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Electron microscopy of frozen biological suspensions

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KEY WORDS. Frozen hydrated specimens, vitrified suspensions, contrast matching, freeze-dried suspensions, electron beam damage, T4 bacteriophage, DNA, catalase.

SUMMARY

The methodology for preparing specimens in the frozen, hydrated state has been assessed using crystals and T4 bacteriophages. The methods have also been demonstrated with lambda bacteriophages, purple membrane of *Halobacterium halobium* and fibres of DNA. For particles dispersed in an aqueous environment, it is shown that optimum structural preservation is obtained from a thin, quench-frozen film with the bulk aqueous medium in the vitreous state. Crystallization of the bulk water may result in solute segregation and expulsion of the specimen from the film. Contrast measurements can be used to follow directly the state of hydration of a specimen during transition from the fully hydrated to the freeze-dried state and permit direct measurement of the water content of the specimen. By changing the concentration and composition of the aqueous medium the contrast of particles in a vitreous film can be controlled and any state of negative, positive or zero contrast may be obtained. At 100 K, frozen-hydrated, freeze-dried or sugar embedded crystals can withstand a three- to four-fold increase in electron exposure for the same damage when compared with similar sugar-embedded or freeze-dried samples at room temperature.

INTRODUCTION

Cryo-electron microscopy was proposed more than 20 years ago (Fernandez-Moran, 1960). In spite of some outstanding results (e.g. Taylor & Glaeser, 1975), progress towards the routine examination of frozen, hydrated specimens for high resolution structural studies has been limited (for a recent review see Dubochet *et al.*, 1981). To a large extent this can be attributed to the technical difficulties of building a stable cryo-stage, to the sensitivity of frozen, hydrated specimens to damage by the electron beam and to the lack of routine specimen preparation methods.

Recently a number of more stable low temperature specimen holders (Homo, 1980; Lichtenegger & Hax, 1980) and a commercially manufactured superconducting lens system (Dietrich *et al.*, 1977) have become available. In this superconducting system the specimen is maintained in a particularly stable state at 4 K. If therefore, the considerable reduction in damage by the beam observed at 4 K on organic specimens (Knapeck & Dubochet, 1980) is also found for hydrated specimens, the largest remaining difficulty concerns the preparation of specimens. We present here as a continuation of a previous paper (Dubochet *et al.*, 1982) some results concerning preparation and assessment methods for the investigation of frozen, hydrated biological specimens.

In order to assess the quality of a frozen, hydrated specimen, it is pertinent to consider how much water is necessary and how this amount can be measured for the specimen to be fully hydrated. It is well known that even partial drying, by say freeze-drying, introduces considerable morphological change. On the other hand, water which plays no role in structural preservation (bulk water) is an additional source of noise in images of frozen, hydrated specimens and should be minimized. Clearly for a specimen to be fully hydrated, the requirement, and thus our definition, is that at least all those water molecules which are necessary to maintain the structural integrity of the material should be present at their structurally important position. For crystalline objects this state is conveniently tested by the quality of the electron diffraction pattern. For non-crystalline specimens all the information must be derived from the image recorded in the electron microscope.

MATERIALS AND METHODS

Preparation of frozen hydrated specimens

Catalase crystals (Sigma, Munich) were grown according to the procedure of Dorset & Parsons (1975), and then dialysed against water. T4 and lambda bacteriophages were a gift from Professor A. Tsugita and Dr V. Pirrotta respectively. DNA was purchased from Sigma and the purple membranes were kindly supplied by Dr R. Henderson. Specimens were prepared with a thin film of the aqueous medium containing the material under study by either of two methods which have been previously described (Dubochet *et al.*, 1982). The work presented here was carried out using the technique which relies on the adhesion of the suspension to a carbon film treated by glow discharge for 10 s in an alkylamine atmosphere at a pressure of 1 Torr. After adhesion of the material, the 400 or 600 mesh grid, mounted on a guillotine-like frame, was partially dried by pressing between filter papers for about 1 s, and the guillotine was immediately released plunging the grid into the cryogen of liquid nitrogen or liquid ethane. After a few trials thin, frozen layers of suspension could be easily obtained. Similar results to those presented here were obtained by spraying microdroplets of the biological suspension on to an air-glow discharged supporting film and freezing rapidly as described previously (Dubochet *et al.*, 1982).

We have also tested an equilibration method (Booy *et al.*, 1981) in which a thin layer of suspension was deposited on a carbon coated grid which was then left for a period ranging from many hours to several days at a controlled humidity (76–98%) maintained in a closed container by a saturated salt solution (O'Brien, 1948). After equilibration, the specimen was rapidly removed from the container and immediately quench-frozen.

Electron microscopy

The electron microscope used for this study was a Philips EM 400 operating at 80 or 100 kV and fitted with an improved, blade-type, anticontamination device. The mechanical stability and temperature regulation of the cryo-specimen holder, type PW 6591/100, have been improved by maintaining a constant pressure in the liquid nitrogen reservoir. Cold nitrogen gas is then drawn through the specimen heat exchanger by a membrane pump at a rate controlled by a needle valve. Frozen hydrated specimens were inserted in the cryo-specimen holder under liquid nitrogen and rapidly introduced into the microscope. Care was taken to avoid condensation of water on the cold parts of the holder. Under our working conditions, contamination by condensed atmospheric humidity was not a disturbing factor and all parts of the specimen were kept below 130 K during the transfer.

Micrographs were recorded on Kodak electron image films 4489 (speed: 0·5) using the low dose unit of the microscope. Electron diffraction patterns were recorded with a dose of 30–70 e^{-} nm^{-2} using a 70 μm condenser aperture and a strongly excited first condenser lens. Electron dose was measured from the optical density of the film which was itself calibrated by direct measurement of the current collected by a Faraday cage.

