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LOW TEMPERATURE ELECTRON MICROSCOPY

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J. Dubochet, F. P. Booy[1], *R. Freeman, A. V. Jones, and C. A. Walter*
European Molecular Biology Laboratory (EMBL), Heidelberg, Federal Republic of Germany

Introduction

In the investigation of biological ultrastructure by electron microscopy the most significant observation to date is a 1 nm repeat in the purple membrane of *Halobacterium halobium* (73). With a high degree of certainty, the spacing can be ascribed to the inter α-helical separation within bacteriorhodopsin molecules. This resolution is only made possible because of the unique properties of the purple membrane and is due in particular to its two-dimensional crystallinity, which results in a repetition of the structural data. For a nonperiodic object, the meaningful resolution often lies between 3 and 5 nm, as illustrated by comparing two models that have been proposed independently for the structure of ribosomes (45, 82). More frequently, it is in even larger structures that uncertainties become apparent. For example, there are good arguments for questioning the validity of the unit membrane as it is seen in most ultrathin sections (62), and electron microscopy has not yet been able to settle the question of whether chromatin fibres are 10, 20 or 30 nm in diameter. In short, the resolution obtained with biological structures is disappointing when compared with the resolving power of modern electron microscopes of between 0.2 and 0.5 nm. It is thus not the performance of the electron microscope that is the limiting factor, but the damage caused during specimen preparation and irradiation by the electron beam (4).

Cryoelectron microscopy has long been considered as a possible avenue to overcome both of these limitations. On the one hand it was anticipated that the beam would have a less damaging effect on cooled specimens (i.e. cryostabilization), whereas on the other hand, freezing

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seems a straightforward method of avoiding preparation damage. Until now this second aspect has received the most attention, as indicated by the continuous publication of papers on freeze etching, freeze fracturing, freeze drying, freeze substitution, or cryosectioning. However, these techniques have two features in common: the specimen is dry when it is finally introduced into the electron microscope, and it is observed at room temperature. These two features, which seem to contradict the basic idea of using freezing methods to reduce damage, can be explained by pragmatic considerations. Frozen-hydrated specimens are technically difficult to prepare and transfer to the microscope and are much more beam sensitive than conventionally prepared specimens. The observation that frozen-hydrated catalase crystals show, by electron diffraction, reflections down to less than 0.3 nm (Figure 1) was therefore

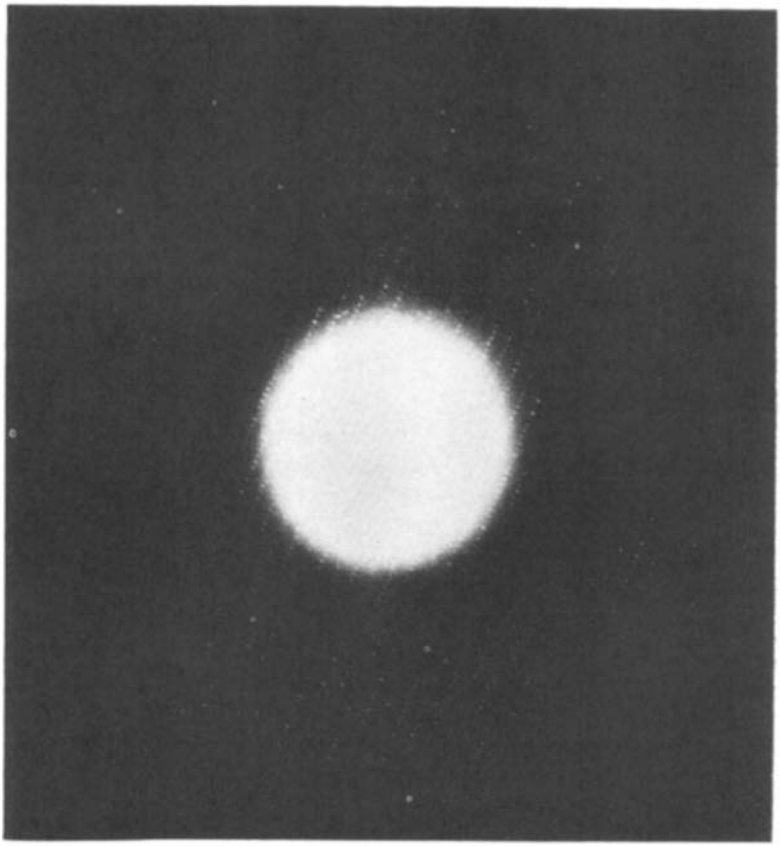

Figure 1 Electron diffraction pattern of catalase recorded from a frozen-hydrated crystal. One cm corresponds to 3.3 nm (courtesy of K. A. Taylor).

