

Electron Microscopy: Imaging Molecules in Three Dimensions

Up to now, x-ray diffraction and transmission electron microscopy have been largely complementary techniques for imaging biological structures. Crystallographers use x-ray diffraction for high-resolution mapping of the structure of biological molecules, ranging from relatively simple amino acids to complex proteins, provided that they can be prepared in crystalline form. Electron microscopy has been useful for directly imaging structural features on a much wider variety of specimens but also at a much lower resolution.

This state of affairs may soon change, however, because in the last year Nigel Unwin and Richard Henderson of the Medical Research Council (MRC) Laboratory of Molecular Biology, Cambridge, England, have successfully combined the mathematical technique of image reconstruction from projections with new methods for preparing specimens and minimizing radiation damage. Thus they were able to reconstruct images of a protein molecule to a resolution of 7 angstroms, nearly three times the best resolution previously attainable. This combination of techniques promises to bring to electron microscopy both high resolution and the ability to image periodic assemblies of molecules that are too

small to yield information by examination with x-rays.

The protein molecules were components of the purple membrane, which itself is a specialized part of the cell membrane of the bacterium *Halobacterium halobium*. This organism has been much in the news recently as the only known example of a cell that does not contain chlorophyll but that can convert sunlight into energy for its own metabolism. Scientists hope that understanding this unique process will clarify such cell processes as the production of adenosine triphosphate, the energy-storing molecule in cells, as well as lead to new solar energy technologies.

Unwin and Henderson's first innovation was to preserve the purple membrane (an oval sheet with a diameter of about 1 micrometer and a thickness of 45 angstroms) in a dilute glucose solution. This procedure is necessary because in the moderately high vacuum [10^{-3} pascal or better (1 pascal equals 7.53×10^{-3} torr)] of an electron microscope, dehydration of an untreated biological specimen normally causes cracking, fragmentation, or other trauma that distorts its structure.

Moreover, since the atoms in biological molecules are light, they do not

strongly scatter electrons. Because of the resulting lack of contrast, microscopists have had to invent staining techniques to produce an artificial contrast. One widely used method, which was a breakthrough in its own right when introduced in the mid-1950's, is to coat the specimen with a dilute solution of a heavy metal salt, such as uranyl acetate. The solution dries and leaves the metal salt to cover the surface and fill crevices on the specimen. This technique is called negative staining because contrast, provided by the metal, is associated with an absence of molecular material.

Negative staining not only provides contrast for imaging but also stabilizes a specimen against the effects of dehydration. Nonetheless, the stabilization is at best incomplete, and only the features of a specimen that have dimensions greater than 20 angstroms are preserved. Moreover, because the stain only coats its surface, features inside the specimen cannot be seen at all. Thus, the 2- or 3-angstrom resolution possible with good electron microscopes cannot be utilized in practice.

According to Unwin and Henderson, the use of glucose alleviates the dehydration problem because, when dried, the surface of the solid glucose looks much like water, but is not volatile. And, since it contains no heavy atoms, glucose does not prevent the internal features of the membrane from being imaged. Electron diffraction patterns taken from purple membrane lattices and also from beef liver catalase crystals indicated that details with dimensions as small as 3.5 angstroms were preserved in the glucose-embedded specimens. This is within shooting distance of what researchers can achieve with x-ray diffraction of materials (including catalase) that crystallize in three dimensions, and indicates that the ultimate resolution attainable with electron microscopy may yet come close to that of x-ray crystallography.

Unfortunately, glucose does not solve the problem of the intrinsically low contrast of biological molecules. The MRC investigators overcame this difficulty by taking advantage of a special property of the purple membrane that also enabled them to solve another perennial problem of imaging biological materials with electron microscopes: Biological molecules are extremely susceptible to radiation