Thickness and contrast

In the range for which the photographic film response to electron dose is linear, the mass thickness t (expressed in mg m^{-2}) of a vitreous ice layer is given by

$$t = 275 \ln D_0/D$$

in which D_0 and D are the optical densities of a well focused bright-field amplitude image in the absence and the presence of an ice layer respectively. The numerical factor which depends on the geometry of the microscope and the composition of the material has been calculated (Eusemann *et al.*, 1982).

In general, the optical density D of a layer with mass thickness w is related to the optical density D_0 without the layer by

$$D = D_0 e^{-\frac{w}{\Lambda}} \tag{1}$$

where Λ is the 'mean free mass thickness' for this material. This calculation also holds for polycrystalline specimens if the area of measurement is taken much larger than the average size of the crystals. For large crystals errors due to coherent scattering can be reduced taking the measurements from regions without bend contours.

The contrast C between two areas of a micrograph exposed at the optical densities D_1 and D_2 respectively is defined as

$$C = \frac{2(D_1 - D_2)}{D_1 + D_2} \tag{2}$$

RESULTS

Frozen hydrated specimens

Three typical examples of frozen hydrated samples are shown in Fig. 1: in (a) catalase crystals are embedded in a hexagonal ice layer with a maximum thickness of 300 nm, (b) lambda bacteriophages are shown embedded in a film of vitreous ice *c.* 100 nm thick and in (c) the water layer hydrating purple membranes is discontinuous. In general the alkylamine and the spray-freezing methods were found easily and consistently to produce sufficiently uniform frozen films. Other methods, for example spreading on air-glow-discharged films, can also be used, but in our hands the yield of successful preparations is lower. If we add the criterion of the preservation of order better than 0·35 nm in catalase crystals (as judged by electron diffraction) both methods lead to *c.* 80% success. When the alkylamine method is used, the thickness of the water layer can be roughly controlled. Below 50 nm however, the layer is generally discontinuous (Fig. 1c). The concentration of material required in the suspension is similar to that required for negative staining. Layers much thinner than 50 nm can be obtained by the spray-freezing method, which also offers a reduction in time between spraying and freezing to the millisecond range. It does, however, require a considerable amount of a highly concentrated suspension of the specimen.

As described previously (Dubochet *et al.*, 1982), water can be frozen in the hexagonal, cubic or vitreous form (or a mixture of these), depending on the freezing speed and the possible cryoprotective effect of the solute. The vitreous form can be obtained by freezing a film less than 1 μm thick in liquid propane or ethane.

The hydrated state

Under optimum conditions, order (to better than 0·2 nm) in catalase crystals can be preserved in the electron microscope. This is normally the case when the crystal is fully embedded in a thin layer of vitreous ice as illustrated in Fig. 2. Upon freeze-drying, which is best done in the microscope, order is lost to about 1 nm (Fig. 3). Accompanying this, the crystal shrinks and the lattice parameters change from 6·9 and 17·4 nm to about 5·5 and 16 nm (Fig. 3) and cracks become visible in the image (not shown).