most encouraging (68). From this and subsequent work it is clear that the technical problem of observing certain types of frozen-hydrated specimens can be overcome and that preparation artifacts can, to a large extent, be prevented as illustrated in Figure 2 (67). Furthermore, cryostabilization at a very low temperature (4 K) has recently been shown to be much more effective than was previously thought (43), thus providing a possible method to overcome the second limitation of low

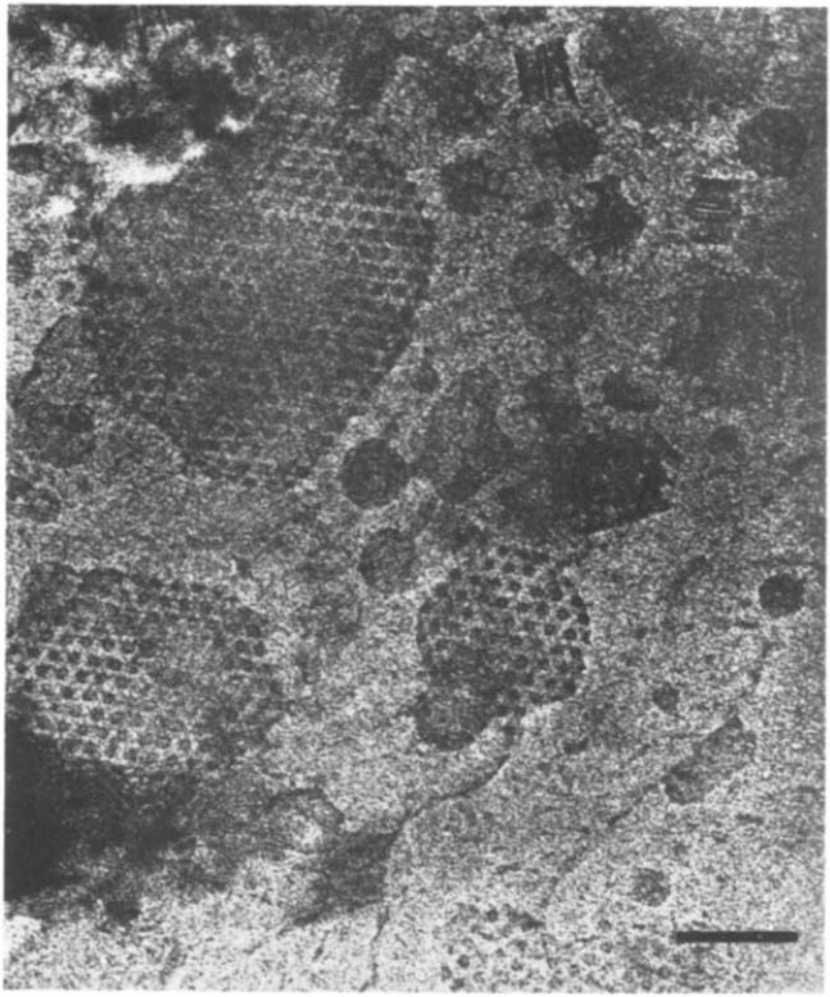

Figure 2 Frozen-hydrated specimen of cell wall of *Spirillum serpens*. The scale bar represents 90 nm (courtesy of K. A. Taylor).

temperature electron microscopy. These findings open new and largely unexplored possibilities for cryoelectron microscopy.

In the first section we consider the problem of beam damage and discuss recent results demonstrating the excellent cryostabilization effect of very low temperatures. In the second section we consider the problem of specimen preparation at low temperature, especially of frozen-hydrated specimens. Finally, we deal with the instrumental aspects of low temperature electron microscopy.

