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III. APPLICATIONS OF IMAGE ANALYSIS TECHNIQUES IN ELECTRON MICROSCOPY

Optical diffraction and filtering and three-dimensional reconstructions from electron micrographs

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This section deals with some methods which have been developed in recent years for processing the information recorded in electron micrographs so that one can extract from them the maximum amount of reliable information about the three-dimensional structures which are being examined. The basic problem is that the electron microscope has a depth of focus of several hundred nanometres, so that all features along the direction of view are superimposed in the image. There is a limit to perception by direct inspection because we are not accustomed to seeing *through* an object. The details one 'sees' in a picture are therefore confused and not easily interpretable without methods which separate the contributions to the image from different levels in a specimen. The methods are particularly powerful when applied to periodically repeating structures (whether with translational or rotational symmetry), but in their ultimate form the methods can deal with the general asymmetric particle. The methods are closely analagous to those used by X-ray crystallographers to measure periodicities or degrees of order and finally to see the arrangement of atoms in simpler substances. The difference, however, is that there is no phase problem here because the phase information is contained in the electron micrograph.

The methods provide a means of assessing the relative reliability of images of different specimens and of averaging over them. It is also possible to filter out the effects of noise, arising from such effects as irregular perturbations of the structure, variability in the staining and the granularity in the supporting films. It has been found in this way (and by comparison with X-ray diffraction where available) that the long-range order in some specimens, particularly those prepared by the negative staining technique, is preserved to a resolution of about 2 nm, which is sufficient to resolve individual macromolecules. The fine details of the arrangement and shape of the molecules in this range of resolution are therefore recorded faithfully in the image.

Optical diffraction and Fourier transforms

The first approach (Klug & Berger 1964) was to use optical diffraction of an electron micrograph, which allows analysis of all periodicities present in the image. Until this technique was introduced the interpretation of pictures of this kind was left to subjective judgement and quite often periodicities which were difficult to discern were not recognized. The diffraction pattern allows an objective analysis of all periodicities to be made for it displays an average of the genuine repeating features in the structure. By this technique we were able to identify and interpret features in negatively stained biological specimens which were not apparent to the

eye (Finch, Klug & Stretton 1964; Grimstone & Klug 1966). A novel application of optical diffraction was that by Thon (1966) to produce a display of the modulation transfer function of the electron microscope at various degrees of defocusing.

The diffraction patterns are interpreted by methods similar to those used in the analysis of X-ray diffraction patterns from crystals, though fortunately they are not plagued by the phase problem. The optical diffraction pattern records only the amplitudes of the Fourier components of the specimen, but more complete information may be obtained by an examination of the phases of the Fourier components. The phases can be generated by computing the Fourier transform from a digitized representation of the image obtained by microdensitometry (DeRosier & Klug 1968). This computer-generated transform contains both the amplitudes and the phases of the original subject. This is an immediate advantage of computed transforms over optical transforms because the phases of the diffraction pattern are readily available. For purposes beyond that of merely measuring periodicities, the phases are more important than the amplitudes, e.g. in determining helical parameters or in assessing the degree of preservation of the symmetry of a structure. An illustration is provided in the paper by Finch & Klug to be presented later in this section (p. 211). When it comes to recombining the Fourier coefficients in a Fourier synthesis, as described below, the phases are, of course, vital.

It is possible to measure phases optically in a holographic system in which the diffracted rays from the subject are allowed to interfere with a strong reference beam, coming, for example, from a point in the same plane as the subject. But this technique requires labour and high precision in the optics, especially when dealing with the very fine scale of periodicities present in electron micrographs, and DeRosier & I did not consider it worthwhile pursuing our preliminary experiments in this direction, when a computer-linked densitometer became available.

It is relevant to ask here why the electron microscope should not be used directly to generate the electron diffraction pattern of the object. This is indeed possible, but there are drawbacks. The first is the fact that a beam cannot usually be obtained small and bright enough to illuminate only the area of interest, but this is presumably a limitation which will eventually be overcome. The more serious limitation is that a diffraction pattern obtained in this way records only the amplitudes and not the phases which, as we have seen, contain the vital part of the information. To obtain these phases it would be necessary to use a reference beam in the microscope itself, that is, carry out electron holography. It is far from clear that a practical 'phase-marking electron diffraction camera', as Gabor (1956) called it, will be attainable in the near future. My view has been that there is already a 'method' of sensing the phases of the diffracted rays, namely by using the objective lens of the microscope to focus the rays into an image, which, of course, preserves the phase information. The information in the form of the Fourier coefficients of the image can be 'released' at a subsequent date by what are nowadays rapid computations, thanks to the development of the so-called 'fast Fourier transform' programs (Cooley & Tukey 1965).