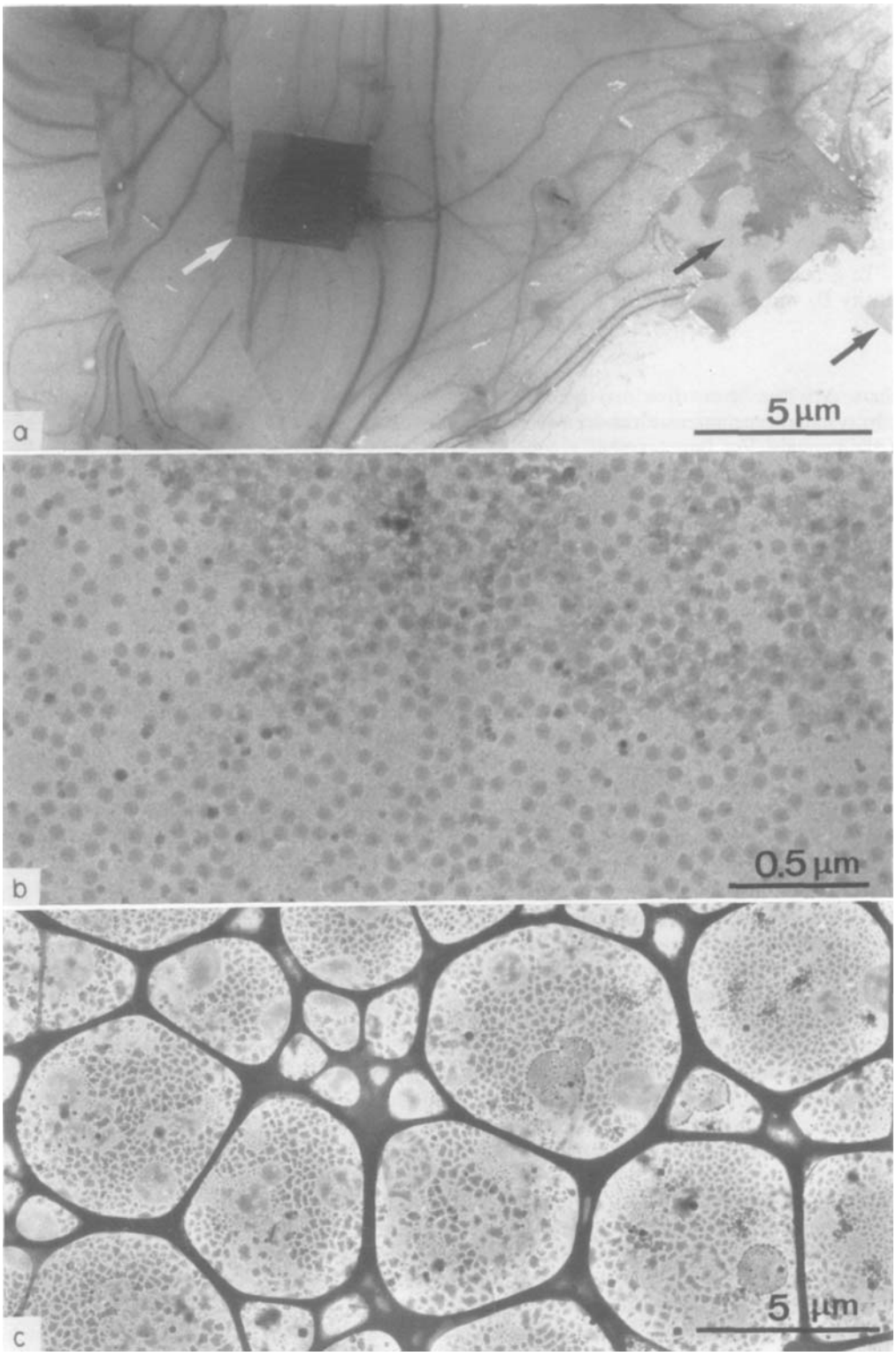

Fig. 1

Good structural preservation can be obtained even when the freezing speed is not sufficient to vitrify the bulk water. This is the case in Fig. 4 (left) showing the diffraction pattern from a catalase crystal surrounded and also covered by a large hexagonal ice crystal, similar to the one marked by a white arrow in Fig. 1(a). For good preservation, the catalase crystals do not need to be embedded in ice. However, in this case and as it is illustrated in Fig. 1(a) (black arrow), the electron diffraction pattern of the crystal is accompanied by the cubic pattern of ice (Fig. 4 (right)) or with the pattern of vitreous ice (not shown). Under these freezing conditions water alone freezes in the hexagonal form of ice. The vitrified ice associated with the crystal cannot be devitrified by warming in the microscope but it sublimes during freeze-drying. The evolution of contrast upon freeze-drying does not depend on whether the initial embedding ice was in the hexagonal or vitreous form. The cubic ice associated with the crystal does not show the typical microcrystalline aspect of the bulk cubic ice. It also sublimes during freeze-drying.

In order to define more precisely the behaviour of interstitial and bulk water, we have slowly freeze-dried specimens of catalase crystals embedded in ice, following the process by photographically recording the contrast of the crystal and its order and lattice constants by electron diffraction. The results from such an experiment are illustrated in Fig. 5 and plotted in Fig. 6. Crystals which are dried before freezing are already severely damaged and remain unchanged during all the drying process (Fig. 6, □). Crystals which are free from bulk water but nevertheless hydrated show a reduction in contrast on freeze-drying corresponding to the sublimation of interstitial water (Fig. 6, △). Crystals fully embedded in ice have low contrast, proportional to the difference in scattering between protein and water. When the specimen is warmed up, all structural changes take place when the water evaporates, that is at *c.* 170 K. The process occurs in three phases (Fig. 6, ○). Firstly no changes are observed in the crystal as long as it is only the water above the crystal which sublimes (Fig. 6, A). Secondly, when the ice surface reaches the level of the crystal, the water around the crystal sublimes more rapidly than the water in the crystal, thus resulting in a rapid increase of contrast until all the embedding ice has left (Fig. 6, B), and thirdly a small reduction of contrast occurs because of the sublimation of the residual internal water (Fig. 6, C). It is early in this final phase that high resolution order is lost, and later that the lattice constant is shortened and the cracks are formed. No further changes take place during subsequent warming to room temperature. From the change in contrast between the hydrated and the dry state it can be calculated that *c.* 40% of the mass of the frozen crystal is water which sublimes during freeze-drying. The evolution of contrast upon freeze-drying does not depend on whether the initial embedding ice was in the hexagonal or vitreous form.

The contrast shown by the fine structure within a crystal also depends on the state of hydration. When embedded in water such intracrystalline contrast is lower than when the crystal is freeze-dried. In practice when images are recorded under normal conditions in the CTEM the periodicity of the lattice is hardly discernable by direct observation. Analysis with optical diffraction shows however that the structure is present and well preserved. In the dry state, the periodicity is directly visible on the image but the order is more poorly preserved than in the hydrated state. As far as can be judged by comparing direct images and electron diffraction, the loss of high resolution order takes place at the same time as the three-fold increase in contrast of the periodic structure within the crystal.

The method of equilibration has been tested in an attempt to maintain interstitial water whilst removing bulk water. For catalase it is known from X-ray studies (Longley, 1967) that the structure is preserved at a humidity of $>92\%$. We therefore hoped that leaving a thin layer of

Fig. 1. (a) Catalase crystals embedded in a layer of hexagonal ice up to *c.* 300 nm thick. The specimen was frozen by plunging into liquid nitrogen. The white arrow indicates an embedded catalase crystal, the black arrow indicates a crystal and part of a crystal where cubic ice is detected by electron diffraction. × 5400. (b) Lambda bacteriophage embedded in an amorphous ice layer *c.* 100 nm thick. The specimen was frozen by plunging into liquid ethane cooled by liquid nitrogen. × 43,000. (c) Purple membranes embedded in a thin discontinuous film of hexagonal ice, supported on a holey carbon film. The specimen was frozen by plunging into liquid nitrogen after a longer contact with the blotting paper than in (a) or (b). × 5400.

