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FREEZING: FACTS AND HYPOTHESIS

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Abstract

Hexagonal ice crystals formed in frozen biological specimens are large and branched. They can produce severe structural damage by solute segregation but there are also cases where they seem to cause only minor damage. When cooling is more rapid, cubic ice crystals can be formed. These are small and in general, they cause little damage. These observations can be readily explained with the hypothesis that large hexagonal ice crystals can originate from the rewarming induced transformation of a large number of cubic ice crystals. This transformation would take place without significant solute displacement.

Key Words: Cubic ice, freezing damage, hexagonal ice, ice crystal, vitreous ice, vitrified water.

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Introduction

Most cryo-methods for preparing biological specimens for electron microscopy rely on the freezing of water. The hexagonal ice crystals formed are, in most cases, very large at least at the scale of the cell ultrastructure (Dubochet and McDowall, 1984). How is it that, in spite of this massive rearrangement, methods such as freeze-substitution or freeze-drying can give the excellent results we know from so many reports in this or in other volumes?

Cubic ice is a low temperature form of ice which can be formed by very rapid cooling (Dubochet *et al.*, 1982; Mayer and Hallbrucker, 1987). The average volume of one cubic ice crystal is typically 4 orders of magnitude smaller than one hexagonal ice crystal. How is it that, when cooling is very rapid and the time given for nucleation is very short, there are many more crystals formed than when cooling is slow with ample time for nucleation?

We propose to address these two apparent paradoxes by: (1) observing that crystallization into cubic ice does not seem to produce severe structural rearrangements in biological material (2) making the hypothesis that one hexagonal ice crystal can originate from many cubic ice crystals.

Observation 1. Hexagonal ice crystals are large

When ice crystals are growing in a cell or in an aqueous solution, solute concentrates in the liquid phase until the residual material can no longer be frozen under these cooling conditions. The separation in two phases produces the typical aspect of 'badly frozen' freeze-substituted, freeze-dried or frozen hydrated specimens in which regions representing pure ice are surrounded by concentrated 'unfreezable' material. Figure 1 shows examples of badly frozen hydrated sections in which the ice crystal domains are well visible. For a long time, it has been thought that each domain is the remnant of one ice crystal. Most studies on ice crystal size are based on

this hypothesis (see Bald, 1987 for references).

One advantage of frozen hydrated sections is that ice is still present in the specimen when it is observed in the electron microscope. On such specimens it was demonstrated by electron diffraction that one hexagonal ice crystal is always relatively large and that it extends over many domains (Dubochet and McDowall, 1984).

Fig. 1 illustrates this observation. Typically, there are only one or few hexagonal ice crystals per cell and their volume in rapidly frozen 10-20% sucrose or gelatine solutions is in the range of 10^3 μm^3. Each domain is therefore only the representation of a section through one branch of a ramified single crystal. A section through a forest could be seen as a useful analogue of this complex structure; it would show innumerable segments, but it would be difficult to decide which belongs to which tree. The large depth of field of the SEM also suggests the complex structure of an ice crystal in badly frozen freeze-dried specimens (Franks, 1990). This view of a ramified large crystal is in accordance with theories of nucleation and crystal growth (Riehle and Hoechli, 1973).

Observation 2. Cubic ice crystals are small and in general they do not cause severe damage to biological structures

Specimens in which ice is in cubic form look very different from those in which ice is hexagonal. There is generally no visible segregation of solute (Fig. 2a) or it is very fine. Furthermore, the ice crystals are always small. Consequently, in the electron diffraction mode, there are many crystals in the observed region, even if the area is only a fraction of a μm^2 (insert). The diffractogram consists of concentric rings, typical for powder diffraction. In general, only sharpness of the diffraction rings puts a lower limit on the size of cubic crystals. They were estimated to be not larger than about 30 nm (Dubochet, and McDowall, 1984). Only a few cases have been observed where cubic crystals are in the μm range. A remarkable example is shown in Fig. 2b, but in this case the elongated shape of the crystals, perpendicularly to the cutting direction, demonstrates that they have not been produced during a normal cooling process but have been formed by the action of the knife.