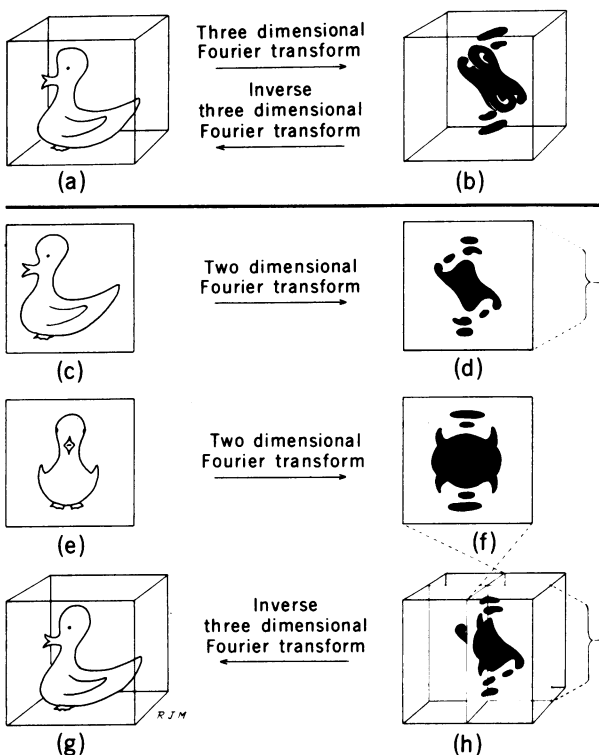


Fig. 1. The mathematical principles of three-dimensional reconstruction. A three-dimensional duck (a) and its Fourier transform (b) are approximated as follows. Required are (c) a two-dimensional projection of (a); (d) the two-dimensional Fourier transform of (c); (e) another projection of the duck; and (f) its two-dimensional Fourier transform. The three-dimensional duck (g) is calculated from an approximate three-dimensional Fourier transform (h) which was reconstructed from the two-dimensional transforms (d) and (f). [Source: James Lake, New York University Medical School]

damage by the beam of high energy (100,000 electron volts) electrons at normal exposure levels. Again, staining provides some relief, but in the words of one researcher, normal electron microscope pictures are not really of biological molecules at all, but rather of their stained ashes.

The special property is that the purple membrane occurs naturally in the form of a two-dimensional periodic array with a thickness equal to the normal thickness of the membrane. By reducing the electron beam current but without reducing the magnification, Unwin and Henderson lowered the exposure of the membrane to about 0.5 electron per square angstrom. This exposure is about a thousand times less than that normally required to image an object at high resolution. This exposure was chosen to be below the radiation dose that would significantly damage the specimen.

The maximum contrast between light and dark areas of the membrane is about 1 percent. At the low exposure required to avoid radiation damage, the signal due to this amount of contrast is much less than that due to statistical fluctuations in the small numbers of electrons forming each point of the image. Thus, no recognizable image is directly obtainable. But, because each unit cell in the purple membrane "crystal" structure is identical, the researchers could use Fourier transform techniques to average out the statistical fluctuations in each of the several thousand unit cells and, in effect, create a clear image of a single average cell.

Diffraction patterns (either x-ray or electron) are naturally interpretable in terms of a mathematically defined reciprocal space or Fourier space. By knowing the amplitude and phase of diffracted x-rays or electrons at enough points in Fourier space, researchers can invert (Fourier transform) these data to obtain an image in direct or real space of the specimen. Unfortunately, diffraction patterns, when recorded, contain only the Fourier amplitudes; the phase information must be obtained in some other way. For large molecules, x-ray crystallographers use a cumbersome and laborious method known as heavy atom, isomorphous replacement. Because molecules in crystals are not perfectly arrayed, the resulting data are limited in spatial resolution to 2 to 3 angstroms. In an electron micrograph, a focused beam of electrons gives rise to an image in direct space, which inherently contains both amplitude and phase information.

What Unwin and Henderson did, therefore, was to obtain Fourier ampli-

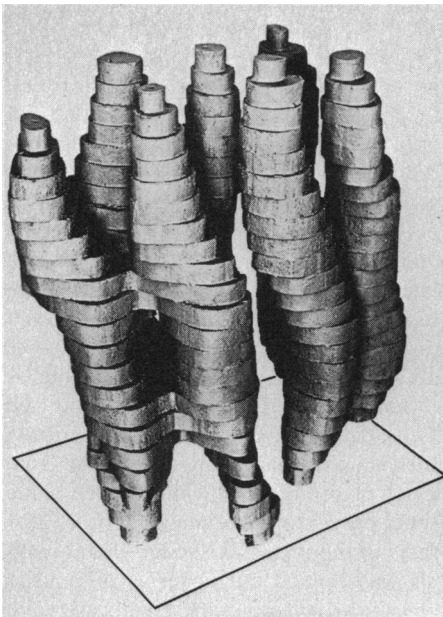


Fig. 2. A model of a single protein molecule in the purple membrane. The molecule is oriented perpendicular to the plane of the membrane. The α -helices are 35 to 40 angstroms long and spaced 10 to 12 angstroms apart. [Source: Richard Henderson and Nigel Unwin, MRC Laboratory of Molecular Biology]