Optical filtering

There is an important range of applications where the Fourier components can be manipulated in an optical system without the necessity for measuring or computing phases. In the case of specimens which consist of two overlapping layers rotated relative to each other, the analysis of the optical diffraction pattern allows the contributions from the front and back to be separated

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and an image of each of them to be reconstructed. This is the method of optical filtering (Klug & DeRosier 1966) and it is possible to carry it out in a simple optical system because it is basically a two-dimensional operation. The method has been successfully applied to structures which are essentially two-dimensional such as shallowly grooved helical particles (Klug & DeRosier 1966), thin-walled tubes (Kiselev, DeRosier & Klug 1968; Kiselev & Klug 1969) or overlapping plane layers (e.g. cell walls, Finch, Klug & Nermut 1967). Other examples of the use of optical diffraction and filtering will be found in the papers by Moody and by the King's College groups in this session.

In optical filtering a two-dimensional Fourier synthesis is performed optically using only the diffracted rays which it is desired to combine. This operation can, of course, be carried out on a computer by a Fourier synthesis using the desired Fourier coefficients. However, in this case the phases of the Fourier coefficients have to be determined first, a step which is not needed in the optical method. An example of filtering by computer may be found in the paper by Moore, Huxley & DeRosier (1970, Plate X*b*).

Three-dimensional reconstruction by Fourier synthesis

In particles or systems more complex than those mentioned above, there is a contribution to the image from the internal structure as well as from the front and back surfaces. To work in three dimensions a generalized form of the optical filtering process must be found. In effect, what is required is a three-dimensional Fourier synthesis. A general method was developed by DeRosier & Klug (1968) which permits the objective reconstruction of a three-dimensional object from a set of transmission pictures of that object. In general, it is necessary to use a selection of electron micrographs viewing the particle from different angles. The important feature of the method is that it tells one how many different views are needed (for a required resolution) and how these are to be recombined into a three-dimensional map of the object. The process is both quantitative and free from arbitrary assumptions. It contains the two same essential steps as optical filtering, namely the formation of the diffraction pattern and the recombination, but they are both carried out as Fourier transformations on an electronic computer.

The method of reconstruction is based on the projection theorem, which states that the two-dimensional Fourier transform of a plane projection of a three-dimensional density distribution is identical to the corresponding central section of the three-dimensional transform normal to the direction of view. The three-dimensional transform can therefore be built up section by section using transforms of different views of the object, and the three-dimensional reconstruction then produced by Fourier inversion. The approach is similar to conventional X-ray crystallography, except that the phases of the X-ray diffraction pattern cannot be measured directly, whereas here they can be computed from an image. The different views may be collected either from a single particle by using a tilting stage in the microscope, or from several particles in different but identifiable orientations. In general, it is desirable to combine data from different particles so that imperfections can be averaged out.

This method is applicable not only to structures containing a regular repeat but to any structure whatsoever. However, for a general asymmetric particle the number of views required is fairly large (e.g. a minimum of 30 views is needed to solve a particle of 20 nm diameter to 2 nm resolution (see figure 1). When the particle is built out of subunits and therefore possesses symmetry, the number of views is reduced. This is because a general view of a particle made up

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of subunits has contained within itself as many different views of the subunit—related in a known way—as there are subunits in the repeat. This symmetry may be included in the reconstruction in the following way. Each view of the particle provides a central section of Fourier space and the symmetry of the particle can be used to generate further central sections related to the first by symmetry. Thus, by inserting data from different views of the particle, and making use of the symmetry, Fourier space is 'filled up' to enable the reconstruction to be carried out to the required degree of resolution.