Biological structures are generally well preserved in the presence of cubic ice. This is not surprising since, in a majority of cases, the formation of cubic ice does not even produce observable solute segregation. The observation has been reported on numerous occasions on a variety of specimens. One published example is shown in figure 3a. According to our knowledge, the only reported case in which structural damage has been attributed to cubic ice formation concerns a hole in the center of myosin filaments of glycerinated insect flight muscle (McDowall *et al.*, 1984) which was thought to be a freezing artefact. This fact was not confirmed in a subsequent study of intact frog muscle (Trus *et al.*, 1988). There are also reports that electron diffractograms extending to ca. 0.3 nm can be recorded on catalase crystals in presence of cubic ice (Lepault, personal communication).

Observation 3. Hexagonal ice formation sometimes causes little apparent damage

As was shown above, the formation of hexagonal ice is frequently associated with massive structural damage. This, however, must not always be the case. Many excellent micrographs obtained by freeze-etching, freeze-drying or freeze-substitution, under preparation conditions by which hexagonal ice crystal must have formed because the hydrated specimen was rewarmed during preparation well above the cubic-hexagonal transition temperature of about -80°C, are witness to the quality of structural preservation. A similar observation was made on some frozen hydrated sections in which, as in Fig. 4, the structural preservation seemed good, lacking in particular any visible pattern of solute segregation, but in which the water formed large hexagonal crystals. At first sight, the general aspect of the section seems typical for a vitrified and well preserved specimen. The overall contrast is low, there are no segregation domains visible and the fine structure of the cell architecture seems intact. However, the presence of large hexagonal ice crystals is revealed by black lines due to Bragg reflections on large hexagonal ice crystals. Electron diffraction confirmed the interpretation (not shown).

Discussion

The above observations raise two questions:

1) How is it possible that the cell ultrastructure can be preserved in spite of the fact that water, which represents the major part of the sample, is transformed into large crystals of hexagonal ice? How can it be that a crystal with overall dimensions in the μm range can form within the cell structure without producing damage larger than some nm?

2) We know that freezing is a two step process (Franks, 1982). Firstly, the ice crystal must nucleate. This means that the water molecules cooled below the melting point must arrange themselves (or with the help of some template) into an ice nuclei. This is a stochastic event which must take place against free energy.