tudes from electron diffraction data and Fourier phases from the low contrast electron micrographs. One micrograph, however, is a projection in two dimensions of what is actually a three-dimensional object, and thus there is insufficient information to reconstruct the full three-dimensional structure. By placing the specimen on a tilt stage which rotates the sample with respect to the electron beam, a sufficient number of "views" or projections can be obtained with enough information to allow reconstruction of a high resolution image (Fig. 1).

This procedure is a specific application of the general mathematical technique known as image reconstruction from projections. The technique has been applied in a variety of disciplines. The most well known of these is computerized axial tomography (CAT) in which computerized x-ray scanners produce cross-sectional images of the head or body (*Science*, 7 November 1975, p. 542). In the case of electron microscopy, however, the picture produced is essentially a contour map of the electron density distribution within a molecule.

The model of the purple membrane constructed by Unwin and Henderson on the basis of this technique indicates that the protein molecules (three of which constitute a single unit cell of the lattice) comprise seven rod segments known as α -helices which are oriented

perpendicular to the plane of the membrane (Fig. 2). In a unit cell, three helices from each of the three protein molecules form a 9-membered inner ring which is surrounded by a 12-membered outer ring of the remaining helices. The spaces between a ring and between neighboring rings are known from this and other information to be filled by lipid molecules arranged in a bilayer configuration. Unwin and Henderson speculate that this structure may be characteristic of many proteins in membranes that act as molecular pumps. Walther Stoeckenius of the University of California, San Francisco, and his co-workers have shown that, under the stimulus of sunlight, the purple membrane pumps protons across the *H. halobium* cell membrane.

Aaron Klug and his colleagues at the MRC laboratory are generally credited with introducing the idea of three-dimensional image reconstruction in electron microscopy, or Fourier microscopy as it is sometimes called. Klug and Jack Berger began by using electron micrographs as diffraction gratings that, upon being illuminated with visible light, give rise to optical diffraction patterns which reveal details of the symmetry of the specimen. Optical diffraction and a related technique called optical filtering combine the many images present in a symmetric specimen to produce an average two-dimensional image of the repeating units of its structure.

In 1968, David DeRosier (who is now at Brandeis University) and Klug reported on three-dimensional image reconstruction of the tail of the bacteriophage T4. DeRosier and Klug obtained all the information needed for a reconstruction from a single electron micrograph, because the helical symmetry of the tail is such that 1 micrograph is equivalent to 21 views at equally spaced angles. In addition to simplifying the reconstruction itself, symmetry is of considerable practical significance because in all likelihood, it would be quite difficult to obtain 21 independent views of a nonperiodic object with well-defined orientations, in part because the orientations of tilt stages are not accurately known.

Fourier amplitudes and phases were obtained by the researchers from the micrographs, with the help of a scanning microdensitometer to digitize the images and a computer to carry out a Fourier transform of the digitized data.

The techniques of three-dimensional reconstruction were extended to electron micrographs of positively stained crystals that had previously been sectioned, when James Lake (now at New

York University Medical School) and Henry Slayter at Harvard Medical School applied them to ribosome crystals. In positive staining, contrast is provided by heavy metal ions bound to the specimen, rather than coating its surface. With a modified version of the Fourier technique of DeRosier and Klug, Lake determined an electron density map of the ribosome at a resolution of 100 angstroms from the 17 different views present in a micrograph of a sectioned helical array of ribosomes.