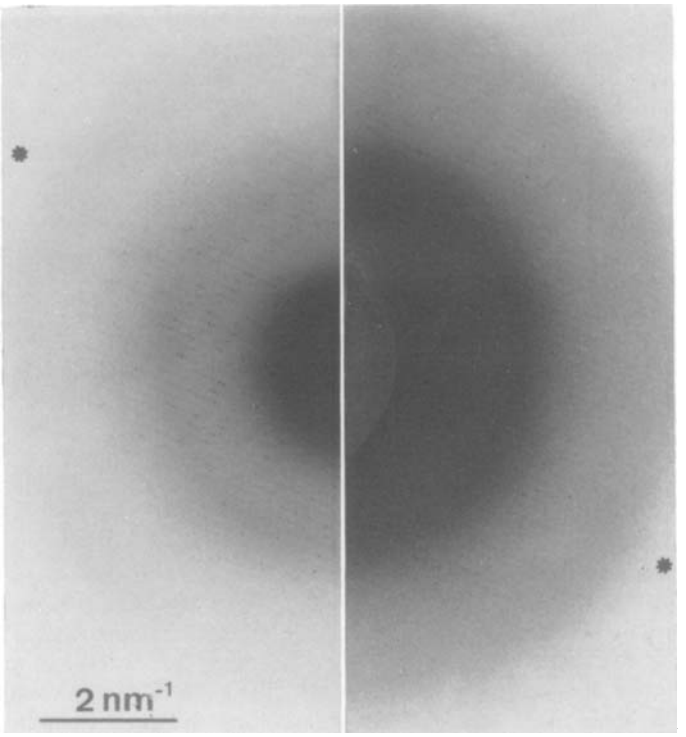

Fig. 2. Electron diffraction pattern of a catalase crystal embedded in an amorphous ice layer. The right-hand side of the pattern has been printed so that contrast of the high resolution spots is enhanced. The two broad rings are characteristic of amorphous ice and are located at 1/0·37 nm^{-1} and 1/0·21 nm^{-1}. The asterisks indicate reflections around 1/0·18 nm^{-1}.

suspension to equilibrate at 98% humidity would lead to hydrated crystals free from bulk water. Although well-preserved crystals (ordered to better than 0·35 nm) could be obtained, the yield of successful preparation was low and, even on good preparations, only a small proportion of the crystals was optimally preserved. The method can possibly be improved by optimizing the equilibration conditions and minimizing the transfer time in a less humid environment just before freezing. Not every biological system has such severe requirements in this respect as catalase crystals. Fibres of DNA in the A conformation can reliably be prepared in the frozen

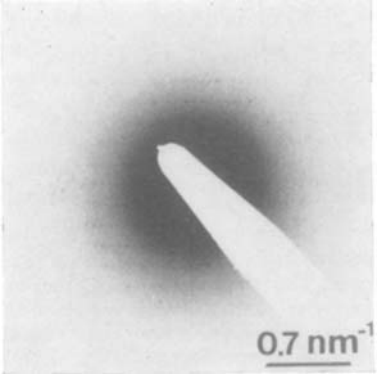

Fig. 3. Electron diffraction pattern of a freeze-dried catalase crystal.

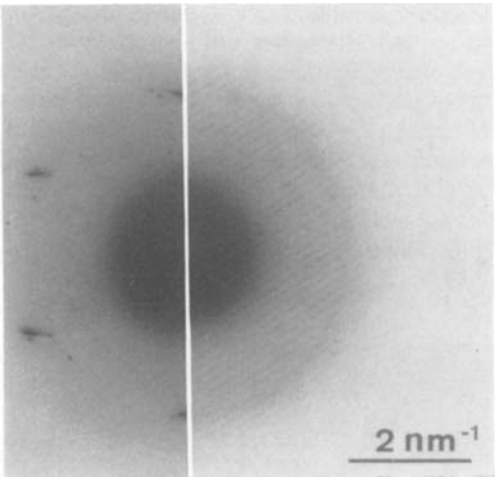

Fig. 4. Electron diffraction pattern of catalase crystals associated with hexagonal ice (left) or cubic ice (right). The two sharp rings on the right side are located at 1/0·36 and 1/0·22 nm^{-1} and are characteristic of cubic ice.

hydrated state by the equilibration method. A fibre-electron diffraction pattern from such a preparation is shown in Fig. 7.

As shown above, the hydrated state of catalase is most reliably conserved when the crystal is fully embedded in water. It is likely that the same is true for any biological object, especially if it is small compared with catalase crystals. With this hypothesis the degree of hydration can

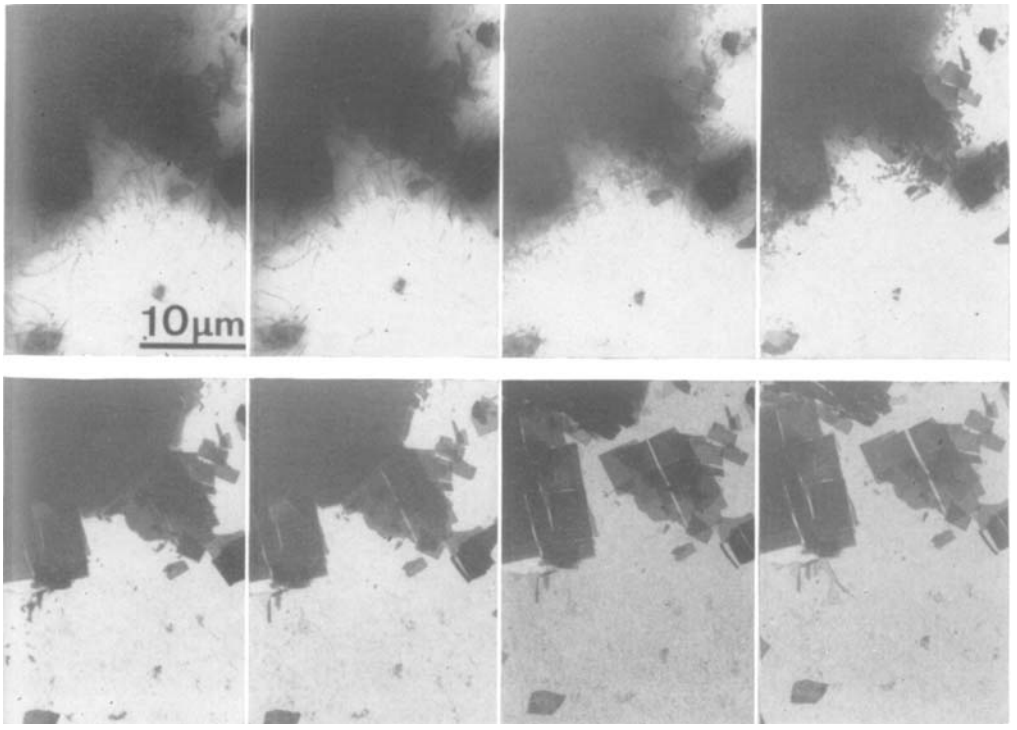

Fig. 5. Images of catalase crystals recorded during freeze-drying by increasing the temperature of the specimen holder from 100 K to 200 K in about 20 min. Images 3–7 have been taken within 5 min. × 1400.

