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The possibilities and prospects of obtaining high-resolution information (below 30 Å) on biological material using the electron microscope

Some comments and reports inspired by an EMBO workshop held at Gais, Switzerland, October 1973

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I. Introduction

Commercially available electron microscopes routinely provide resolution of some 2–4 Å, as determined on the spacing of crystalline lattices of certain stable, small-molecular substances. On biological material – either macromolecules or macromolecular assemblies – 'biologically significant' details below some 20 Å have hitherto not been observed. We consider as 'biologically significant' those structural details observed or contained in electron micrographs which are consistent with, or confirmed by, other data obtained from biochemical or functional experiments or by other physical methods (optical, magnetic, electric).

It was the goal of this workshop to discuss and weigh the different limitations and the prospects for overcoming them. Three sessions were therefore devoted to considerations of specimen destruction by the electron beam, the different degrees and forms of distortion introduced by the techniques of specimen preparation, and the possibilities and limitations of the different procedures for expressing the information carried by the electrons after interaction with the specimen. Thus, procedures leading to more-or-less truthful imaging (conventional transmission microscopy and transmission scanning microscopy) or electron diffraction were discussed. The last session was devoted to mathematical and optical methods for processing the information contained in an electron micrograph. By such procedures, relevant information may be extracted out of a background of different 'noises', or bettered by adding the information contained in many repeated identical elements.

Why is the range between 5 and 35 Å so important for the biologists as to justify efforts to improve electron microscopy? Are other methods like X-ray diffraction and other physical techniques not sufficient?

X-ray-diffraction has the required resolution, but it is confined to relatively large three-dimensional crystals or very precisely oriented gels. Soluble proteins, like enzymes and haemoglobins and soluble tRNA are among those which have been demonstrated to be crystallizable and to yield biologically significant information. Such crystals are fundamentally different from small molecular crystals in that they have a very high water content (up to 80%). The interactions holding the crystals together are weak and of the same nature as those holding them apart in solution (charge, polarity and hydration shells). Because of this particular situation, the conformation is maintained in a biologically significant state. Conformational changes related to biological

functions and their regulation could thus be demonstrated in many instances. In some cases, regulatory substances can act on the proteins even within the crystal lattice; they penetrate through the water-filled spaces of the crystals.

Other biological macromolecules are specifically designed so as to form regular aggregates held together by specific interactions which are much stronger than those in the above-mentioned crystals. They are characterized mainly by an exclusion of water in between the interacting surfaces. Examples of this are the naturally occurring and biologically functional biocrystals in which the so-called hydrophobic interactions play a major role. A variety of two-dimensional biocrystals exist, of which the biological function is most interesting, while all naturally occurring three-dimensional crystals hitherto known have only storage functions. Two-dimensional protein biocrystals are the basis, for example, of virus shells, microtubuli, flagella, cilia. They have the distinctive fundamental feature that, once their assembly is complete, the equilibrium constant of the aggregate (with its free subunits) is so strongly modified that the biocrystal becomes virtually insoluble under physiological conditions. It seems obvious that it will be most difficult to produce three-dimensional regular arrays of such two-dimensional biocrystals. It was suggested that their sub-units could be crystallized in three dimensions out of organic, non-aqueous solutions. Since it was shown that proteins undergo strong conformational changes (denaturation) in such solvents (Tanford *et al.* 1962), it is likely that, even if obtainable, such crystals will show the macromolecule in a conformation which bears little, if any, resemblance to its biologically functional state.

Proteins of another class are those of the biological membranes, where they are responsible for major specific functions such as active transport and signal transmission. These proteins are partly buried in the lipid bilayer, by virtue of strong lipid–protein interactions. It has already been demonstrated that most, if not all, of these proteins lose their biological activity as soon as they are freed from the lipid. Their functional conformation can exist only in the presence of lipids or their like.

A third class of functional macromolecular combinations are those based on nucleic acid–protein interactions. Enzymes involved in replication, transcription, repair, etc., of nucleic acids, are obviously of highest interest, since they are part of strongly regulated systems.

Again, their biologically functional conformation is likely to exist only in contact with the nucleic acid. Hence, very large obstacles have to be overcome when producing a three-dimensional crystal in which the subunits still show a biologically significant conformation.

Some of these proteins and protein complexes might be studied by optical methods (ORD, CD and others) when in aqueous solution. These methods are, however, intrinsically limited and, unlike electron microscopy, seem to have no potentiality for further important extensions. Other physical methods mainly give information on bonding, and are, therefore, complementary to, and dependent on, structural studies.

Finally, specific heavy metal staining of the different purine and pyrimidine bases of nucleic acids or of the specific interaction sites on proteins is another very important approach in the resolution range of 5–10 Å. Obviously, it is correlated with the possibility of imaging sufficiently many significant details for the location of the stained site to be identified relative to the overall structure of the macromolecule.

From these considerations, it might become clear why electron microscopy in the range of 5–30 Å deserves so much attention and effort. Indeed, its potential capabilities suggest that it might be useful exactly in those numerous cases where the method of X-ray diffraction is severely limited. By virtue of their low penetrative power, electrons are particularly suitable for studying single molecules or monolayers thereof. In particular, two-dimensional biocrystals are of the highest interest, because as we will see later, they might provide an instance in which the electron dose per subunit could be kept sufficiently low as to restrict specimen destruction to within tolerable limits. As with X-ray analysis of three-dimensional crystals, the information contained in many identical subunits of a crystalline array can be pooled by image-processing or by direct electron diffraction.