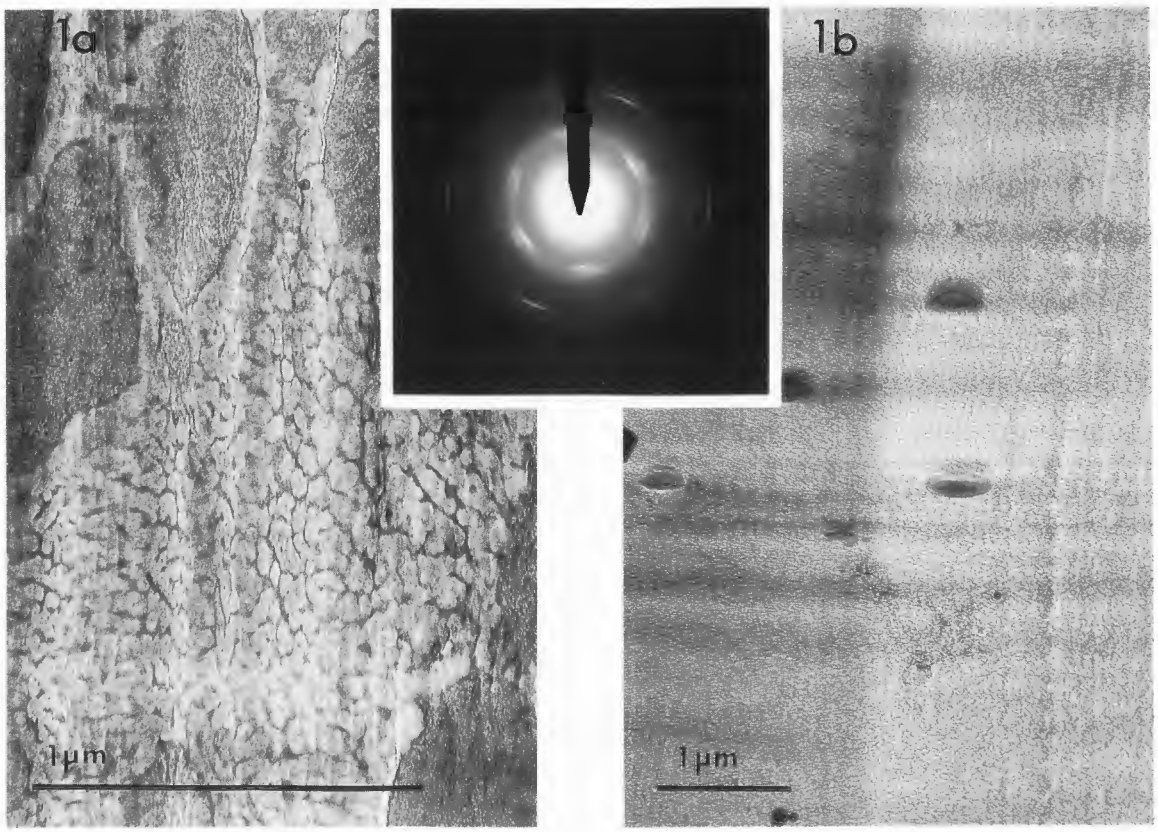


Figure 1. Frozen hydrated section of rat liver (a) and of 20% gelatine/sucrose solution (b). Domains due to ice crystal formation are marked (D). Electron diffractograms of the corresponding regions are shown in the center. They demonstrate that there is only one hexagonal ice crystal in the field. The specimens have been prepared and observed as described elsewhere (Dubochet *et al.*, 1988).

Secondly, the nucleated crystal must grow. This is a thermodynamically favorable process which can proceed very rapidly.

Cubic ice crystals form during rapid cooling. They are very small. This means that during the short cooling time, a large number of nucleation events have taken place and no crystal can grow very large before encountering another growing crystal.

How is it then possible that, when cooling is much slower and, one would first think opportunities for nucleation are even greater, this number of ice crystals is reduced by 4 or more orders of magnitude and their size increased in the same proportion?

These two paradoxes are resolved with the hypothesis that hexagonal ice crystals are the result of the transformation of a large number of preformed cubic crystals. In other words, it is hypothesized that water crystallizes first into cubic ice. The better known hexagonal ice is obtained by conversion of the primer freezing form.

With this hypothesis, the good preservation of frozen biological specimens, as is shown in figure 4, would be explained as follows: during the rapid cooling of the sample, water crystallizes into many small cubic ice crystals. This transformation is rapid and is terminated long before the temperature is reached, at which cubic ice becomes a stable form of ice. In this state the water of the biological sample is dispersed in innumerable very small crystals and no long range rearrangements of solute have taken place. While the specimen is cooled further, one small cubic crystal may suffer a phase transition towards the more stable hexagonal ice. Perhaps by contact nucleation on neighboring crystals, perhaps also with the help of heat released during its own phase transition, the cubic ice crystal nucleates the transformation of its neighbors and extends its growth over a large volume. The transformation can take place without requiring any significant displacement of ma-