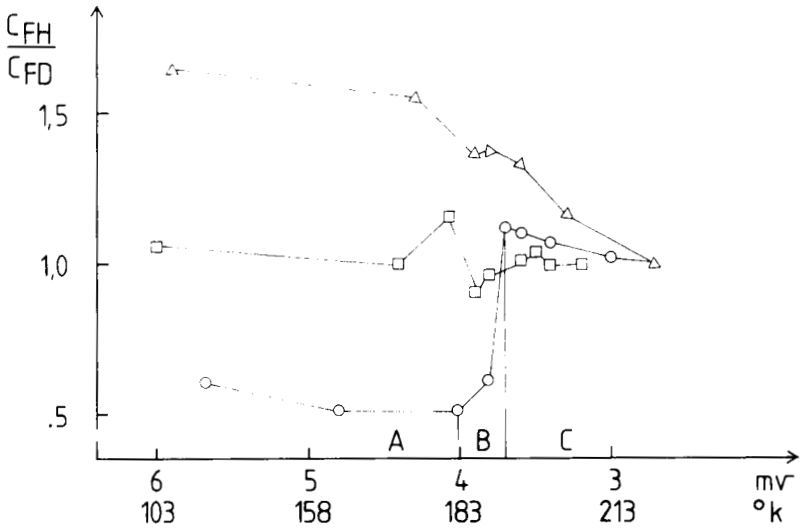

Fig. 6. Contrast variation during freeze-drying of embedded (○), hydrated but non-embedded (△) and dried (□) catalase crystals. The regions marked A, B and C correspond respectively to the contrast of embedded, hydrated but not embedded and freeze-dried crystals. For each crystal the contrast has been normalized to the value found after drying. The voltage read, at the moment the photograph is taken, from the thermocouple situated close to the specimen is given in abscissa. The temperature of the specimen corresponding to this voltage at equilibrium is also given.

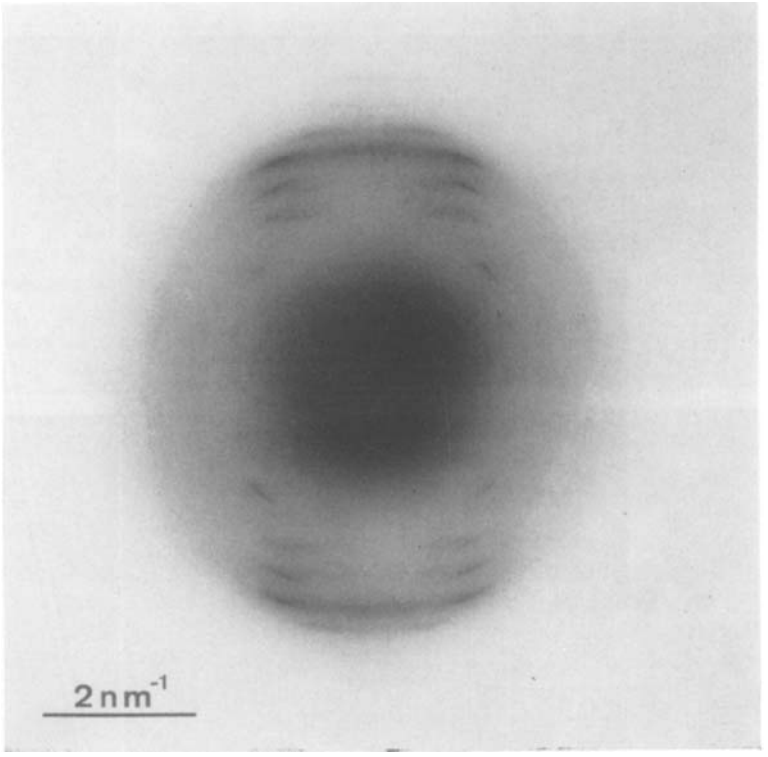

Fig. 7. Electron diffraction pattern from a fibre of DNA (sodium salt) equilibrated at 76% humidity, then quench-frozen in liquid nitrogen and observed at *c.* 100 K.

easily be tested by contrast measurements alone. Preliminary observations on viruses confirm that severe shrinkage and distortion take place as soon as they are no longer fully embedded.

The presence of water close to or superimposed upon biological particles is not proof of being embedded. In particular, when water freezes in large hexagonal crystals, particles have a tendency to be expelled and become concentrated on the surfaces. This can be illustrated with polystyrene spheres in vitreous ice which have one-tenth of the contrast of the dried state. When frozen in crystalline ice, this contrast is variable but generally it is not much lower than in the dry state. The accumulation of particles on the surface of the ice can be confirmed by stereo-views (not shown). Figure 8(a) shows a similar case with T4 bacteriophages frozen with crystal-