In order to cover the four different aspects mentioned above, the participants were recruited from among biophysicists and biologists on the one hand, and electron opticians and specialists on information treatment on the other. Care was also taken that the contacts between researchers from the old and the new continents could be intensified for improving world-wide collaboration.

As we shall see in greater detail in the following four sections, some rather encouraging outlooks emerged from the workshop. Despite the fact that the interaction of the electrons with the specimen necessarily

leads to destruction of biological macromolecules, it was shown for a most extreme case that even the remaining 'heaps of ashes' – as they were called in exaggeration – might provide biologically significant information below 20 Å.

It became also very clear, that – besides diffraction – two modes of image formation will be pursued, one of which aims at a maximum of coherence of the electron waves; the resulting very complicated interference patterns of the image (when considering levels below 30 Å) are then analysed mathematically by computer. In the other approach, one tends rather to a maximum of incoherence, so as to obtain directly a 'true' image, which is obviously easier to observe and interpret. Indeed, below 30 Å the 'image' obtained by the coherent procedure will become understandable only when reconstructed from the superposed interferograms of all the component atoms, while in the second, the image at 3–5 Å resolution will be an average over the light atoms and only single heavy atoms will be depicted discretely. The future will show whether both types of imaging will be necessary for further progress. To some of us, however, it seems likely that the approach through the incoherent type of image formation will be much less demanding in terms of instrumentation and computation and therefore will be more accessible to biological research. Here indeed we need large, statistically significant numbers of observations to ensure reproducibility of results. The 'pseudo-imaging' method, based on coherent waves, will perhaps be applied only on biological structures which have been pre-screened by 'true' images. In any case we have to await further research efforts about the restrictions imposed by the action of the beam on the specimen.

II. Specimen damage due to the beam

The beam of the electron microscope must be regarded not only as a flux of short wavelength radiation that can be focused to give a high-resolution image, but also as an intense flux of ionizing radiation which is capable of causing severe damage to biological specimens.

Specimen damage has been measured from a wide variety of experimental standpoints, including degradation of electron diffraction patterns, mass-loss, changes in the energy-loss spectrum of transmitted electrons, and changes in the infrared spectrum. In addition, it has been evaluated by using optical diffraction on electron micrographs obtained before and after additional beam exposure.

Much of the radiation-damage literature (e.g. Glaeser, 1971) was reviewed by Glaeser. He presented estimates of the smallest object detail that could be seen with beam exposures that do not exceed the critical exposure for complete damage (estimated according to the various damage end-points). To make these estimates, the Rose equation was used with an assumed contrast value of 0·1 and utilization factor of 0·25. The limits quoted were: ~ 15 Å for stained specimens (catalase); ~ 50 Å for unstained, saturated bond molecules; between 1 and 15 Å for unsaturated (aromatic) compounds.

Experimental work has been carried out to determine whether new methods or techniques can overcome these limitations. Glaeser expressed the opinion that one can largely exclude ideas based upon (*a*) high voltage, (*b*) ultra-high vacuum, (*c*) very low specimen temperature, (*d*) image intensifiers, and (*e*) giant pulse electron microscopy. Ideas that remain to be tested are (*a*) use of the information redundancy inherent in periodic objects, (*b*) use of energy 'scavengers', (*c*) development of more resistant stains and/or embedments, and (*d*) use of heavy atom-labels under experimental circumstances where migration of the atom-label cannot occur.

It is not yet certain that a change (damage) that occurs on the atomic or chemical bond level necessarily results in major changes at a higher (molecular) level, the one of primary interest to the molecular morphologist. This point was raised by Parsons and was repeated in written comments submitted by Bahr. In addition, Bahr has noted that the degree of mass-loss depends substantially upon the initial current density. Lower current densities result in less loss of total mass, presumably because they tend to favour cross-linking over the mass-loss pathway. Fixation by cross-linking may not be so effective for free-standing molecules as for those in a dense film. This may explain why Williams was unable to find shadows cast by single molecules of nucleic acid after electron irradiation, in spite of the fact that Bahr reports that the nucleic acids lose only about 5% of their mass.

Mass-loss was also studied by Dubochet, using the autoradiography method of Thach & Thach (1971). Results obtained by this method have been quite variable, ranging from no loss at all to as much as 35% mass-loss. The higher figures are given by Bahr (Bahr, Johnson & Zeitler, 1965) and by Wall (1972). Dubochet suggested that the quality of the high vacuum might affect mass-loss studies significantly, and cited unpublished studies of Hartman on the effects of ultra-high

vacuum. It was agreed that processes of etching and contamination are important at high beam currents. But Glaeser asserted that these should be regarded as secondary effects in relation to those from direct beam damage, since the latter occur at exposures that are small compared to those needed to produce etching or contamination effects.