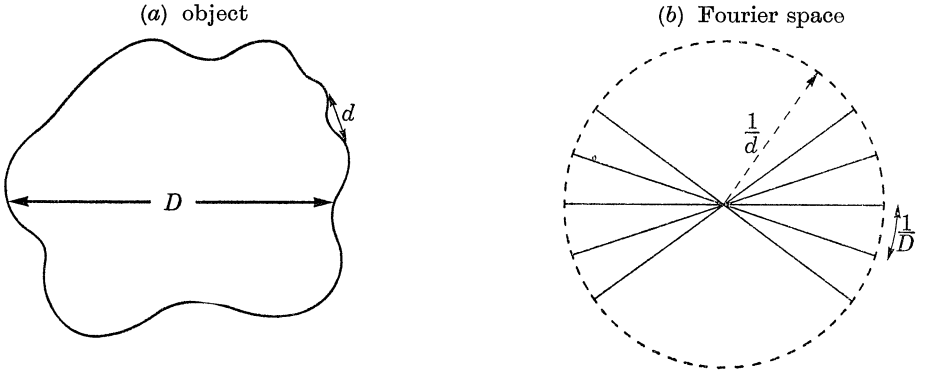

FIGURE 1. (*a*) An asymmetric particle of maximum diameter D containing features on a scale d. (*b*) Two-dimensional illustration of the 'filling-up' of Fourier space by central sections. Data must be collected to a radius of $1/d$ if the reconstruction is to show detail to a resolution d. The angular separation is decided as follows. The Fourier transform of a particle of diameter D does not, on the average, change appreciably over a distance $1/D$ in a Fourier space. There will thus be a sufficient number of sections if their intersections with a circle of radius $1/d$ are spaced at intervals at least as small as $1/D$. The minimum number of views, n, is therefore equal to $n = \pi(1/D)/(1/d) = \pi D/d$. For a particle of diameter 20 nm, to be solved to a resolution of 2 nm, $n \approx 30$. The value of n will in practice need to be greater since the calculation assumes that all the data can be collected by a set of tilts about a single axis covering a range of $\pm 90°$. This is impossible in practice. Limited ranges of tilts about more than one axis will have to be employed, and the strategy of data collection can be worked out by the methods given by Crowther, DeRosier & Klug (1970).

In the case of a particle with helical symmetry, a single view may often provide sufficient information to reconstruct the object at least to limited resolution. The reason for this is that successive stretches along the particle, each of length equal to the translation parameter of the helical screw, contain different views of the asymmetric unit turned through an angle equal to the screw angle of the helix. If the screw angle is not a simple rational factor of 2π, it will be a considerable distance along the particle before the views begin to repeat. In other words, a helical particle has built-in tilts of the asymmetric unit and this fact is incorporated into the mathematics of the reconstruction which is carried out by a Fourier–Bessel synthesis. This approach was first used to produce a three-dimensional map of the structure of the tail of bacteriophage T4 (DeRosier & Klug 1968) and further applications are described by DeRosier and by Finch later in this session. If the helical parameters are unfavourable, that is, when the axial repeat contains a relatively small number of asymmetric units, then more views of the particle must be obtained, for example by tilting.

For non-helical particles it is necessary to combine the data from a number of different projected views and this in turn requires data reduction and interpolation. While simple in principle, the implementation of the method raises difficulty in the detailed mathematical analysis and computational steps. My colleagues and I have developed procedures for implementing the method (Crowther, DeRosier & Klug 1970) and these have been applied to produce

reconstructions of a number of spherical viruses (Crowther, Amos, Finch, DeRosier & Klug 1970). The procedures are described in more detail by Crowther in this Symposium. Spherical viruses were a natural choice for the first application of this extended method of reconstruction because their high symmetry meant that only a small number of independent views of a particle were needed. There is, however, in principle, no reason why the method should not be extended to systems with lower symmetry, for which many more views must be combined to achieve the same degree of resolution.

Objections are sometimes raised to the premise that an image of a particle of moderate size embedded in negative stain, or of a positively stained thin section, essentially represents a simple projection of the distribution of stain. It is asked whether multiple scattering might not vitiate this assumption. It is pertinent, therefore, to add that the excellent agreement which has been obtained between observed images and projections from three-dimensional reconstructions based on this assumption, would seem to justify the original assumptions, when the scale of detail is not less than 2 nm. A gallery of such comparisons is given by Crowther. Another example of good agreement will be found in the paper by Moore *et al.* (1970) on actin. Second-order effects due to multiple scattering were noted earlier in a study of negatively stained cell walls (Finch, Klug & Nermut 1967). They represent only a small correction to the image predicted on the first-order theory of image formation. The experimental investigation described by Erickson at an earlier session demonstrates quantitatively that the imaging of a typical biological specimen can be adequately described by a linear or first-order theory, with only small deviations due to second-order effects. Thus for the present range of applications, it is valid to interpret the image as a simple projection of the object.

Direct reconstruction

Since the methods proposed for reconstruction begin with a density distribution and finally produce a density distribution, it might be asked whether there is any need to use the Fourier transform as an intermediate step. It might be thought possible to reconstruct the density by some simple form of back projection or three-dimensional triangulation from the various available projections, arranged in the correct relative orientations (figure 2). There is no doubt that this method would work for a finite idealized object consisting of point atoms, provided the relative directions of view can be chosen at will (figure 2*a*). Difficulties, however, arise in the case of a continuous density distribution or in the case of atom images of finite size, because of the way the density overlaps when back-projected (figure 2*b*). This limitation appears to apply to the method proposed by Hart (1968). Back projection may nevertheless be useful in giving a first approximation to the density, which can then be refined by an iterative method. For example, the spurious feature in figure 2*b* can be recognized and eliminated by comparing the reconstruction with each of the original projections in turn. In more complicated examples, an iterative procedure could be used to produce a reconstruction consistent with the given projections. However, this reconstruction will not necessarily represent the true density unless sufficient projections have been included.