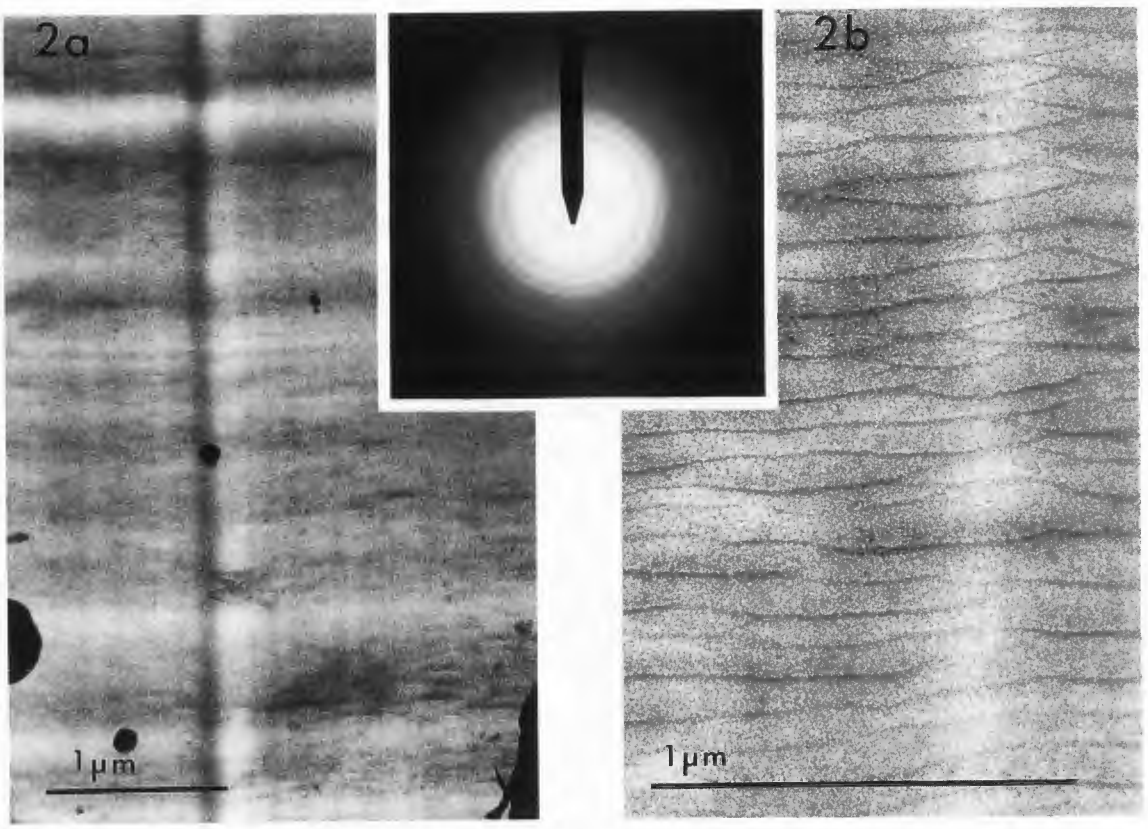


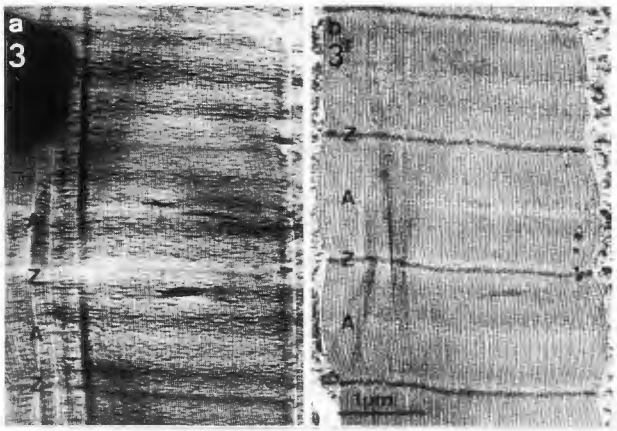
Figure 2. Frozen hydrated section of a 20% gelatine/sucrose solution. The water is in the form of cubic crystals. The electron diffractogram corresponding to fig. 2a. is shown in insert. Solute segregation due to crystal formation is not visible in a) but it is in b). In the latter case, the ice crystals originate from the cutting process.

terial; the water molecules must only rearrange locally; proteins or membranes do not need to be displaced. Although the water now forms μm large crystals, solute segregation is still minimal and the ultrastructure of the cell seems intact.

The hypothesis has the merit to explain the two apparently counterintuitive observations mentioned above. It is further supported by the observation made in thin frozen layers that, between vitrified regions and areas where water is in hexagonal crystals, there is generally a border of cubic ice crystals (Dubochet and McDowall, 1984). This supports the idea that cubic ice antecedes hexagonal ice formation. One must however say that there are also published observations where this does not seem to be the case (e.g. in Dubochet *et al.*, 1988).

The apparent paradox that water succeeds in nucleating many more cubic crystals when it is cooled rapidly than it has time to nucleate hexagonal crystals when it is cooled more slowly could also be explained by considering that both phenomena are very different: during slow cooling, nucleation is a rare event, taking place in water which is not severely undercooled. It leads to large hexagonal crystals. When cooling is rapid, undercooling is important and nucleation becomes much easier.

The test of the hypothesis that hexagonal crystals can result from the transformation of a number of small cubic crystals and that this type of freezing produces minimum structural damage to biological structures will probably not be easy. High speed video recording of rapidly cooled samples with a polarizing microscope would probably bring an answer, but installing the instrumental set up for the experiment would be a major challenge. Valuable information would also be obtained if a specimen frozen in cubic ice could be rewarmed in the electron microscope in order to observe how cubic ice is transformed into hexagonal ice. This experiment is unfortunately impossible as water, in thin specimens, evaporates long before the transition from cubic to hexagonal has taken place. We hope however that this experiment will be realized, though in a less direct way, when freezing conditions allowing that a given state of frozen water, whether hexagonal, cubic or vitrified will be obtainable in a reproducible way. It will then be