It was debated whether studies based upon degradation of electron diffraction images were a good measure of the damage effect to be expected in images. Crewe pointed out that for aromatic amino acids the diffraction-loss effect is more rapid than the mass-loss effect, which in turn is far more rapid than the loss of characteristic ultraviolet spectral features. Crewe advocated the measurement of damage cross-sections for the process of interest in a given experiment, and then a comparison of the damage cross-section to the elastic scattering cross-section in order to test the feasibility of molecular imaging at any given resolution. An alternative to the use of diffraction data was suggested by Frank. This method requires the calculation of the point-by-point modulus of the difference between image functions for the damaged and the undamaged molecule. Glaeser emphasized that the loss of the diffraction patterns, as normally measured, was precisely the observed quantity needed to determine the useful resolution that would be had after a given exposure.

In terms of practical microscopy, Williams showed several demonstrations of the improvement in image information that results from using only as small a dose as that which is needed to blacken the photographic plate. Among the examples shown were micrographs of T_4 bacteriophage, tobacco mosaic virus, bushy stunt virus, and aspartate transcarbamylase. Further insight into the damage that occurs in stained specimens for exposures above 0·025 C/cm^2 was produced in the work of Unwin, who noted that changes occur in the relative intensity of some peaks in the optical diffractogram of TMV (stacked disc) protein. With the help of three-dimensional reconstruction techniques, Unwin was able to demonstrate that these changes were due to migration of the stain about the more stable radiation-product (the 'ashes') of the underlying protein. The stability of this product has also been noted by Bahr.

III. Limitations due to specimen preparation and ways to improve this

The second session (of the EMBO workshop) was concerned with limitations arising in the specimen. The biologist is interested in the detailed structure of macromolecules and their complexes suspended in an aqueous medium which also contains small solutes. In general the wet specimen cannot simply be inserted into the electron microscope since in the vacuum the aqueous phase would evaporate and during the drying the macromolecular structure would collapse. Three types of approaches have been used to avoid this problem: (1) negative staining where the aqueous matrix is replaced by a non-volatile electron dense substance; (2) methods involving freezing of the sample followed by partial or complete removal of the water; (3) use of special chambers which permit high humidity in the immediate vicinity of the specimen. These procedures were discussed as well as the special problems encountered when tissue must be sectioned. Finally the properties of ideal and real supporting films were presented.

Horne described new variations (Horne & Ronchetti, 1974) in the negative-staining procedure which lead to regular arrays of spherical virus particles with good preservation of structure down to 20 Å.

It appears that, if the specimen is dried before negative staining, only information about the surface of the specimen is obtained. However, stain applied to a wet specimen penetrates and gives insight into internal organization. This was illustrated by Elliott who worked with muscle filaments (Elliott, 1974).

Possible distortions accompanying negative staining were illuminated by Crowther who examined tomato bushy stunt virus in the electron microscope (Crowther & Amos, 1971) and compared his results with the X-ray diffraction data on wet suspensions (Harrison, 1971). Negatively stained specimens show reproducible structural features down to 25 Å; during preparation, the particles shrink by about 30% in diameter but retain their icosahedral symmetry.

Similar comparisons were reported by Unwin, who used stacked discs of TMV protein (Unwin, 1972). The results indicated that radial and longitudinal structural features were preserved to perhaps 10 Å while azimuthal distortions were somewhat greater than this.

Bachmann reported on a re-investigation of preservation of biological structure through rapid freezing (Bachmann & Schmitt, 1971; Bach-

mann & Schmitt-Furnian, 1973; Williams, 1952). The best procedure is to drop tissue blocks into freon or propane at the temperature of liquid nitrogen, or – better still – to spray droplets on very cold surfaces and then to dry them under vacuum (Williams, 1952). Contrast is obtained through fine-grained tantalum or tungsten shadowing (Abermann, Salpeter & Bachmann, 1972). The polyhedral molecular shape is preserved in ferritin, and the dimensions found for fibrinogen and serum albumin are correct to within 10 Å.

An attractive possibility for freezing with minimum damage is suggested by the work of Hoppe *et al.* (1973), who showed that protein crystals frozen at very high pressures – where a high density form of ice is obtained – give X-ray diffraction patterns essentially the same as those derived from wet crystals.

Ottensmeyer discussed the critical-point method of Anderson (Anderson, 1951; Fromme *et al.* 1972) for avoiding the surface tension artifacts produced during drying. Flattening of phage heads and damage to tail fibres could be avoided in this way.

For these methods designed for three-dimensional preservation, one has to keep in mind that smaller parts are susceptible to 'thermal collapse' (Anderson, 1954): fibres or fibrous protrusions of diameters less than some 30–40 Å will collapse on to nearby surfaces.

Parsons described electron microscopy of wet unfixed material – perhaps the best procedure for preserving biological structure (Parsons *et al.* 1973*a*). Specimens are kept hydrated inside a special compartment within the electron microscope. Here they are separated from the vacuum except for small openings for the entrance and exit of the beam. The device allowed high-quality electron diffraction of catalase crystals with reflexions to 2 Å (Matricardi, Moretz & Parsons, 1972). High-resolution imaging is at present hampered by mechanical instabilities (which presumably will be overcome). Also, the beam must traverse vapour and liquid and the resulting multiple collisions impair the image. This problem is somewhat diminished by the use of High Voltage (Parsons, Matricardi & Uydess, 1973*b*). Finally, the contrast is low. This latter difficulty may be eased in the future through microscopy with energy analysers which provide a novel, largely unexplored, approach to contrast improvement.

