrast, which remains of the same type regardless of the material and is simply made stronger by attenuation of the primary beam.

The reason for the more complex behaviour of the amplitude contrast stems from differences in electron scattering properties: the carbonaceous material, being essentially amorphous, scatters electrons more strongly within phase plate aperture

than it does outside; on the other hand, the negative stain, which is generally composed of very small crystals having a small unit cell, tends to scatter electrons relatively weakly within this aperture, but strongly outside. Assuming 100 KV electrons and a phase plate of the dimensions described in the caption to Fig. 1, it can be shown that, mainly because of these differences, a fraction as large as 3/4 of the electrons scattered by a carbonaceous object is likely to contribute to its image, but that a fraction of less than 1/5 of the electrons scattered by the stain is likely to contribute to its image.

How these differences in scattering properties lead to differences in amplitude contrast behaviour for the two materials is most easily shown in the following way: define p as the fraction of the electrons incident on an object that are scattered by it, and q_s and q_0 as the fraction of these and of the undeflected electrons which contribute to its image. The total fraction of the original number of electrons incident on the object that contribute to its image is thus $q_s p + q_0(1-p)$, or:

$$q_0 + (q_s - q_0)p$$

as compared to the background fraction, q_0. Clearly then, when $q_0 > q_s$ the object must appear darker than the background (dark amplitude contrast – as in bright field conditions), when $q_0 = q_s$ it must be of the same intensity, and when $q_0 < q_s$ it must be brighter (bright amplitude contrast). Now q_0, which is easily determined from the geometry at the diffraction plane, is such that $3/4 > q_0 > 1/5$ under normal operating conditions; under these conditions, therefore, q_s is greater than q_0 for the carbonaceous material ($q_s \cong 3/4$) but less than q_0 for the negative stain ($q_s < 1/5$). These two materials must accordingly appear in the normal phase plate image in bright and dark amplitude contrast respectively.

The reason why the phase plate method preferentially enhances the biological material is now quite clear: it makes the two contrast mechanisms work cooperatively for this or other carbonaceous material (both phase and amplitude contrast will be bright) at the same time as making them work in opposition for the negative stain (where the phase contrast will be bright and the amplitude contrast dark).

The degree of enhancement of the biological material and weakening of the contrast of the stain relative to bright field conditions will, of course, depend on the relative contributions made by the two contrast mechanisms and hence on the spatial frequency and on q_0. This question has been discussed in detail in Reference 3 and it is sufficient to note here that the lower the value for q_0 the closer the image approximates to one of being of the carbonaceous material alone; furthermore, the high contrast that one obtains in the fine detail in images formed with the low values for q_0 must still be mainly due to phase contrast because of the shape of the phase contrast transfer function and the more powerful nature of this contrast mechanism.

Figure 4 gives examples of the two types of image of some negatively stained specimens – a T4 bacteriophage and a rod of the stacked disk aggregate of tobacco mosaic virus protein, the phase plate image being formed with sufficiently low value for q_0 ($\cong 0.4$) to make the contrast in the stain fairly weak compared with that in the carbonaceous material. Note that amplitude contrast can only have made a major contribution to the relatively gross features in the phase plate image (for example to the difference in intensity levels between the thin carbon support film and the thicker carbon film seen on the right hand side of this picture) and that phase contrast must be mainly responsible for the finer scale detail associated with the regular parts of the specimens. Only in gross features, therefore, does this image bear any comparison at all with a dark field or strioscopic image.

Operating Procedure

The form of electric field required for optimum functioning of the phase plate is one which is cylindrical in the very small central region through which the primary beam passes, but almost spherical over the remaining area (see Reference 2). Such a field is generated when the central portion of the thread is raised to a uniform positive potential of about one volt, and the primary task in operating with the phase plate is to achieve just this potential distribution.