Cryostabilization

The pioneers of electron microscopy were fully aware that biological specimens are damaged by the electron beam. It is, however, only in the last two decades that beam damage has been studied quantitatively. From these studies, we know that, typically, the molecular structure of biological materials is destroyed by a dose of 100 electrons/nm^2, and that further irradiation can lead to substantial mass loss from the specimen. Unfortunately, it is not possible to reduce the electron dose arbitrarily. Because of the corpuscular nature of the electron, only those structures with which a certain number of electrons have interacted can be detected. To obtain a resolution of around 1 nm in a nonperiodic thin object the theoretically required dose is at least several thousand electrons per nm^2. The problems of beam damage and of the minimum dose required to form an image are discussed in recent reviews (28, 40).

There is already much evidence to suggest that electron beam damage is reduced at low temperature. For example, radiochemical studies have shown that many reactions are slowed down or suppressed in cooled samples (10). In the electron microscope, at low specimen temperature, heavy atoms are less mobile (79), mass loss is reduced (23, 30) and contamination (77) is suppressed. However, measurements of *structural* damage to organic samples made in the electron microscope have been disappointing. When compared with the damaging effect at room temperature, it was found that there was little improvement at low temperature. More details of these observations can be found in recent reviews (16, 29).

Results obtained using a cryoelectron microscope equipped with superconducting lenses recently put into operation in the research laboratory of Siemens AG, in Munich, are much more encouraging. Preliminary observations of bacterial cell walls, and on layers of polyhydroxybutyric acid, indicated that the sensitivity to the electron beam is reduced at 4 K by three to four orders of magnitude (20). The cryostabilization effect was then investigated by an electron diffraction study of a number of small organic crystals (43). This was done by measuring

the dose D_e required to reduce the intensity of the first order diffraction spot (around 0.5 nm) to 1/e of its initial intensity. Figure 3 shows a series of diffractograms of phenylalanine crystals recorded in a conventional electron microscope at room temperature (*a–d*) and in the superconducting electron microscope (*e–h*). In each case, the dose is given in electrons/nm^2 normalized for 80 kV electrons. These images

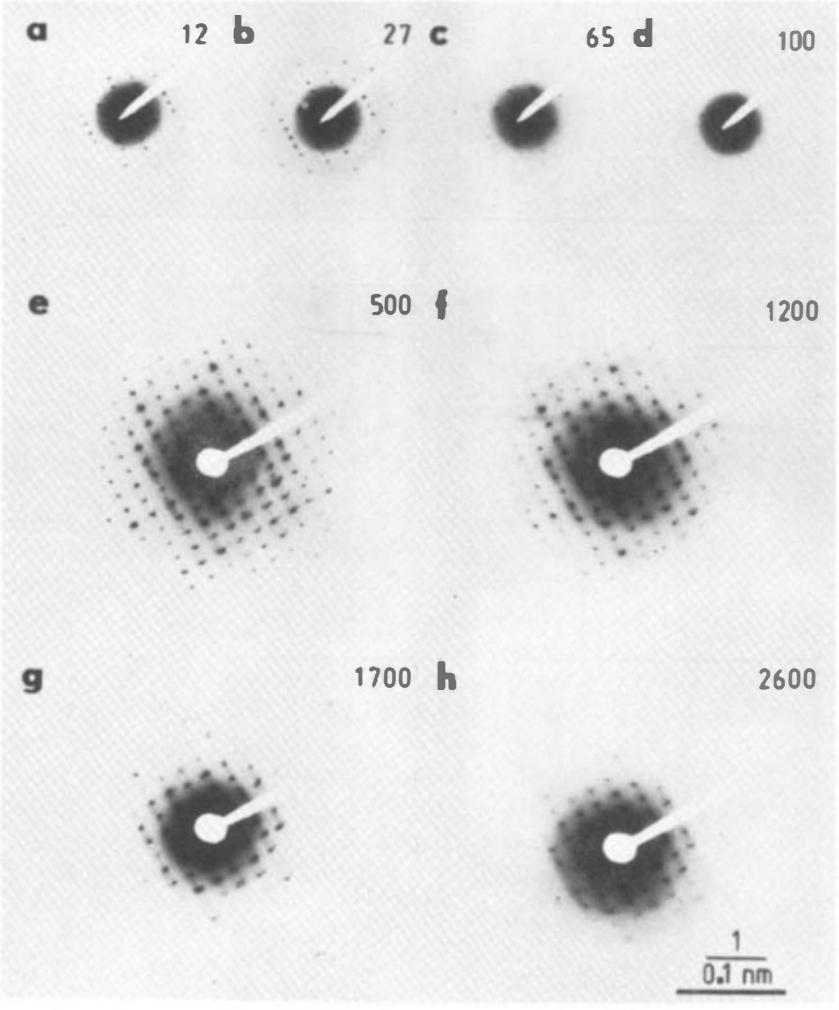

Figure 3 Electron diffractograms of phenylalanine crystals recorded at room temperature (*a–d*) and at low temperature (*e–h*). The dose, normalized for 80 kV electrons is expressed in electrons/nm^2. The prints are all made at the same scale [from Dubochet & Knapek (24), with permission of the Royal Academy of Sciences, Sweden].