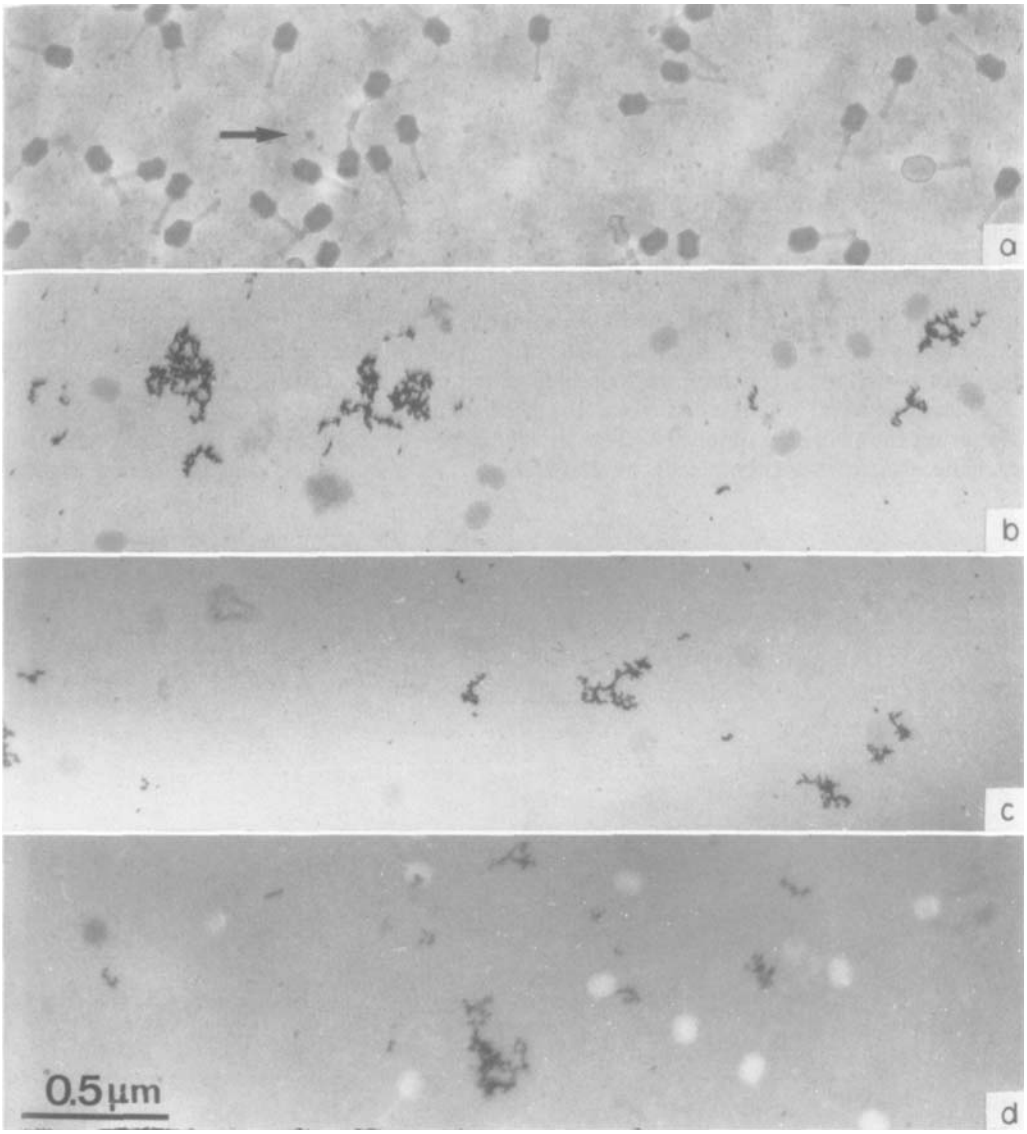

Fig. 8. (a) T4 bacteriophages in a hexagonal ice layer. The arrow indicates a base plate. (b) T4 embedded in a vitreous ice layer. (c) T4 embedded in a 10% metrizamide solution. (d) T4 embedded in a 20% metrizamide solution. × 40,000. The dark particles in (b), (c) and (d) are gold islands used as focusing aids.

line ice. As compared with freeze-dried samples the contrast of the heads is too high for them to be embedded in water. Furthermore the clear visibility of such small structures as tail fibres or base plates (arrow) demonstrates that they are not embedded and the contrast is possibly reinforced by the deposition of salts. The phenomenon of expelling particles from a crystallizing ice matrix does not take place when the suspension is vitrified (Fig. 8b–d).

Staining and contrast matching

The density of ice being 933 kg/m^3, most embedded biological particles will appear with low but positive contrast. This is illustrated in Fig. 8(b) for T4 bacteriophage in vitreous ice. The fact that solutes are not segregated in vitreous ice makes it possible to increase the density of the medium, thus changing the contrast of embedded particles. Any salt can be used for this purpose, for example sodium phosphotungstate, uranyl acetate or caesium chloride. Depending on the concentration of the salt, any intermediate state between ice-embedding and standard negative staining can be obtained. Charge interaction at low concentrations of solute may lead to a non-uniform distribution of ions around the particles. For example, DNA molecules appear positively stained in a 2% solution of uranyl acetate. This effect will not take place with non-ionic molecules such as metrizamide (a tri-iodinated benzamido derivative of glucose, Nyegaard & Co.) frequently used for ultracentrifuge gradients. Figure 8(c) shows bacteriophages T4 in a 10% solution of vitrified metrizamide. The contrast of the proteic tail has vanished whereas the nucleo-proteic head remains with positive contrast. Figure 8(d) shows the same bacteriophage in 20% vitrified metrizamide. Tail and head appear here with negative contrast. Figure 9 gives the average contrast of the head of the bacteriophage for various densities (and thus concentrations of metrizamide). The dependence of the contrast on the concentration is roughly linear at low concentrations. At high concentration, positive staining and/or disintegration of the particles as well as metrizamide radiodecomposition make the curve irregular.