Sjöstrand discussed the special problems involved in preserving structure in tissues which must be embedded and sectioned. Among other innovations, he introduced the use of ethylene glycol as dehydrat-

ing agent (Sjöstrand & Barajas, 1968, 1970) since this alcohol is known to produce relatively small denaturation artifacts on proteins (Tanford *et al.* 1962). The quite different appearance of the tissue – particularly in the case of mitochondrial membranes – emphasizes that the phenomena occurring during dehydration in organic solvents have not been adequately accounted for so far.

A compelling need exists for the production of smooth specimen-supporting films since, in the final image, the structural features of the object and the substrate are inextricably superimposed.

Müller reviewed the work on single crystals of beryllium by Komoda, Nishida & Kimoto (1969), of graphite by White, Beer & Wiggins (1971), Riddle & Siegel (1971), and Hashimoto *et al.* (1973) and their co-workers, and vermiculite by Baumeister & Hahn (1973*c*). These appear very uniform in the electron microscope. Nearly as good are the 'Alox' films recently developed by Koller and his collaborators (Müller & Koller, 1972). Finally, recent times have seen progress in the technology of producing exceedingly thin evaporated carbon support films perhaps down to only a few Ångstroms (Williams & Glaeser, 1972). Recent advances in this area have allowed the visualization of single heavy atoms and even the carbonaceous parts of macromolecules (Whiting & Ottensmeyer, 1972).

In developing supporting films, adequate smoothness is not the sole criterion. Procedures must be found which lead to the deposition of the specimens of interest in suitable form and abundance (Dubochet & Kellenberger, 1972). Koller illustrated this by his thorough study of the deposition of double-stranded DNA on various substrates (Koller, Sogo & Bujard, 1974). Different substrates responded remarkably differently with good deposition obtained on mica and evaporated carbon in the presence of ethidium bromide. Of course, smooth substrate will be useful only if procedures are found for depositing samples on them.

The delicacy needed in choosing the conditions for applying specimens to substrate is illustrated in the finding by Elliott & Offer (1974) that solutions of ammonium acetate sprayed on to mica do not spread, but solutions of ammonium formate do.

The image contributions due to crystalline substrates can be eliminated in dark-field microscopy by judicious placement of the objective aperture so that the diffracted orders are excluded from it as shown by Hashimoto. Alternatively the image can be filtered optically as pointed out by Baumeister.

IV. Information transfer by means of different types of imaging

Main discussion points of the preceding sessions were the artifacts in the electron micrographs of biological objects caused by radiation damage and by preparation. It was the goal of this session to discuss artifacts and the lack of information resulting from imperfections of the optical imaging process.

(*a*) *Bright field* (*linear transfer*)

The elastic scattering process as well as the inelastic process give essential information about the atomic structure of the microscopical specimen. In conventional electron microscopy, only the elastically scattered electrons are employed for image formation. In the case of weak objects, the optical process can then be described by a linear theory, whereby phase-contrast transfer functions can be established. By means of these functions, the information content stored in the image can be clearly described.

Transfer functions depend on spherical aberration and astigmatism of the objective lens and also on defocusing. Parsons reported on investigations by Engler and himself into measuring the axial component of the magnetic lens field with a Hall effect microprobe (sensitive element $100 \times 100\ \mu$m). The small probe gave smaller field values, resulting in larger values of the spherical aberration constant than are commonly assumed. Further improvements should also allow measurement of the coefficient of astigmatism. Siegel (Krakow, Downing & Siegel, 1973, 1974) reported on experiments using a tilted carbon foil for determining the transfer function. A modified light-optical diffractometer equipped with a cylindrical lens was used to obtain the 'one-dimensional' Fourier transform of the micrograph of this foil, giving a continuous representation of the defocusing dependence of the transfer functions. The tilt technique provides a rapid means of obtaining the large amount of data required for determining the optical transfer characteristics of the imaging system with phase plates (see below). In the case of linear transfer, the images can be improved by spatial filtering and by holographic reconstruction methods. Particularly, filtering by means of phase plates is of great practical importance. Unwin (1971, 1972) showed that the charge distribution generated at

the centre of his electrostatic phase plate (a 0·3 μm thread in the back focal plane) provides the necessary phase-shifting properties and partial interception of the unscattered electrons. It is thus analogous in function to the absorbing phase plate in light microscopy. It produces a strong contrast in thin biological specimens over the useful spatial frequency range $\sim \frac{1}{50} - \frac{1}{5}$ Å^{-1}. This method makes it possible to gain information about specimens which cannot be detected in conventional bright field. This advantage is attributed to preferential contrast enhancement of the biological material (rather than of the stain as in the conventional case) as a result of absorption and phase contrast effects working co-operatively for this material, but in opposition for the stain. Krakow & Siegel (1972) have produced an Unwin-type phase plate (0·25 μm Pt wire, coated with Au). Their important result is that there is an additional term in the phase-shift which is proportional to the logarithm of the scattering angle. Hence the unscattered beam is strongly phase-shifted even for slight divergences ($\sim 10^{-4}$ rad), reversing the relative contrast in the image.