Table 1 Cryostabilization measurements on organic crystals.

Crystals	D_e[a] 4 K	300 K	Gain[b]
Adenosine monophosphate	20000	60	330
Adenosine triphosphate	23000	60	370
Cromoglycate	70000	120	58
Paraffin	11000	380	29
Phenylalanine	2600	80	33
Stearic acid	7300	140	52
l-Valine	2000	30	67

[a] Electron dose D_e required to reduce the intensity of the first order reflection to $1/e$ (37%) of the initial value, at low temperature (4 K) and at room temperature (300 K). The doses are expressed as electrons/nm^2 normalized for 80 kV electrons.
[b] Ratio of doses required to produce comparable damage at 4 K and 300 K.

show that the order in the crystal is improved at low temperature and, more important, that beam damage is considerably reduced. Table I gives the value of D_e at low temperature and at room temperature for various small crystals mounted on a thin carbon film, together with the ratio of these two numbers expressing the gain due to cryostabilization.

Compared with previous results by other workers, the results obtained in the Munich microscope are outstanding, but at present only a partial explanation for this is possible. Further experimental work with this and other cryomicroscopes will be necessary for a full understanding of this cryostabilization phenomenon. Part of the answer can be deduced from the construction of the superconducting microscope itself. In this instrument the whole region around the specimen is fully contained in a large liquid helium cryostat. The specimen has no direct contact with the warm part of the microscope. All the electrical circuits inside the cryostat are superconducting, thus carrying no energy into it. Consequently, the inside of the cryostat, including the specimen, is in thermal equilibrium at liquid helium temperature, namely 4.2 K. Previously, most measurements of beam damage were made using a specimen holder cooled with liquid nitrogen, and in these cases the temperature was barely below 100 K. The few measurements made by workers with helium-cooled specimen holders were probably at temperatures which were above 10 K. The best hypothesis at present to explain the results obtained in Munich is therefore that cryostabilization becomes efficient only at a temperature close to that of liquid helium.

The electron beam is in itself a source of energy that may heat the specimen. The lack of data on the thermal conductivity of specimens at very low temperatures does not allow us to calculate the heating of the

specimen as a function of the flux and of the beam geometry. The most reasonable estimate, however, suggests that this heating could be of the order of a few degrees under the operating conditions used in the electron microscope (20, 44). Preliminary experimental results confirm the flux dependence; for example, paraffin crystals mounted on a plastic film are destroyed with a smaller dose when irradiated with a higher flux (3). This effect, which may be very considerable for thick crystals, was observed only for specimens mounted on plastic film and probably occurs because this support has a smaller thermal conductivity than a carbon film. This may also account for the only previous data from specimens that were definitely observed at the temperature of liquid helium. When monitoring beam damage to organic crystals mounted on a plastic film, Siegel (60) found that the gain due to cryostabilization was not great (1.5 to 5-fold). From the point of view of beam heating, the Munich microscope has a considerable advantage. Because of the absence of a temperature gradient in the vicinity of the specimen, drift and vibration of the specimen are kept at an unprecedented low level. It is thus possible to record images with very long exposure times, reducing accordingly the flux and the heating of the specimen.

Most of the results obtained with the Munich microscope have been determined on organic crystals, whereas our real interests are more complex biological structures. However, we do not believe that cryostabilization will be fundamentally different in biological specimens. This view is supported by preliminary electron diffraction data from crotoxin crystals, the rattlesnake venom protein where cryostabilization appears to be equally effective (W. Chui and E. Knapek, unpublished observations). Hydrated specimens have not yet been tested at this very low temperature. Previous experiments (61) that show that atomic hydrogen produced by irradiation of ice is fixed in position at 4.2 K but becomes mobile and reactive as soon as the specimen is warmed to 15 K support the hypothesis that cryostabilization will occur in hydrated material.