The adjustments involved in producing these electrical conditions are described below, but firstly it is pointed out that the creation and subsequent stability of the charge at the centre of the thread is due mainly to a secondary emission process[2]: when

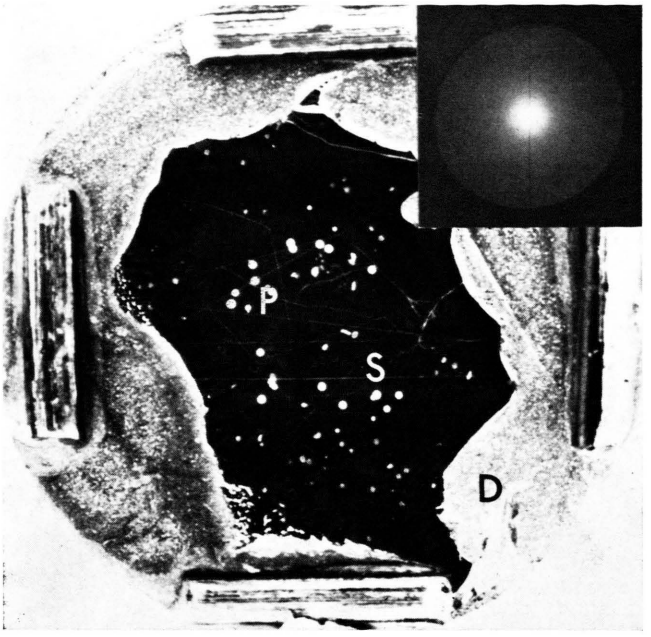


Fig. 1. The construction of the phase plate and its mounting. The mounting consists of a thin metal foil (S), and a supporting 3 mm diaphragm (D) to which four projecting rods are attached to protect the phase plate during handling. The phase plate itself (P) is also shown against the electron scattering pattern of a carbon film in the inset.
The construction procedure is (i) latex spheres (optimal diameter, about 30 μm — see Ref. 2) are spread uniformly on the convex surface of a clean glass plate; (ii) silver is evaporated over the spheres and glass so that a thin (~ 0.5 μm thick) film containing numerous small circular openings is formed on blowing the spheres away; (iii) $0.3-0.5$ μm thick thread of a small spider is wound many times around the silver-glass composite so as to span a fair proportion of the openings in the film; (iv) the film is cut into small squares which are floated off on distilled water and collected on 3 mm diameter diaphragms; (v) those squares containing openings having single threads spanning their diameter are selected with the aid of an optical microscope, mounted more firmly on their diaphragm support with a conducting adhesive and coated with a thin ($\sim$150 Å thick) discontinuous layer of gold, using a rotary shadow caster to produce a more or less uniform thickness around the thread. This procedure has the advantages that only minor technical difficulties are involved and that it enables a considerable number of phase plates to be made at any one time.