'Linear transfer' does not mean that only 'real object structures' are present in the image. In most cases electron micrographs with linear transfer are subject to strong 'parasitic structures' (extended systems of Fresnel fringes) (see Hanszen, 1973*a*). Linear transfer means, however, that structures of this kind can be eliminated by reconstruction methods. The size and shape of the effective illumination source has a great influence on the shape of the transfer function (Hanszen & Trepte, 1971; Hanszen, 1973*b*; Hanszen & Ade, 1975; Frank, 1973*c*) and thus on the appearance of parasites. A 'triangular' transfer function would suppress the parasites entirely (Hanszen, 1973*a*). A transfer function of this shape can be very closely approximated by a ring condensor (Hanszen & Trepte, 1971). It will be pointed out below that an illumination of this kind might also play an important role in dark field microscopy. According to theoretical investigations of Rose (1974) phase-contrast images can be recorded in the scanning transmission electron microscope with electron doses of the same order of magnitude as in the conventional microscope. For this purpose, an annular detector of appropriate geometry and an additional circular detector should be located behind the object inside the illumination cone. The arrangement is closely related to the above-mentioned bright-field ring condensor in the conventional microscope. Only atoms lying in a thin layer of the specimen will be shown with significant contrast. This arrangement will

therefore be appropriate for detecting single atoms in relatively thick molecules.

The imaging of many biological specimens can be described by the linear transfer theory. According to Erickson & Klug (1971), thin negatively stained catalase crystals are imaged as predicted by this theory. These specimens are typical of biological objects and confirm that the slightly underfocused image is a valid picture of the projected mass density of the object. Recently, Voter and Erickson investigated the imaging of thicker, higher-contrast crystals which demonstrate a comparatively high resolution beyond 15 Å. Significant departures from linear theory were found which imply artifacts and spurious structures in the image. The departures appear to be second-order interactions but are not explained in detail by the second-order theory (Erickson, 1973). The interpretation of high-resolution images will require careful considerations of these effects. Dorset and Parsons investigated kinematic versus dynamic scattering (i.e. the scattering of strong objects) in high-resolution electron diffraction patterns of wet catalase microcrystals. By using an electron microscope hydration chamber it was shown that much higher resolution diffraction patterns could be obtained from wet unstained, unfixed catalase crystals than from dry fixed or stained crystals (Matricardi *et al.* 1972). These wet crystals are not more sensitive to radiation damage than dry crystalline amino acids. Dorset and Parsons analysed some principal intensities from over 400 patterns of this kind having spots going out beyond 2 Å. Several approaches indicated that the diffraction intensities were essentially kinematic.

The report of Hoppe (Hoppe, 1972; Hoppe *et al.* 1973) dealt with the question of whether an improvement in resolution is of much value in biological microscopy, since radiation damage restricts the useful resolution to $\sim$ 5–10 Å. It is usually overlooked that a specimen in every degradation phase still consists of atoms. The Fourier transform therefore does not 'fade out' (as in the case of non-sharp objects). Improvement of resolution therefore also means improvement of the signal-to-noise ratio. An improvement of the order of 50% over the conventional resolution has recently been achieved (Feltinowski & Hoppe, 1973). The image improvement described by Hoppe (1971) is of special interest as it has been shown recently (Hoppe & Köstler, 1973) that this scheme also corrects the chromatic aberration (Hoppe, 1970).

(*b*) *Dark field*

During the workshop it became clear that the dark-field method will play the most important role in high-resolution microscopy. According to the concept of Fourier optics ('spatial frequency approach'), imaging always becomes non-linear when the unscattered beam is eliminated as is done in dark field microscopy (see Hanszen, 1969). There is always the danger in these cases that higher spatial frequencies ('smaller structures') which are not present in the object may appear in the image. Spurious structures of this kind cannot be eliminated by the above-mentioned reconstruction processes. The intensity of these structures in dark-field micrographs depends to a considerable degree on the illumination mode: coherent axial or tilted illumination or partial coherent hollow cone illumination (ring condensor with narrow or broad ring-width – the latter case is often called 'incoherent dark field'). In bright field with axial illumination, the image is built up of the spatial difference frequencies as well as of the sum frequencies, in dark field with ring condensor, however, only of the difference frequencies (Hanszen, 1969; Hanszen & Ade, 1974*a*, *b*; Ade, 1975). In the strict sense of Fourier optics, it is not possible to establish transfer functions in all these cases of dark field. However, it should not be overlooked that the illumination with a broad ring condensor realizes incoherently radiating object points to a very good approximation. Therefore, this dark-field illumination mode is a powerful tool in high-resolution electron microscopy, regardless of whether it conforms to linear transfer theory or not. Crewe reported on recent results with the scanning transmission electron microscope using a broad ring detector which is the direct analogue of the above-mentioned ring condensor in the conventional electron microscope. With this arrangement he was able to obtain a resolution of 2·5 Å when imaging single atoms. Willasch (Thon & Willasch, 1972; Willasch, 1973) showed dark-field images of three Hg atoms at a mutual separation of 7 Å. These micrographs were taken in a conventional microscope using a ring condensor having a small ring-width. The advantages of this illumination mode, compared with the other ones discussed below, are high current density in the object – allowing short exposure times – and rotational symmetry of the ray tracing.