Although the mechanism of cryostabilization has been studied by radiation chemists, their conclusions cannot be directly applied to the problem of the electron microscopist. This is because only those radiation-induced reactions that produce atomic displacement of the order of the resolution aimed at, are significant for the electron microscopist. Furthermore, doses normally used by the electron microscopist are so enormous that most bonds are broken, complex interactions have occurred, and the situation is unrelated to an ideal radiochemical system. We refer the reader to a recent publication (50) for a general view of the problem of cryostabilization in the electron microscope and for exam-

ples of the complexity and multiplicity of the phenomena involved in beam damage at low temperatures.

From general considerations it is nevertheless possible to gain some idea of the mechanism of cryostabilization. The first event is the inelastic scattering of an electron from the beam by an electron in the specimen. During an interaction time of about 10^{-13} sec some tens of eV are transferred to the specimen electron, causing, in most cases, its ejection from the atom. Inelastic scattering events are independent of the temperature, and therefore cryostabilization does not have any effect. The system having been disturbed by this sudden energy input becomes rearranged in a succession of chemical reactions until it has found a new equilibrium. Each reaction involves the movement of atoms or molecules, adding, therefore, to the overall structural change. It is in this second phase that cryostabilization is likely to occur. The very low temperature may trap the system in an intermediate state, which would be unstable at room temperature. This mechanism could explain the observations that the final damage measured at room temperature is, to a first approximation, independent of the temperature at which the specimen was irradiated (50, 60).

Another mechanism of cryostabilization is probably related to the reduction in atomic and molecular diffusion at a low temperature. This does not prevent chemical rearrangement but prevents any gross structural changes. Support for this type of mechanism is illustrated by the suppression of mass loss and contamination at low temperature, which are large-scale effects but which probably involve an inhibition of movement at the molecular level.

Frozen-Hydrated Specimens

A living cell is composed mostly of water (generally 70–80%). Part of it is intimately associated with macromolecules forming the so-called hydration shell that is essential for maintaining structural stability (27). Consequently, changes in the aqueous environment as a result of dehydration or replacing the water by different chemicals cause structural rearrangements to occur. The conventional procedures such as fixation, dehydration, and embedding that are necessary preparational steps in the processing of biological material for electron microscopy of thin sections invariably alter the molecular organization of the specimen (46). Even in negatively stained preparations that reputedly allow excellent preservation of isolated biological particles, numerous artifacts can occur. Furthermore, the resolution attainable is limited by the size of the grains of the sustaining salt layer to about 2 nm. For further information and discussion on specimen preparation damage, the reader

should consult Hayat (31) and a recent review by Kellenberger & Kistler (41).

Clearly, the best method to avoid most processing artifacts is to observe the specimen in an aqueous environment. This approach is already being pursued, but despite some excellent results (47) it is not adequate for high resolution observations because at room temperature hydrated material is very sensitive to the electron beam and the environmental cells need to be relatively thick. A more promising alternative is to examine specimens in the frozen-hydrated state. Fernández-Morán (26) was the first to actively consider the possibility of examining quench-frozen biological specimens in the electron microscope. This idea was not developed further until Chanzy et al (14) used this technique for observing rapidly frozen single crystals of cellulose triacetate in which some molecules of the mother liquor are required to preserve the crystalline structure. Subsequently, the technique was used in order to prevent the dehydration and thereby maintain the crystalline structure of a variety of quench-frozen specimens (2, 8, 9, 12, 13, 70). At present, there are some 20 reports on the observation of quench-frozen specimens in the electron microscope. Heide & Grund (33, 34) and Taylor & Glaeser (69) have independently developed more sophisticated techniques for observing frozen specimens. With the advantage of a more stable stage, Taylor & Glaeser have been able to record images of frozen-hydrated catalase crystals to a resolution of 1.15 nm (68). Attempts have also been made to image frozen-hydrated sections, particularly of unfixed striated muscle (38, 76). However, the resolution so far obtainable is at least an order of magnitude poorer than that of either epoxy resin–embedded sectioned muscle or cryosections of glutaraldehyde-fixed muscle examined after thawing and staining (63).

Freezing biological specimens can have serious structural consequences. A considerable amount of work has now been done in attempts to avoid the damaging effects of ice crystal formation during freezing. In particular, the use of freeze substitution (54), freeze fracturing/etching (51, 65), and freeze drying (41, 49) methods are valuable sources of information on how to prevent freezing artifacts. From the large amount of accumulated data on biological freezing we know that the damaging effect of freezing on biomaterials has to be considered at both the cellular and the molecular level.