The contrast of a particle (such as the head of bacteriophage T4) is due to the electron scattering from all its components. These include the exchangeable (e.g. water, salt) and non-exchangeable in the volume seen in the electron micrograph. The density of such particles

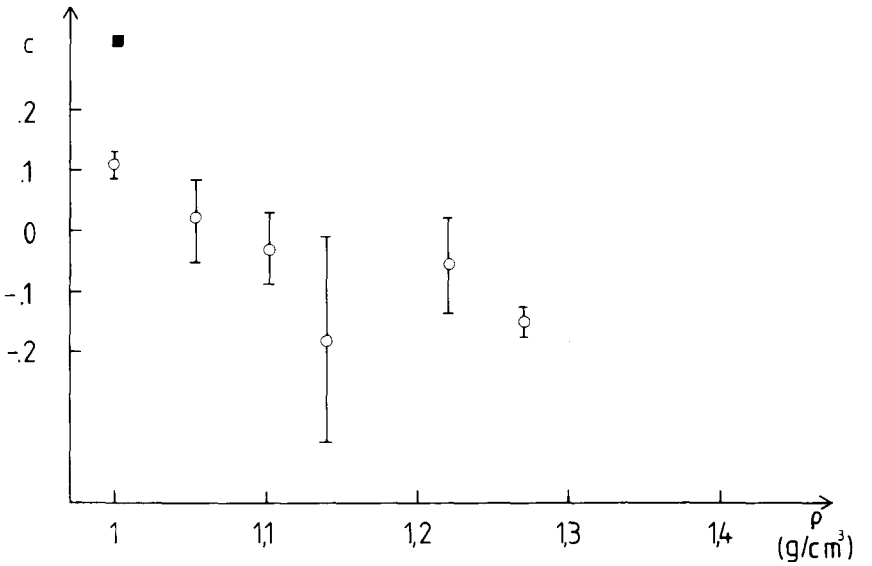

Fig. 9. Contrast of T4 bacteriophage in solutions of metrizamide of different density. ○, Vitrified solutions, ■, hexagonal ice. The densities are those for solutions at 298 K. For Figs. 9, 11 and 12, each point is the mean of several measurements, error bars represent standard deviations.

(which is used to compute the contrast (formulas 1 and 2)) is different from the density derived from ultra-centrifugation studies.

Beam damage

The fading of the electron diffraction pattern of biological crystals is an excellent test for beam damage (Glaeser, 1971). It is illustrated in Fig. 10 where the electron diffraction pattern of the same hydrated catalase crystal maintained at 100 K is shown after irradiation by 70, 140, 210 and 280 $e^- nm^{-2}$. The dose D_e required to reduce the reflections to $1/e$ of their initial intensity is shown in Fig. 11 as a function of the temperature. For hydrated specimens reflections corresponding to dimensions smaller than 1 nm have an average D_e value around 150 $e\ nm^{-2}$ which slowly decreases with increasing temperature (Fig. 11, ○). Above 170 K they disappear because the specimen rapidly freeze-dries. Glucose embedded catalase crystals (Unwin & Henderson, 1975) have a very similar D_e value which also decreases with increasing temperature (Fig. 11, △). This temperature dependence of D_e is compatible with the measurements made on the purple membrane of *Halobacterium halobium* at room temperature and low temperature (Hayward & Glaeser, 1979). For dimensions larger than 1 nm, the D_e value is the same for hydrated, freeze-dried and glucose embedded specimens (Fig. 11, ■). These data indicate that damage is not significantly affected by the embedding medium.

As shown in Fig. 12, the D_e value is independent of the electron flux between 0·5 and 50 $e\ nm^{-2} s^{-1}$. The phenomenon of bubbling that we have attributed to the accumulation of molecular fragments unable to diffuse out of the specimen (Dubochet *et al.*, 1982) is characteristic of organic material in hexagonal or vitreous ice. As is the case in sugar solution, bubbling appears above 1000 $e\ nm^{-2}$ in ice-embedded biological material. The nucleation leading to bubbling is likely to start at a lower dose. The dose for bubbling increases with decreasing

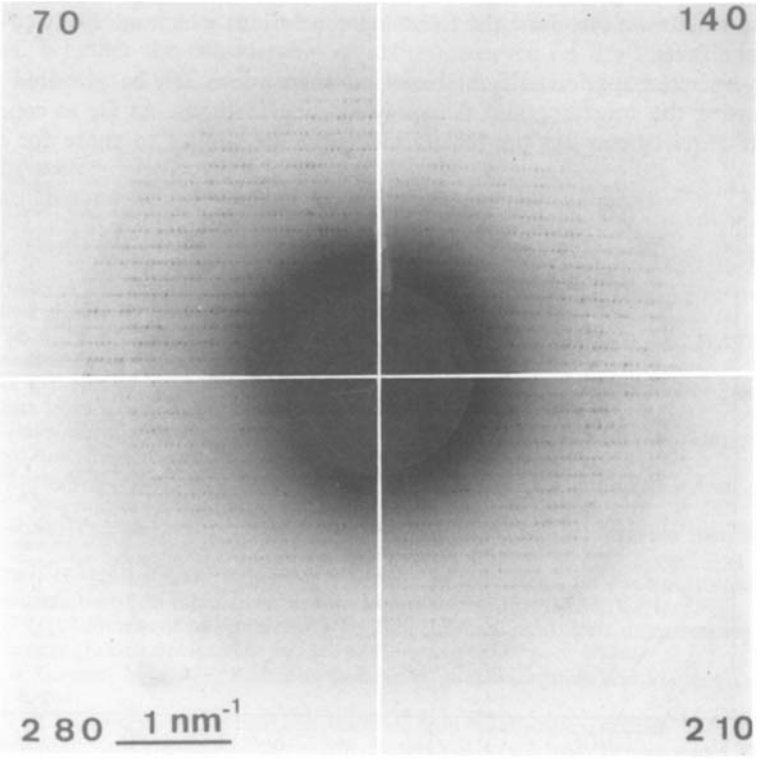

Fig. 10. Montage of different electron diffraction patterns recorded from the same catalase crystal and with the same exposure time but with accumulated doses of 70, 140, 210, 280 $e^- mm^{-2}$ respectively.