This problem of overlap has been considered elsewhere in terms of the Fourier transform (Crowther, DeRosier & Klug 1970) which enables an analysis of the difficulties to be made. We have also discussed a method of direct reconstruction without the use of transforms which avoids the difficulty just mentioned. The method depends upon solving the linear equations relating the projected two-dimensional density distributions to the three-dimensional object

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from which they were derived. The relative merits of the density space method and the Fourier space method are at present under investigation in our laboratory. The density space method has certain advantages particularly in cases where it is desired to include only a part of the data included in the projection (e.g. when a part of the image is distorted or obscured by another particle). However, our present view is that the method is in general less practicable than the Fourier approach, but might be valuable in certain special cases.

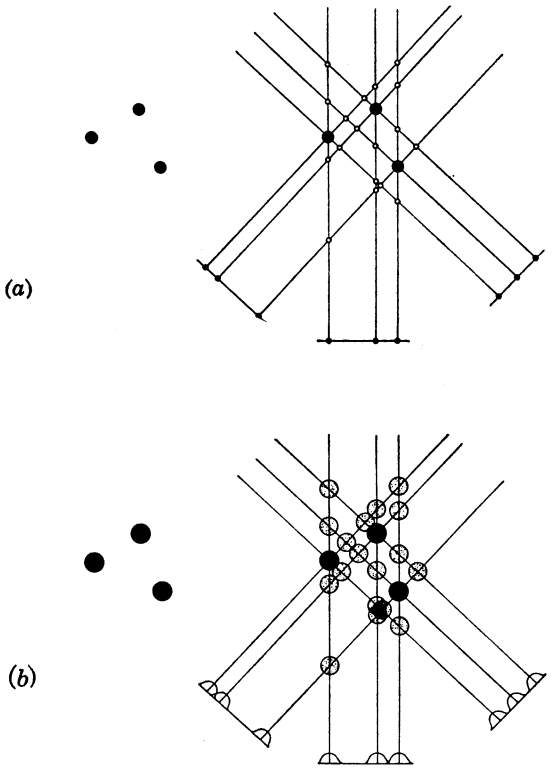

FIGURE 2. Two-dimensional illustration of density space reconstruction by back-projection. The 'object' is shown at left. On the right, its structure is reconstructed by back-projection from three projected views of the object. (*a*) Object consisting of 'point' atoms. The reconstruction consists of the original object plus subsidiary features of smaller weight which can be discriminated against by some form of threshold. (*b*) Object consisting of atoms of finite size. Even in so simple an example, the reconstruction shows a chance overlap producing a spurious feature of weight equal to those in the true structure.

Of course, there must be a formal counterpart in density space to any method developed in Fourier space and vice versa, but the corresponding method in the two spaces will differ considerably in the practicability of implementation. A relevant example in two dimensions is the question of the relation between optical filtering using diffraction methods and the photographic integration method developed by Markham for enhancing image detail in electron micrographs of objects with a regularly repeating structure (Markham, Hitchborn & Hills 1963; Markham, Hitchborn, Hills & Frey 1964). Markham's rotational and linear integration techniques were the first to be proposed for extracting significant detail from images of periodic structures. When the object consists of two superposed lattices rotated relative to each other, an image of one side of the structure may be produced by repeatedly translating the electron micrograph in certain directions and making a photographic superposition of the set so obtained. If the translations have been chosen correctly the structure of the object will be revealed.

In the latest form of the method (Hitchborn & Hills 1967, 1968) the directions and distance to be employed are determined by optical diffraction. It may be shown that there is an exact relation between averaging by this technique and optical filtering (DeRosier & Klug, paper in preparation). If translational averaging were carried out over *all* the repeats of *all* the lattice vectors in the subject—something which cannot easily be done in practice—then one would obtain a picture which is equivalent to an optically filtered ideal image, but with a larger noise level. This is because the 'other side' of the structure is not being removed as is done in filtering, but is being smeared out. It would, therefore, seem that, in this instance, the filtering or Fourier method has advantages not only because it is easier to carry out but because it leads to equivalent or better results for a given amount of work.

However, it is by no means certain—indeed it is very unlikely—that the last word has been said in a new subject like image reconstruction which has developed so rapidly. It is hoped that one of the outcomes of this Discussion meeting will be that unresolved questions will be further explored and that we can look forward to additions to the armoury of practical methods available.

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