Hitherto unexplained bright spots in dark field having sizes as low as 3–5 Å were discussed by Brakenhoff (1974). They were found to

appear at an overfocus of the order of 2750 Å with respect to the bright field image. A physical model was proposed according to which the bright spots are associated with electron wave-fronts Bragg-reflected on small crystals in a carbon foil.

Hashimoto *et al.* (1973) showed dark-field micrographs of single atoms in thorium pyromellitate molecules and thorium oxide crystals. Tilted illumination brought a resolution which was two times better than axial illumination. The molecules and crystals were supported by thin graphite films having extremely low noise. Ottensmeyer succeeded in achieving sufficient contrast to image single atoms as light as Pd directly (Whiting & Ottensmeyer, 1972) or as light as S by combining this dark-field illumination with the technique of superposing many micrographs and applying optical filtering methods (Ottensmeyer, Schmidt & Olbrecht, 1973). As early as 1969, this approach indicated the possibility of imaging unstained and unshadowed biological macromolecules (Ottensmeyer, 1969). The results presented at the workshop indicate that despite extensive radiation damage to such specimens, images can be obtained which indicate a resolution of better than 10 Å and which bear a remarkable resemblance to the accepted structures in cases where a comparison can be made with X-ray diffraction data. Moreover, the atom-imaging capability of the applied technique permits the identification of specific portions of the image corresponding to regions in the macromolecule which had been specifically labelled with clusters of heavy atoms.

(*c*) *Inelastic scattering*

Over the last few years considerable efforts have been made to evaluate the object information stored in the electron beam due to inelastic scattering in the object (see Misell & Burge, 1973). The inelastic electrons are lost in the coherent beam behind the object and cannot produce contrast by interference with the unscattered beam (which is the essential contrast mechanism for weak objects). Since these electrons are missing there, the wave function of this beam is amplitude-modulated. In the case of weak objects, this modulation is proportional to the number n_i of inelastic electrons. The phase modulation b caused by the elastically scattered electrons, however, is proportional to the square root of the number n_e of these electrons. Since, for a given material, the ratio n_e/n_i is constant, the elastic interference contrast b (linear phase contrast) is proportional to the square root of the linear amplitude contrast

a, which is associated with the inelastic scattering. For weak objects ($a, b \ll 1$), the elastically scattered electrons therefore always play the predominant role in bright-field contrast formation (see Hanszen & Ade, 1974*a*).

The situation is quite different in dark-field microscopy, where the interference between scattered and unscattered waves does not contribute to the image. In this case, the inelastic electrons can play an important role in image formation (see Misell & Burge, 1973). Brakenhoff pointed out that dark field geometries are mostly of such kind that inelastically scattered electrons make a dominant contribution to the image of biological material. The optimum focus for these inelastic electrons (energy loss $\approx$ 25 eV) would then be expected at about 5000 Å under-focus with respect to the Gaussian image plane. Pictures of biological material were shown which confirmed these expectations. The resolution of the inelastic image seems to be of the order of 15 Å. Ottensmeyer reported on the use of an energy filter in the imaging of thick unstained specimens such as polyoma virus (Henkelmann, 1973). Energy filtering virtually removes the chromatic aberration which normally precludes the use of dark-field technique in imaging thick specimens. the resolution improvement in filtered dark-field images is such that one is tempted to say that, for a large class of biological specimens, the problem of resolution and contrast no longer exists but has been replaced instead by the more fundamental problem of specimen preservation.

According to Krakow and Siegel, the inelastic background in a central-beam-stop dark-field micrograph of a thick specimen ($\sim$ 300 Å) is greatly attenuated by taking one micrograph at 'optimum focus' for the elastic image as defined by Misell, and a second micrograph at a defocus where the 'elastic image' is greatly attenuated, but the broader 'inelastic image' has not changed appreciably. A reverse contrast image of the second micrograph is made on a photographic transparency and the two superpositioned transparencies are printed to give a print of essentially the 'elastic image' alone with enhanced resolution and contrast.

As is commonly known, the scanning transmission electron microscope offers excellent possibilities for processing the elastic as well as the inelastic signal. The ratio of the signals can be used to visualize single atoms on the background of a carbon foil (see for example, Crewe, 1970). Lenz reported on some new calculations of differential and total elastic

and inelastic cross-sections (between 40 and 100 keV) which have been carried out by Schwertfeger (1974) for C, Ge and Pt. He treated the atoms as being packed in an amorphous solid. The calculated cross-sections are in much better agreement with experimental data than previous cross-sections calculated for free atoms. They were used to estimate the elastic/inelastic signal, especially with respect to its monotonically decreasing dependence on the detector angle. However, the relatively good agreement between scattering cross-sections for atoms packed in a solid and cross-sections measured with solid specimens does not necessarily imply that the scattering cross-sections of isolated heavy atoms bound in organic structures come closer to those calculated for atoms packed between other atoms of the same kind than to cross-sections for free atoms.