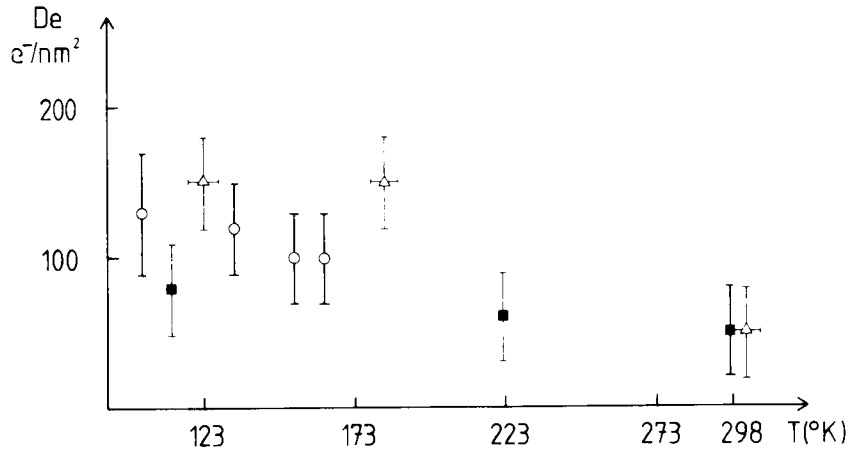

Fig. 11. Doses (D_e) required to reduce the intensity of the catalase crystal electron diffraction pattern by a factor equal to e^{-1} for different embedding media and temperatures. ○, Frozen hydrated catalase crystals; △, glucose embedded catalase crystals (for these specimens the doses D_e have been determined for the reflections corresponding to less than 0·8 nm); ■, freeze-dried catalase crystals (doses D_e determined for the reflections corresponding to more than 1 nm).

temperature. Bubbling does not depend strongly on the concentration of the biological material or on the electron-flux.

CONCLUSIONS

From these results we can draw the following conclusions which are likely to be valid for most biological objects.

(1) Frozen-hydrated specimens from biological suspensions can be prepared rapidly and reproducibly using the spreading and the spray-freezing methods. As far as concentration is concerned, the requirements for the former technique are similar to those for conventional

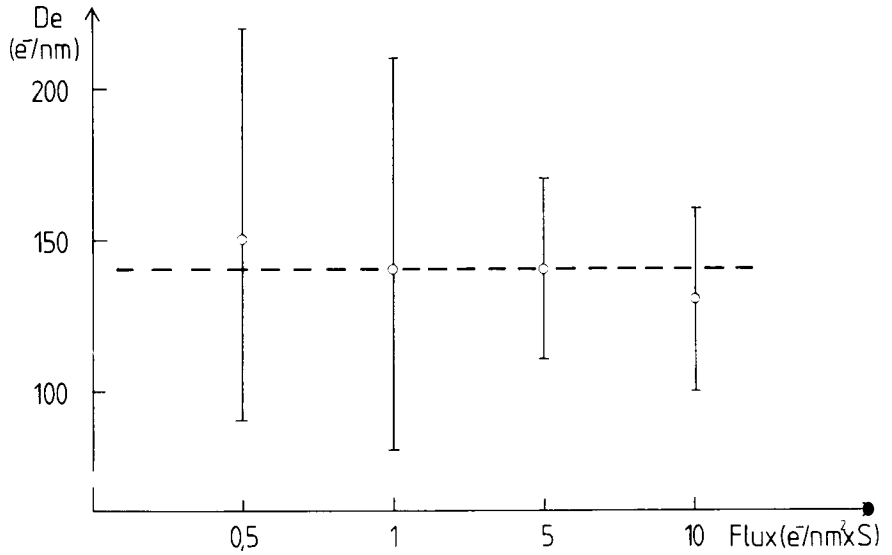

Fig. 12. Dose D_e determined on frozen hydrated catalase crystals maintained at 110 K for different electron fluxes.

staining whereas for the latter a very high concentration of biological material is needed. For both methods a high concentration of salt or solute is not a disturbing factor. The equilibration method is likely to give good results easily for specimens whose structural integrity is maintained by tightly bound water. When a humidity higher than say 90% is required for preserving the structure, the techniques of spreading and spray-freezing become more useful. However, for such specimens as fibres of DNA, which dissolve in water, the equilibration method is the only one at present available.

(2) For optimum preservation of the specimen the freezing rate should be sufficient to vitrify the water since no solute segregation then occurs. However, in suspensions with a low solute concentration good structural preservation can sometimes be obtained with the water in the form of hexagonal or cubic ice.

(3) It is difficult to remove bulk water reliably by controlled drying whilst preserving hydration. Consequently, one can be certain of good structural preservation only for particles completely embedded in ice. This results in low contrast. One should also be aware that crystallization of ice in the hexagonal form frequently pushes the particles to the surface of the specimen. They then appear with higher contrast but may loose their hydration water.

(4) The contrast of the electron microscopical image is related to the amount of material represented by each image element. Quantitative measurements lead to the determination of the mass distribution in the specimen. By comparison with freeze-dried specimens, the water content of biological particles can be calculated. Varying the density of the embedding medium should allow the determination of the density distribution within the particles.

(5) When the particles are embedded in stain of various concentration, any situation ranging from no staining to standard negative or positive staining can be achieved.

(6) Electron beam damage to frozen-hydrated biological specimens is not significantly different from that occurring in sucrose embedded or dry samples when measured at 100 K. At this temperature a gain of a factor 3–4 is obtained when compared with room temperature measurements. Whether the considerable reduction observed on dry organic material at 4 K (Knapeck & Dubochet, 1980) will also take place on hydrated specimens remains an open question.

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