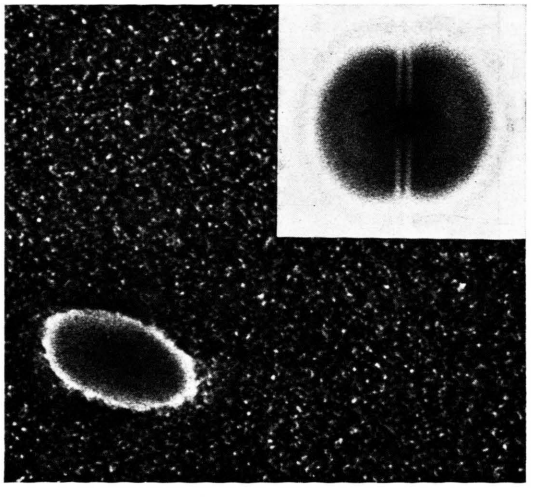
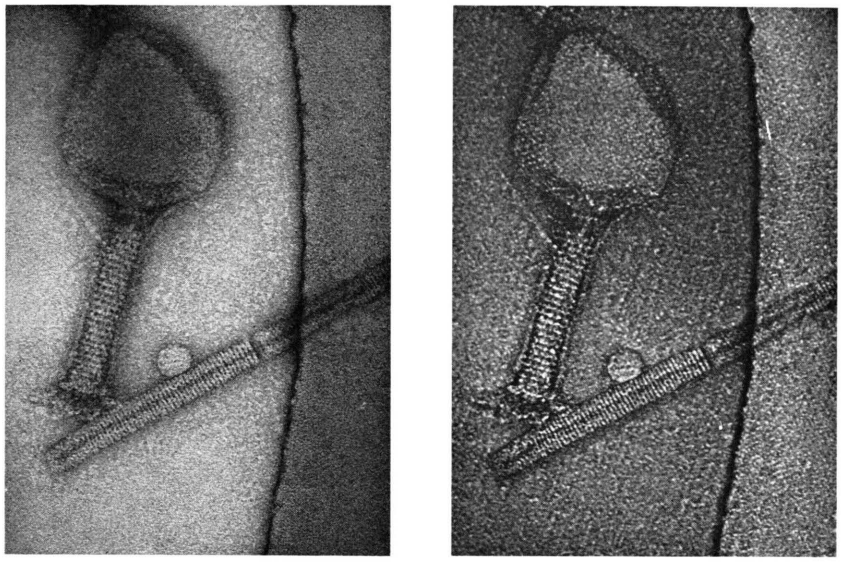


Fig. 2. Phase plate image and optical diffraction pattern (inset) of a thin carbon film containing a hole. (Magn. $\times$1,150,000; inset: 0.1 Å^{-1} = 6.2 mm.)



a b

Fig. 4. (a) Bright field image and (b) phase plate image of a T4 bacteriophage and a rod of the stacked disk aggregate of tobacco mosaic virus protein. The specimens have been stained with uranyl formate which is clearly visible in (a). In (b) the absorption conditions have been adjusted ($q_0 \cong 0.4$) so as to weaken the contrast from this material and enhance the contrast from the biological material. A carbon film much thicker than the ~ 50 Å thick film supporting the specimens is shown at the right hand side of each micrograph. (Magn. $\times 210{,}000$.)

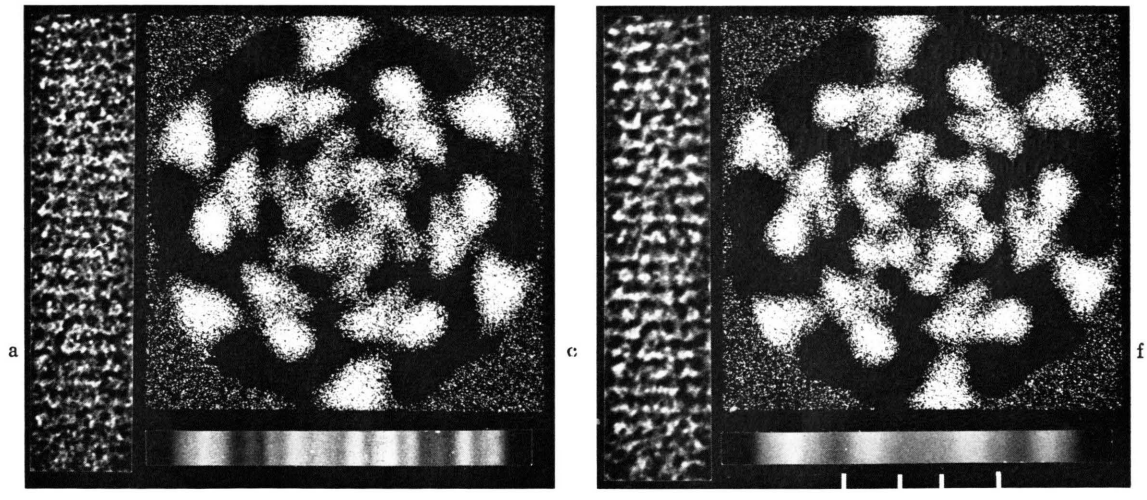


Fig. 5. Showing (a) a bright field image of a T4 bacteriophage tail, (b) the view formed by optically compressing this image vertically, and (c) a computer display of a cross-section of the three dimensional model reconstructed from (a) after correcting for the effects of non-uniform contrast transfer; (d), (e) and (f) are the corresponding phase plate pitcures for the same tail, (c) and (f) also being of identical cross-sections; the distances between the inner and between the outer pair of markers on (e) correspond to the inner and to the outer diameters of the core.