Although Fourier transform methods seem to many researchers to be the natural way to go about image reconstruction, there are other possible approaches. For example, in 1970, Richard Gordon (now at the National Institute for Arthritis, Metabolism, and Digestive Diseases, Bethesda, Maryland), Robert Ben-

der, and Gabor Herman of the State University of New York at Buffalo proposed a method called the algebraic reconstruction technique (ART) that did not use Fourier transforms. Instead, ART is an iterative technique that searches for the minimum discrepancy between actual projections (micrographs) and computed projections of an estimated image. Although ART and its variants are of much interest to researchers in other disciplines, most electron microscopists are sticking with the Fourier technique.

Also at the MRC laboratory, Klug and Tony Crowther have developed criteria for obtaining maximum information from a set of projections while, at the same time, preventing the introduction of artifacts in the image. Although their analysis was carried out in terms of Fourier space, the investigators' results are valid

for other reconstruction methods. And Harold Erickson and Klug demonstrated how to recover the maximum contrast in an object by defocusing the electron microscope and using a computer to compensate for the distortion in the resulting image. This procedure was an essential ingredient of Unwin and Henderson's success.

Despite its potential efficacy in imaging biological molecules, three-dimensional image reconstruction has not yet become a widely practiced technique. In the United States, for example, Lake and his associates at the New York University Medical School, who have been studying ribosomes, and David Eisenberg, Frederick Eiserling, and their colleagues at the University of California, Los Angeles, who have been combining

(Continued on page 400)

The Coldest Planet: Methane Ice Found on Pluto

Pluto is 39 times farther away from the sun than the earth is, and at that distance it appears fainter than many stars. The sun's radiation is so weak at Pluto that temperatures close to absolute zero have long been expected.

The difficulties in observing such a faint object are great, and so it was with much excitement that planetary astronomers received the recent news that the surface composition of Pluto had been measured for the first time, and found to be frozen methane. The finding means that the surface must be colder than 50°K, the temperature at which pure methane condenses at very low pressure. It also lends credence to certain theories of the origin of the solar system, and suggests that perhaps Pluto is the only planet that has survived in a pristine state, preserving a memory of the conditions at which it was formed 4.6 billion years ago.

Using the 4-meter telescope at the Kitt Peak National Observatory, three astronomers from the University of Hawaii showed by reflectance spectroscopy that the surface of the outermost planet is at least partially covered with methane ice. Dale Cruikshank, David Morrison, and Carl Pilcher observed the infrared reflections of Pluto through two very narrow band filters—one selected to admit bright reflections from water ice and the other to admit reflections from methane ice. "On Pluto, the response of the filters was exactly as expected for methane ice," says Cruikshank. The filters were also used to observe several moons of Saturn, already known to have the spectral signature of water ice, and the opposite response was found. Only Pluto exhibited the band ratio expected for methane ice.

Although methane was known in the atmospheres of Jupiter, Saturn, Uranus, and Neptune, and had also been identified as an obvious component of the atmosphere of Titan, a moon of Saturn, the recent measurements were the first detection of methane in solid form. The data were obtained during five nights in mid-March, using one of the world's largest telescopes, an instrument that is normally used for stellar and extragalactic astronomy.

Of course, the measurement alone can only be a hint at the complete description of Pluto, but it suggests that Pluto is small and icy, with low density—more like the satellites of the outer planets than the outer planets themselves.

Previously, Pluto was thought to be about 6000 kilometers in diameter, but refinements based on the methane discovery will tend to lower that estimate, according to the Hawaii astronomers, perhaps to the size of the moon (3500 kilometers). Such small planetary bodies are the ones most likely to radiate away their internal heat without undergoing chemical change. The measurement of Pluto's methane coat, says Morrison, is an insight into the "surface composition of objects small enough to retain some memory of the conditions at which they formed."

If solid methane composes more than the surface of Pluto, the planet should have a very low density. But its density is virtually unknown. The diameter is in question and some astronomers think no limits at all can be put on the mass with the data available. Thus its bulk composition is also unknown, but the general considerations of the chemistry of the outer planets give some clues.

Chemical models of the outer solar system predict that it should be rich in water, ammonia, and methane—as well as their combinations—because they would be the last substances to condense out of the early solar nebula. Laboratory chemistry, with the help of computer calculations, shows that among these icy materials, pure methane should be the last to condense. Thus it is something of a confirmation to find it on the outermost planet, which was presumably formed at the lowest temperature. But Pluto could also have a more complex structure. It could have a core of water ice, formed first, followed by layers of methane clathrate, ammonia clathrate, and finally pure methane ice.