V. Image improvement†

The results of the first two sessions may be summarized in two rather pessimistic sentences. The staining, although stabilizing and somehow protecting the object from radiation damage, destroys the high-resolution information. Unstained specimens, on the other hand, provide high-resolution information which is worthless because it is unrelated to the structure of the undamaged object. It may well be, therefore, that the original goal of electron microscopy – the visualization of individual molecules at high resolution – will never be achieved. Thus it is the more important that maximally efficient use be made of all information that appears, distorted and hidden in various ways, in the image, and of additional *a priori* information.

The imaging and image improvement steps cannot be considered separately. It seems odd, for instance, to increase the coherence of the conventional electron microscope further and thus to make the image into a jungle of fringes unless the effective action of the instrument, combined with a suitable restoration procedure, is considered. Unfortunately, pictures that are most suitable for image improvement by the use of linear superposition theory are least intelligible to the observer, and vice versa. Obviously, the conventional electron microscope places a heavier burden on subsequent processing than does the scanning transmission electron microscope, where the information appears localized in time and space, and can be partly processed on-line.

† We acknowledge helpful comments made by Dr P. W. Hawkes.

A number of restoration methods and filtering techniques that were discussed in this session are essentially attempts to suppress the substrate noise. Ottensmeyer pointed out that effective substrate noise suppression can also be achieved by a combination of rather simple photographic techniques, such as averaging, compensation (Lohmann, 1959) and ground-glass filtering (Ottensmeyer *et al.* 1972).

According to a proposal by Glaeser (Glaeser, Kuo & Budinger, 1971) the electron dose can be minimized by using minimum exposure conditions (Williams & Fisher, 1970) combined with spatial averaging (Markham, Frey & Hills, 1963) over a number of images of identical objects; ideally, over many repeats of the unit cell of a crystal lattice. Glaeser illustrated this idea by test computations using a simulated input picture. Theoretically, spatial superposition of the Markham type (Markham *et al.* 1963) is equivalent to filtering in Fourier space with infinitesimally small filter holes (Smith & Aebi, 1973). Fourier processing can be done either optically (Klug & De Rosier, 1966) or with the computer working on a digitised representation of the micrograph. Erickson (1974) and Smith (Aebi *et al.* 1973) reported on new results obtained by filter-averaging of two-dimensional biological lattice structures (microtubule surface lattice and T-layer of *Bacillus brevis*, respectively). Even though optical and computer filtering cannot be expected to be equivalent for various reasons (e.g. finite size of optical filter holes, aliasing error of the digital transforms, etc.) the results are, in fact, very similar (Aebi *et al.* 1973). Another interesting result reported by Smith was that micrographs of negatively stained objects seem to show indications of signal-dependent noise.

It is sometimes stated that, as opposed to the case of X-ray crystallography, there is no phase problem in electron microscopy. This means that, by measuring the bright-field intensity, both the modulus and the phase of the Fourier transform of the object can be derived directly, a fact that is of vital importance for the three-dimensional synthesis (De Rosier & Klug, 1968) of electron-microscopic objects from micrographs. However, the matter is more complicated in the high-resolution range, where the phase-contrast mechanism becomes dominant. The usual theoretical approach is to make a weak-scattering assumption, which permits the image formation to be described in terms of linear transfer theory (Hanszen, 1971).

The image and object transform are related by the phase- and amplitude-contrast transfer functions. If these functions are known,

either by inspection of the optical diffraction pattern (Thon, 1965) of a carbon film area on the micrograph or from a least-squares analysis (on the computer) of the digitally obtained transform (Frank *et al.* 1970), then the object can be recovered by Maréchal-type restoration (Frank *et al.* 1970; Maréchal & Croce, 1953; Erickson & Klug, 1970, 1971; Thon & Siegel, 1970; Baumeister & Hahn, 1973*a*, *b*; Hahn, 1972; Hahn & Baumeister, 1973). In a more sophisticated approach, studied by Welton, the filter function is tailored to *a priori* information about the statistics of the noise and the object function (Wiener, 1949; Welton, 1971, 1974), as Welton was able to demonstrate with the aid of model calculations (Welton, 1971). The restoration of weak phase/amplitude objects has recently been treated in a rigorous way (Hoenders, 1972; Hoenders & Ferwerda, 1973*a*, *b*; Ferwerda & Hoenders, 1974*a*, *b*; Ferwerda, 1974) with the aid of prolate spheroidal wave functions (Slepian, 1964) and related expansions. Ferwerda showed that the restoration procedure can be designed in such a way that it no longer relies on the isoplanatism condition or on uniform sampling (Ferwerda & Hoenders, 1974*a*, *b*; Ferwerda, 1974). Unfortunately, these considerations do not yet take the noise into account and are thus, at present, of little practical value.

Some Maréchal-type restorations of phase objects have been done using the computer (Frank *et al.* 1970; Erickson & Klug, 1970, 1971) and by optical filtering (Thon & Siegel, 1970; Baumeister & Hahn, 1973*a*, *b*; Hahn, 1972; Hahn & Baumeister, 1973). Hahn described a sophisticated optical filtering unit (Hahn, 1972) which he and Baumeister used to process electron micrographs of periodic monomolecular layers labelled with heavy atoms (Baumeister & Hahn, 1973*a*, *b*). The filter was a zonal phase plate designed so as to make up for the phase reversal of the diffraction amplitudes in certain Fourier zones.