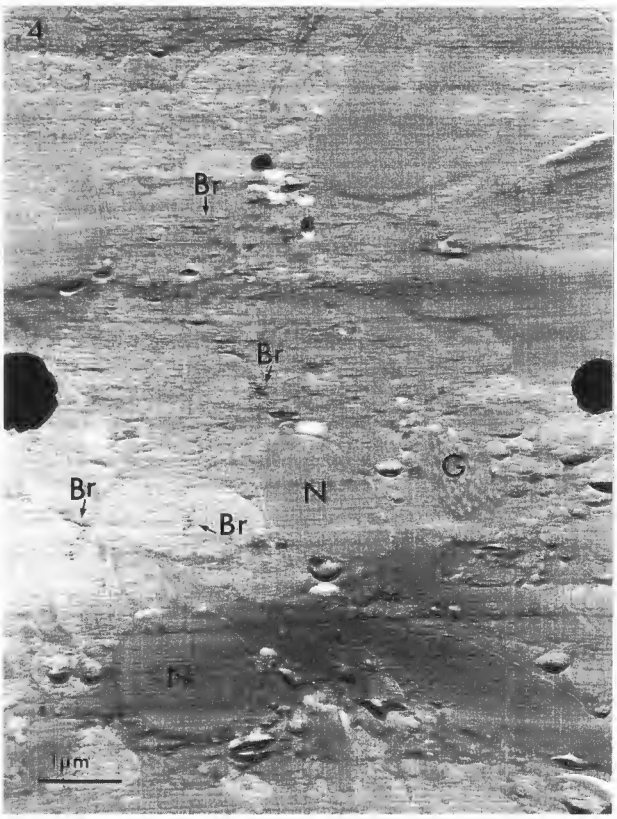


Figure 3. An apparently well preserved frozen-hydrated section of glycerinated insect flight muscle in which water is in form of cubic ice (From McDowall *et al.*, 1984, with permission).

Figure 4. An apparently well preserved frozen-hydrated section of rat liver tissue in which water is in the form of large hexagonal crystals. Some Bragg reflections (Br) on the large distorted crystal are marked.

possible to observe in frozen sections, the transitional effect that cubic to hexagonal ice has on frozen biological structures.

Acknowledgement

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Discussion with Reviewers

T. von Zglinicki: Did you consider the possibility that the hexagonal ice detected in Fig. 4 has been formed during sectioning?

Answer: Yes this is a possibility that cannot be ruled out completely. We show in figure 2b a case where cubic ice is thought to originate from sectioning. Nevertheless, we know that, in general, sectioning is not a major factor in determining the state of the ice in a section. The reasons are as follow. On one hand, experience

shows that the state of the ice in a section can generally be related to the freezing conditions and to the position of the section in the block. Hexagonal crystals in a section are related to poor cooling conditions, to a lack of cryoprotectant or to a relatively large distance to the surface of the block. An experienced observer can also recognize the crystalline state, even before sectioning, and it confirms it by observing the section. On the other hand, no reproducible relationship has yet been made between the cutting conditions (speed, angles, quality of the knife) and the state of the ice in the section.

T. von Zglinicki: Is the liver shown in Fig. 4 untreated with chemical fixatives or cryoprotectants?
Authors: Yes.

P. Frederik: Where does the transition temperature of -80°C come from? The data from Mayer and Hallbrucker (1987) are showing that it takes 30 minutes to transform 70% of crystalline ice (Ic) into hexagonal ice (Ih) at 230K and 240K.
Authors: The value of -80°C comes from the classical article by Dowell and Rinfret (1960), Nature 188, 1144. (Discussed in Dubochet *et al.*, 1982 and 1988). However, we are aware that several recent measurements, in particular by the group of Mayer, show that the devitrification temperature and also the temperature of Ic to Ih transition is significantly higher than measured previously. Our measurements of the devitrification temperature have always been in good agreement with those of Dowell and Rinfret (Dubochet *et al.*, 1982 and 1988). Can this be due to the fact that temperature measurement in the electron microscope is difficult and possibly quite wrong, or that, the devitrification temperature depends on other factors as for example, the freezing conditions? For the Ic to Ih transition, the measurement cannot be done in the electron microscope since the specimen sublimates before this temperature is reached. We still believe that the temperature of -80°C is approximately correct since we have tested it in the cryo-ultramicrotome (a vitrified specimen with minimal amount of cryoprotectant leads only to hexagonal crystals in frozen-hydrated sections obtained after it has been rewarmed for a moment at -80°C). Furthermore, we have observed, as probably many others, that the vitreous state (with a small amount of cryoprotectant) is not preserved in a freezer at -80°C.