Referring to Pluto's genesis from the solar nebula, David Morrison says, "We have at last found one point where it was cold enough to produce solid methane."—W.D.M.

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RESEARCH NEWS

(Continued from page 362)

x-ray diffraction and electron microscopy to determine the structure of glutamine synthetase, are among the few who have reported results with this technique. Groups at Brandeis, the University of California, Berkeley, and the University of Wisconsin-Madison also have research under way. Outside the United States, there are active groups at the Biozentrum of the University of Basel, the Institute of Crystallography in Moscow, and the Max Planck Institute for Biochemistry in Munich.

Reasons cited for this state of affairs do not include the expense of equipment. A good quality electron microscope, a scanning microdensitometer, and computation facilities are neither cheaper nor more expensive than much equipment common in other types of experiments in structural biology, such as protein crystallography and nuclear magnetic resonance spectroscopy. But, three-dimensional image reconstruction is not a trivial technique. Rather, a group of researchers willing to forego the possibility of quicker results from other types of experiments must spend considerable time mastering the various aspects of the method (especially finding and preparing suitable specimens) before they will have publishable results.

Seen in this light, the spectacular results of Unwin and Henderson may be just as important for the boost they may give to others to take up image reconstruction as for their scientific value. There is, however, one outstanding question mark, namely, how generally applicable is their method?

Besides the glucose fixation method of the MRC investigators, there are at least two other ways to prepare hydrated, unstained specimens for electron microscopy. Donald Parsons and S. W. Hui of the Roswell Park Memorial Institute in Buffalo have devised an environmental chamber in which it is possible to maintain a specimen in its natural hydrated state by differential pumping; that is, near the sample, the vacuum is poor, but elsewhere the vacuum is typical of all electron microscopes. And at the University of California, Berkeley, Kenneth Taylor and Robert Glaeser carefully freeze specimens in liquid nitrogen. The frozen samples do not dehydrate when under vacuum. Electron diffraction patterns of catalase crystals prepared by both methods indicate that structural details with dimensions of 2 to 3 angstroms are preserved. In addition, there is accumulating evidence that radiation ef-

fects are much less severe at cryogenic temperatures than at room temperature.

But how many biological molecules are there that naturally align themselves into two-dimensional periodic structures or that crystallize into such a form? Glaeser and Donald Caspar of Brandeis think there may ultimately be quite a few. Besides membrane proteins which would seem to be natural candidates for crystallization into two-dimensional forms, Caspar is enthusiastic about the possibility of imaging large animal viruses. Such viruses may be too formidable for x-ray crystallography to tackle, in part because of the phase problem, but would be easier subjects for image reconstruction because both amplitude and phase information are available. Robert Horne at the John Innes Institute, Norwich, England, has recently reported on a method for preparing highly ordered two-dimensional arrays of viruses that may be applicable to image reconstruction.

Glaeser points out that, within cell membranes, many apparently crystalline materials exist, but as yet no one has been able to crystallize them outside the cell in a form suitable for electron microscopy. Glaeser also feels that, because image reconstruction from electron micrographs is a faster and less arduous technique than x-ray diffraction, it may be the method of choice in cases where both two- and three-dimensional crystals are available, provided that the resolution of electron microscopy can be increased to that of x-ray diffraction.

To this end, Glaeser and Ivy Kuo at Berkeley have been experimenting with image intensifiers and with nuclear track photographic emulsions in order to increase the sensitivity of the electron recording process. In Unwin and Henderson's experiments, perhaps six or seven electrons struck one picture element in the digitized micrograph. The Berkeley researchers want to decrease this to one electron, and foresee that by so doing, they may achieve a spatial resolution of 3 angstroms.

Some scientists think it may be possible to prepare two-dimensional arrays of molecules (for example, elongated molecules could be tied to a substrate at one end), but each molecule could be oriented slightly differently at its binding site. Joachim Frank of the State of New York Department of Health, Albany, has been exploring theoretical methods for averaging data from arrays of identical objects that are not periodic. If such methods were to be perfected, then, in the words of one scientist, the sky would be the limit.—ARTHUR L. ROBINSON