Restoration of the complex object function is being pursued in attempts to enhance the contrast of atoms used as markers of biological objects (Hoppe, 1970; Frank, 1973*a*, *b*, *c*). In practice, data have to be collected from at least two electron micrographs unless a tilted reference beam method is used (Ferwerda, 1974; Hoppe, Langer & Thon, 1970). Two methods of data collection and restoration are presently being pursued: the 'single sideband' method (Hoppe *et al.* 1970; Hoppe, 1971), using two electron micrographs taken with complementary half-plane apertures (Downing & Siegel, 1973*a*, *b*; Hanszen, 1973*a*, *b*) and

the Schiske method (Schiske, 1968) using a defocus series of at least two micrographs (Frank, 1972, 1973*a*, *b*; Bussler, Feltynowski & Hoppe, 1972).

Previous 'single sideband' experiments have not been successful because of electric charge building up on the edges of the half-plane apertures (Hanszen, 1970, 1973*a*; Downing, 1972). Recently, however, a method has been developed to determine the additional phase-shift caused by these charges by optical correlation (Downing & Siegel, 1973*a*). Siegel reported on new optical restoration experiments where this method was successfully employed (Downing & Siegel, 1973*b*): the contrast of a heavy-metal-stained DNA strand came up strongly enhanced in the restored amplitude component of the object function.

It seems, on the other hand, that the Schiske restoration method in its original form (Schiske, 1968) requires more than two input pictures (Frank, 1973*a*) unless special weighting functions are used (Schiske, 1973). Hoppe presented new results obtained from a defocus series of test molecules containing heavy atoms ($(Ta_6Br_{12})Br_2$ and $Th(NO_3)_2$+ perylentetracarbonic acid) which could be identified in the restored amplitude component of the object.

When compared with the heavy-atom discrimination of the scanning transmission electron microscope (STEM) (Crewe, 1970), discrimination by consideration of anomalous scattering has the advantage that it performs well at very high resolution. On the other hand, it may be argued that the STEM could easily be converted to allow for efficient phase-contrast detection with the same theoretical resolution limit as the conventional microscope (this could be done by using a zonal detector arrangement described by Rose in the session on image contrast). Moreover, the discussion in the previous sessions established that it is of questionable biological significance to know the position of a heavy atom in the 'heap of ashes' to within a fraction of an Ångstrom, even though the heavy atom may have had a well-defined position in the undamaged object. Only by combining the anomalous scattering discrimination method with minimum exposure (Williams & Fisher, 1970) and averaging techniques (Glaeser *et al.* 1971; Markham *et al.* 1963; Erickson, 1974; Aebi *et al.* 1974) can one hope to extract significant information in the high-resolution range (i.e. below 5 Å).

Hanszen studied another approach to object restoration, Fraunhofer holography (Hanszen, 1973*a*; Hanszen & Ade, 1974*a*), where the object transform is sampled by a rapidly oscillating transfer function (defocus

range 40 μm). Although light-optical experiments are successful, the application in electron microscopy yields only low resolution since this method requires an impractically high degree of coherence.

The weak-scattering assumption does not hold for a variety of biological objects. There are a number of proposals (which were reviewed by Misell) on how to obtain the image wave from the image intensity in those cases. The iterative Gerchberg–Saxton algorithm (Gerchberg & Saxton, 1972; Gerchberg, 1972) uses intensity data from both diffraction and image planes, but is practically restricted to periodic objects. It has recently been shown to produce a unique solution under assumptions that are not very restrictive (Schiske, 1974). A direct approach by Frank, which uses data from a bright-field dark-field micrograph pair (Frank, 1973*b*) works for aperiodic objects as well, but leaves some ambiguity in the solution. A method very similar to the Gerchberg–Saxton algorithm uses data from a defocus series in an iterative way to restore the object function (Misell, 1973). Finally, it has been proposed that the Hilbert transform relationship that exists between the real and imaginary part of the image wave function in the half-plane aperture ('single sideband') experiment should be exploited for phase determination (Misell, Burge & Greenaway, 1974; Saxton, 1974).

It is very difficult to assess the performance of the various restoration methods from the point of view of the radiation damage limitation. Clearly, what is relevant is not the number of exposures but the total number of electrons passing through the object during the experiment. However, one might fail to use a given number of electrons efficiently from an information-theoretical point of view; for instance when a parameter (the defocus in the Schiske restoration, say) is varied within too small limits, thereby supplying redundant rather than additional information.

Finally, radiation damage also imposes an ultimate limit on the resolution of three-dimensional reconstructions (Klug, 1971; Crowther De Rosier & Klug, 1970; Klug & Crowther, 1972; Crowther, Amos & Klug, 1972). Crowther pointed out that three-dimensional reconstruction requires a minimum number of different (and statistically well-defined) views of the object to be recorded (Crowther *et al.* 1970). It is doubtful, therefore, whether three-dimensional reconstruction could ever be extended to high resolution beyond 20 Å. At 3 Å, for instance, the number of equally spaced views required for reconstruction is approximately equal to the diameter in Å of the molecule to be reconstructed (Crowther *et al.* 1970).

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