1. Cellular level. When the medium surrounding cellular and organized structures is frozen, it becomes segregated into two phases: the growing ice phase and the remaining water with an increasing solute concentration that eventually forms a eutectic phase of concentrated solutes. Depending on the conditions of freezing, the formation of ice crystals

constitutes the biggest source of induced artifacts in low temperature preparation techniques.

2. Molecular level. The separation of the ice phase during freezing is likely to perturb the water layers surrounding structures at the molecular level. To what extent this will lead to adverse structural rearrangements and whether or not it will limit the achievable resolution remains to be shown. This problem is further complicated because the water surrounding macromolecules possesses different physicochemical properties from that of the bulk water. However, if freezing procedures that are conducive to preserving the structure of biological samples in the frozen state are used, the already published high resolution data (68) suggest that for some kinds of specimen, even at the molecular level, damage will be negligible. Further encouragement is offered by the many successes obtained by cryobiologists in maintaining the viability of a variety of different cell types during the more complex and difficult procedures of freezing and thawing (52, 80, 81).

The most fundamental point in common to all cryotechniques is how to freeze the sample in such a way so as to avoid damage due to the formation of ice crystals. For cryomicroscopy of unfixed, uncryoprotected material this usually means freezing as fast as is practically possible. In the absence of cryoprotective agents such as glycerol or dimethylsulphoxide a freezing rate of about 10^5 K/sec or higher is required to obtain the so-called vitrified state in which the ice crystals are so small that they do not cause structural damage at the cellular level. Even so, only very small samples can be vitrified as thin films ($\sim$10 μm) or droplets ($\sim$20 μm), providing they are cooled efficiently (48). The low thermal conductivity of ice and of the specimen itself also imposes severe restrictions on the depth of the sample that can be frozen rapidly enough to prevent ice crystals growing to a damaging size.

In the preparation of frozen-hydrated specimens, the nature and difficulty of the technical problems encountered are very dependent on the size of the specimen. Large specimens, such as cells and tissues, that are too thick for direct visualization in the electron microscope need to be sectioned after freezing. We will discuss the problems relating to cryosectioning later and first consider only those specimens that are already thin enough for electron microscopical examination.

Particulate specimens are relatively easy to deal with, and satisfactory results can be achieved by freezing the specimen as a thin layer in suspension on a support film. Quench-freezing in liquid nitrogen is usually adequate enough for structural preservation to a high resolution. If necessary, faster freezing rates can be used by quenching in nitrogen

slush, liquid propane/butane or helium II for dealing with more delicate specimens. It is, however, technically very difficult to form a thin enough suspension of particles embedded in an aqueous matrix that is less than 100 nm thick and to preserve it, without some loss of water by evaporation, until it is frozen. Even at 173 K the frozen bulk water evaporates at a rate of 10 nm/sec (76). The fact that Glaeser and co-workers in subsequent publications have changed their method of preparing frozen-hydrated specimens—from using sandwich grids (68), to humidity boxes viewed by optical microscopy (69), to techniques involving stearic acid and piston oil (32)—emphasizes that a simple and easily reproducible method has not yet been established and extreme persistence and patience is required.

Another approach is to ensure that the specimen is quench-frozen in the presence of a slight excess of water that is then allowed to sublime within the microscope by controlled heating of the specimen. For certain specimens, including protein crystals, this partial freeze drying results in excellent preservation of structure and an increase in contrast (J. Lepault, personal communication). If the water that is required to maintain the structural integrity of the specimen is tightly bound, during warming all the excess water will be removed at a lower temperature than the bound water. The ease with which this can be performed depends on the nature and the extent to which the water necessary for preserving the structure differs from the surrounding bulk water. Once the excess water has been removed, the specimen may be recooled and examined at a temperature low enough to prevent further evaporation.

The technique of subliming excess water within the microscope places stringent demands on the vacuum system. If the vacuum becomes saturated with water vapor it may become impossible to observe specimens at low temperature because of water condensing as ice on the specimen. Furthermore, this reprecipitated ice may well destroy the structure that was present in the partially freeze-dried specimen. Well-dried photographic materials and the use of an effective anticontamination device are important in controlling these difficulties.