the primary beam first strikes the thread, large numbers of secondary electrons are ejected from this region causing its potential to rise; but more replacement electrons (e. g. secondaries generated by the scattereed electrons on striking the aperture edge and electrons provided by leakage of charge along the thread) are then drawn to it and captured. Very quickly an exact balance between the two types of secondaries is achieved, and as a result, the potential conditions become stable.

With this mechanism operating and for the potential to be spread uniformly over the central region of the thread, it is clearly necessary for this region to be illuminated by a primary beam which, in cross-section, is uniformly intense. The condition of critical illumination (in which the beam is focused on the object so that an image of a more or less uniformly illuminated second condenser aperture is formed at the plane of the phase plate) is therefore employed. With this type of illumination the extent of the charged region is also governed by only one parameter – the size of the second condenser aperture.

The requirement that the potential stabilise at precisely that magnitude which produces the correct phase shifts is met by making some minor adjustments to the illumination conditions. (It is only possible to make these adjustments with a thread which has been coated with a thin layer of metal as described in the caption to Fig. 1, otherwise it ejects secondaries at a very high rate and tends to stabilise at far too high a potential to be useful; the thin metal coating, it seems, both drastically reduces the rate of secondary emission from the thread and increases the rate of replacement by providing for some leakage of charge along it.)

The adjustments routinely used to generate the correct potential consist merely of making small changes in the strength of the first condenser lens and additional compensatory charges in the strength of the second condenser lens to maintain conditions of critical illumination. The principle is that by altering the strength of the first condenser lens, the intensity of the electron scattering pattern is altered; replacement processes thereby assume greater or less importance and cause the potential at the centre of the thread to fall or rise. Increasing the strength of the first condenser lens weakens the intensity of the electron scattering pattern and therefore causes the potential to fall.

These adjustments do not alter the form of the electron scattering pattern (i. e. the sizes of the scattered and primary beams), which is important since it means that the absorption properties of the phase plate – determined solely by the relative diameters of the primary beam and of the thread – remain independently adjustable. The inavoidable consequent changes in the area of object that is illuminated constitute the only disadvantage of this procedure.

To judge when the correct magnitude of potential has been achieved, one studies the appearance of the fine detail in the image in a similar way as one would study it, normally, to determine the position of exact focus and to correct for astigmatism. The standard procedure that has been adopted for taking micrographs is as follows. Astigmatism correction and focusing are first accomplished with the primary beam passing approximately midway between the thread and the aperture edge; the phase plate is then moved into the symmetrical position and the change in appearance of the image is studied closely during the period (5 – 10 seconds) whilst the potential is rising to its stable value. In general, with the first condenser lens only weakly excited, the image will first improve very obviously in sharpness and contrast, reach a distinct maximum, then deteriorate to take on an appearance which is very similar to that of an overfocused bright field image. The maximum in contrast and sharpness coincides with the conditions of optimum phase contrast transfer, as can be shown by recording the image of a thin carbon film at this stage and examining its optical diffraction pattern afterwards. The aim then is to lower the stabilising potential until the maximum represents the last stage in the changing appearance of the image; this is done by increasing the excitation of the first condenser lens as has just been described.

Once these electrical conditions have been set up they are easily maintained provided that all precautions are taken to keep the contamination rate at the centre of the thread very low. My experience is that contamination build up still may limit the operating lifetime of the phase plate to about thirty hours.

The absorption conditions are conveniently varied by choosing any of a number of second condenser apertures (ranging in diameter from ~ 50 to 120 μm). In this way it is possible to cover most of the absorption range available between the extremes

of bright field ($q_0 = 1$) and dark field ($q_0 = 0$) conditions without having to change phase plates.

Application to Negatively Stained Specimens

The advantages of the phase plate method in being able to emphasise the detail in the specimen itself, rather than in the surrounding film of stain, appear to be two-fold. First there is the general advantage that structural irregularities in the stain receive less emphasis, so that the tendency for *relevant* detail becoming obscured is reduced; this is important, particularly with the weaker highest resolution Fourier components of the image. Secondly, there is now the possibility of obtaining information about regions of the specimen which, because of imperfect matching, the stain is not able to outline.

These advantages have been demonstrated by making careful comparisons of the Fourier transforms of bright field and phase plate images of the same specimen [2, 3]. The comparisons have also indicated that the matching between the contours of the stain and of the specimen can be quite poor in the deep grooves or intrusions as exist between the subunits of a structure. It is thought that the two materials to some extent come apart both in preparation as the stain dries around the specimen and during the early stages of irradiation in the microscope.

Images of the tail of the T4 bacteriophage provide a good illustration of the ability of the phase plate method to disclose more information in the case where the matching is poor. The tail is made up of two main structural components: a hollow central core and a surrounding contractile sheath, which is normally extended so as to envelope the whole length of the core. Imaged in bright field conditions, this structure shows considerable detail in the outer sheath region, but is relatively featureless in the region of the core, particularly on the surface bounding the central tunnel (see Reference [6]). This inner surface is unlikely to be smooth in reality, however, and could appear to be so simply because the negative stain, on drying, is not able to completely fill the tunnel – which is only about 30 Å in diameter – and remain penetrating such grooves or intrusions as actually exist.

As was noted above, such a situation should not constitute a disadvantage with the phase plate imaging method. Indeed, that this surface is shown up much more distinctly by this method than by the bright field method, can be demonstrated directly by compressing the corresponding images of a given tail axially by means of a cylindrical lens.

An example of such a pair of images and their 'compressions' is given in Figure 5. The phase plate 'compression' (Fig. 5 e) shows the core as two bright zones immediately on either side of a central dark zone corresponding to the tunnel (the three zones are each close to 30 Å in extent, as would be expected from the known dimensions of isolated cores). The representation is very clear. In contrast with the bright field 'compression' (Fig. 5 b), the core is difficult to distinguish and only the approximate positions of its outer surface can be established with any certainty.

The difference between the two images becomes more obvious on reconstructing three-dimensional models of them by the method of De Rosier and Klug [6], and computer displays of identical cross-sections taken from such a model are reproduced in Figs. 5 (c) and (f).

These displays show the cross-sections for the outer sheath region to look very similar in both cases, and this is not surprising since there is no reason why the stain and protein should not form a reasonably good match in the outer region, where the intrusions are larger and more accessible. As was to be expected, however, the cross-sections do differ very significantly in the core region: the inner surface of the core as reconstructed from the bright field image is fairly smooth, but the same surface as reconstructed from the phase plate image displays well defined intrusions; these intrusions are moreover arranged in such a way as to suggest that the core instead of being comprised of a continuous structure, can reasonably be divided up into elongated subunits having six-fold symmetry, helically arranged down the axis.

Evidence for this feature has been obtained on applying the above methods to other phase plate images, but not convincingly so far on applying them to bright field images (even the corresponding ones) of this type of bacteriophage. There is consequently little doubt that it is a characteristic structural feature that is only able to be clearly distinguished by the phase plate method of observation.

In terms of general application of this method to the study of negatively stained biological specimens, the example of the bacteriophage tail therefore substantiates the conclusion drawn from earlier studies [2, 3], namely that it can be used to disclose important information which may be difficult or impossible to detect by conventional means. In many circumstances the value of this extra information may well be such as to make this method the most worthwhile, even though it is not quite so simple to use in practice.

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