Biological structures that are intrinsically too thick for direct observation in the electron microscope after freezing must first be freeze sectioned. The availability of commercial cryomicrotomes and a steady improvement in the basic methodology of preparing cryosections over the past fifteen years has made cryoultramicrotomy an important alternative technique to sectioning resin-embedded material in morphological studies (5, 6, 39, 71). In particular, cryosectioning is becoming of increasing importance in the fields of immunolabeling (72) and X-ray microanalysis (64).

So far, the best results obtained with cryosections have been on cells or tissues that have been first stabilized with aldehydes, and frozen in the presence of a cryoprotective agent, but
microscope after thawing. The results from unfixed, uncryoprotected material that has been freeze dried have not been so satisfactory. Nevertheless, it is mainly from studies such as these and the sparse information on observations of fully hydrated specimens (38, 76) that we have to base our judgement on how to prepare and handle unfixed biological material. At present, this is not easy to do routinely.

A primary requisite is that the specimen must be frozen in such a way as to keep ice crystal formation to a minimum. Freezing by impinging the sample against a highly polished copper (18, 74) or silver (75) block at the temperature of liquid nitrogen or liquid helium, or injection into the cryogen
provide the most practical means of obtaining an ice crystal–free region in the superficial layers (3–15 μm) of the sample alone. Adapting the rapid-freezing apparatus (75) modified by Heuser et al (36) to specimens that can then be transferred to a cryomicrotome may prove to be beneficial. However, to avoid excision traumas and adverse movements of water and electrolytes, it may be necessary to cryofix the sample in situ (58) or utilize a rapid excision and simultaneous freezing method (11). A second important requirement is that during sectioning and all subsequent maneuvers,
zation temperature and that there is adequate temperature control of the specimen, knife, and cryochamber to meet this demand. The lower limit for ice recrystallization in biological tissues appears to be 203 K, from the investigations carried out by Dempsey & Bullivant (18). In water alone, recrystallization occurs around 150 K (22).

Glass knives are generally suitable for cutting cryosections, but it has been suggested that below 1
Hodson & Williams (37) used diamond knives at 148 K and noted a consistent severe ripple defect in their sections. They believe that the elastic properties of ice are inappropriate for sectioning with knives that were primarily designed for cutting resin-embedded material.

It remains unclear whether cryosectioning occurs as a result of a true cutting action or a fracturing process. Saubermann et al (57) have analyzed the forces produced during sectioning and have suggested that at 193 K and 153 K both a fracturing and a cutting process were involved, whereas at a temperature of 243 K only cutting occurred. Kirk & Dobbs (42) have also provided evidence that at temperatures warmer than 203 K the sections are indeed cut, but are fractured at lower temperatures. From a review of the literature it appears that cutting

may be accompanied by partial melting of the section. As it is necessary to section in the temperature range where knives may be less efficient and there is a higher probability of fracturing rather than cutting, the sections may be unsuitable for high resolution work. Although cryosection thickness cannot readily be determined from interference colors, it is clear that the thinner the section, the more difficult it is to manipulate onto a grid. The available techniques of using an eyelash probe, vacuum suction device (1), or merely touching the sections with a grid, can result in the loss of a high percentage of good sections. Furthermore, because there needs to be close adherence of the section to the grid, it is essential to flatten the sections in a controlled manner. The original method described by Christensen, using a copper rod (15) or a variation of this method (56, 59), seems to be the most favorable way of doing this at present. It is also advisable to collect the sections onto grids soon after they are sectioned and store them under liquid nitrogen until examined so as to minimize partial freeze drying in the cryochamber.

Instrumentation for Cryoelectron Microscopy

After the preparation of frozen specimens, the subsequent problem is to transfer them into the microscope without thawing and without contamination by condensation and then to maintain the specimen at the required temperature during microscopy. Also, it may be necessary, depending on the method of specimen preparation, to provide a means of controlled sublimation of the excess ice in order to achieve optimal contrast.

This range of capabilities is, unfortunately, not available in any commercially produced electron microscope. Almost all modern electron microscopes have available, as an accessory, a liquid nitrogen–cooled specimen stage, although this does not necessarily imply that the specimen actually reaches liquid nitrogen temperature. This temperature is sufficient to inhibit mass loss and to prevent, almost completely, the build up of contamination by surface diffusion, but is not sufficiently low to produce a significant cryostabilization effect. A common feature of all commercially available cryostages is that only part of the stage is cooled. The greater part of the stage mechanism is in intimate contact with the objective lens pole piece and is kept at pole piece temperature to minimize thermal drift. The fundamental design problem is to achieve a compromise between heat transfer to the cooled portion of the stage (which limits the lowest attainable temperatures) and adequate mechanical stability of the structure. These problems increase with decreasing temperature because of the large temperature gradients between the "hot" and "cold" components. Such stages are therefore not generally

capable of high resolution imaging but can be used for diffraction studies of periodic structures.

Almost all serious cryogenic studies have been carried out with user-designed stages. When temperatures below that of liquid nitrogen are required, liquid helium or cold helium gas is used as a coolant. To obtain the lowest temperatures, a complex arrangement of thermal shields (often liquid nitrogen cooled) is necessary to reduce radiative heat transfer. This method of construction is generally not possible in conventional high resolution microscopes because of a lack of space in the objective pole piece region, but it has been successfully applied in high voltage electron microscopes (25, 35, 66). To date, no such stage has been built to match the performance of the best room temperature stage.

The ideal solution is to eliminate thermal gradients by maintaining all contiguous mechanical components—objective lens, stage, and specimen holder—at the same temperature as in the case of room temperature operations. Such an approach raises the technical problems to a new level of complexity, since it involves a total redesign of the objective lens. Systems based on this principle have, however, been constructed for liquid nitrogen (7), helium gas (78), and liquid helium temperatures (19).

Operation at liquid helium temperature has the significant advantage that the objective lens windings can be made superconducting and will therefore contribute no heat input to the system. An additional bonus results from the greatly reduced thermal expansion coefficients of all materials at these temperatures. This, together with careful design, has enabled the Siemens group to achieve a "world record" in mechanical stability (0.01 nm/min) for their cryolens/cryostage assembly. This stability is a critical factor in minimizing electron beam heating of the specimen. However, such an isothermal system does not permit the specimen temperature to be varied. This has no serious consequences for cryostabilization but does result in additional complications in the specimen transfer system.

As described at the beginning of this section, some means of regulating the ice content of the specimen must be provided. If the microscope has a temperature-controlled stage, the specimen temperature can be varied to control the sublimation rate while simultaneously observing the specimen at a magnification low enough to reduce beam damage to a negligible level. Naturally, adequate pumping capacity at the specimen must be provided to prevent recondensation of the water sublimed during rewarming.

In the fixed temperature instrument, this operation must be carried out prior to insertion in the microscope, preferably in the microscope

airlock to avoid subsequent contamination. The ice content can in principle be monitored by periodically inserting the specimen into the microscope, but it would obviously be more satisfactory to provide continuous monitoring by, for example, optical interference microscopy in the airlock itself.

No fully satisfactory solution to this problem has yet been found. An interesting specimen transfer system has been constructed for the liquid nitrogen–cooled isothermal system previously mentioned (7). This permits a number of specimens to be maintained at low temperature in the microscope airlock. Most other systems developed to date rely to an unacceptable degree on the manual dexterity and/or fleetness of foot of the user.

Conclusion

To realize more fully the resolving power of modern electron microscopes in the study of biological material, improved methods of preparing and observing specimens are required.

Electron beam damage to biological structures is considerably reduced by examining specimens at liquid helium temperature. We have called this effect cryostabilization. The most urgent task is to define those parameters that influence cryostabilization and to build microscopes to exploit the advantages. Extrapolating from the results shown in Table I, we predict that cryostabilization will generally permit an increase of 100 in the acceptable radiation dose. This in turn should result in a 100-fold reduction of the smallest detectable *volume* of the specimen on which significant observations are limited by beam damage. We thus expect that cryostabilization will improve the significant resolution attainable on thin nonperiodic objects from the present value, of somewhere between 3 and 10 nm, to closer to 1 nm.

Ultrafast freezing overcomes almost all preparation artifacts and has the added advantage that dynamic cellular activity is stopped virtually instantaneously. This opens up the possibility of examining a whole range of structure-function relationships in cell biology. Frozen-hydrated specimens prepared as thin films from particulate suspension are already providing useful data. Preparing suitably thin specimens from bulk material is more problematic. The cryosectioning technique is still very much in its infancy and its full potential will not be realized until there are further improvements in cryomicrotome stability and the development of new techniques for handling cryosections.

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