Journal of Microscopy, Vol. 207, Pt 1 July 2002, pp. 5–26
Received 13 December 2001; accepted 21 March 2002

Freeze-dried and resin-embedded biological material is well suited for ultrastructure research

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Key words. Cellular water structure, cellular ultrastructure, cryofixation, freeze-drying, gelatine, hydration of macromolecules, immunocytochemistry, ion localization, Lowicryl, mitochondrial membranes, Spurr's resin, transmission electron microscopy.

Summary

Transmission electron micrographs of different biological material, cryofixed, freeze-dried and embedded in Spurr's resin, in Epon, or in Lowicryl, are presented. The structure preservation obtained either without or with application of chemical fixatives after drying showed that freeze-dried embedded specimens are particularly well suited for new morphological, immunocytochemical and microanalytical studies aimed at detecting the life-like subcellular distribution of mobile macromolecules and ions. The results also indicate that the removal of cell water by freeze-drying from the areas of best cryofixation is relatively slow. Ultrathin sections of well cryofixed biological material embedded after freeze-drying in Spurr's resin or Epon reveal cellular plasma phases with very fine granularities and well defined membranes in negative contrast. This may be due to the preservation of the original structure of cellular macromolecules with a considerable amount of their hydration water. Sublimation studies with differently hydrated and cryofixed macromolecules are suggested to settle this issue.

1. Introduction

Ultrastructure research of biological material is aimed at evaluating its structure and function. Different features have to be investigated: the architecture of living cells, the structure of macromolecules at work in living cells and the relationships between proteins (and other macromolecules), ions and water during different physiological activities. All cellular components should be detected and correctly localized with the highest possible spatial resolution reflecting the dynamic living state. Therefore, electron microscopy appears to be indispensable for this purpose. However, the highly dynamic and labile biological material has to be stabilized before it can be investigated with the electron microscope. By using stabilizing and dehydration procedures one introduces artefacts and only suitable control experiments may eventually lead to an understanding and a systematic prevention of these artefacts.

Ultrathin sections of resin-embedded material are well suited for transmission electron microscopy (TEM) but the artefacts introduced by the conventional preparation technique are severe. During chemical fixation of the biological material (first step) the physicochemical properties of macromolecules change: ion shifts, water shifts, and structure changes are inevitable. Furthermore, physiological dynamic processes cannot be captured due to the slow action of the chemical fixative. During dehydration with organic solvents at room temperature (second step) the structure of macromolecules is further modified; water, ions and parts of the macromolecular meshwork are redistributed and extracted. In the third step of embedding and polymerization of the biological material in a resin, additional changes in structure and antigenicity may occur.

By being aware of these possible sources of artefacts several different preparation techniques have been developed aimed at minimizing one or another artificial change. For example, by using the progressive lowering of temperature (PLT) technique (Carlemalm *et al.*, 1982; Robertson *et al.*, 1992) the concentration of the chemical fixatives may be reduced and complete dehydration and embedding are carried out at low (subzero) temperature. Much less extraction and, in many cases, an improved immunolabelling of macromolecules is observed. More realistic TEM micrographs of resin-embedded biological material have been obtained after the development and acceptance of cryotechniques (e.g. Steinbrecht & Zierold, 1987; Echlin, 1992). In a first step all components of the native biological material are immobilized by cryofixation, i.e. by rapid freezing at ambient pressure (Sitte *et al.*, 1987), or by high pressure freezing (Moor, 1987). Afterwards the cryofixed

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specimen is dehydrated at low temperature followed by embedding in a resin and polymerization at low or high temperature (Steinbrecht & Müller, 1987). Dehydration of the cryofixed material is possible by using an organic solvent to replace the water molecules of the specimen (freeze-substitution, FS, e.g. Van Harreveld & Crowell, 1964) or by sublimation of the water molecules in a suitable vacuum chamber (freeze-drying, FD, e.g. Edelmann, 1978).

FS combined with resin embedding and polymerization at different temperatures has uncovered many artefacts produced by the conventional technique and is now widely used for morphological and immunocytochemical studies. The FD-embedding technique, on the other hand, has not yet received attention, probably because of the prevailing opinion that FS is easier and that the morphological results after FD are inferior to those obtained by FS. The purpose of this paper is to present new electron microscopical results from different biological material after FD and embedding under different conditions and to discuss the advantages and problems of FD as well as whether new information on biological material can be obtained by using the FD technique.

2. Materials and methods

The following material was processed: rat kidney, rat liver, human voluntary muscle, human blood platelets, human red blood cells (RBCs), Jurkat cells, and also gels of 20% gelatine–80% water either without or with mild chemical fixation (3% paraformaldehyde, 0.2% glutaraldehyde in 0.1 M cacodylate buffer for 1 h, washing in 0.1 M cacodylate buffer 3 × 20 min). The biological material was not treated with a chemical fixative before cryofixation except one additional preparation of platelets, which was chemically fixed as described for the chemically fixed gelatine gel.

2.1. Cryofixation

Cryofixation was performed either by metal mirror freezing using the Leica MM 80 (Leica, Vienna, Austria) or by high pressure freezing (HPF: HPM 010, Balzers, Fürstentum, Liechtenstein and Leica EMPACT). Kidney and liver slices, as well as slices of gelatine gel, were frozen with the Leica MM80 as described by Sitte *et al.* (1987). Suspensions of human blood platelets and RBCs were frozen as described by Edelmann & Ruf (1996) (spacers: rings of anti-adhesive plastic with an inner diameter of 2.4 mm and a thickness of 0.3 mm). Suspensions of blood platelets and RBCs in blood plasma were prepared as described by Morgenstern *et al.* (1985). Fine needle muscle biopsies were obtained as described by Hohenberg *et al.* (1996) and frozen with the HPM 010. Jurkat cells were frozen either with the HPM 010 in dialysing tubes as described by Hohenberg *et al.* (1994) or with the EMPACT in copper tubes as described by Studer *et al.* (2001). Rat liver tissue was also cryofixed with the EMPACT.

2.2. Freeze drying

The cryofixed specimens were transferred to the LN_2 cooled object table of the Leica CFD as described by Sitte *et al.* (1994). The following preparation was carried out inside a Styrofoam box partially filled with LN2 by using a stereo light microscope and fibre optic illumination. With a scalpel the cryofixed samples were cut into smaller pieces with the shortest side not exceeding 0.3 mm (microcapillary tubes and copper tubes used for high pressure freezing have to be cut open because closed tubes cannot be freeze-dried). For later embedding in Lowicryl the specimens were transferred into plastic moulds on an object table as described by Edelmann (1994b). For later embedding in Spurr's resin or Epon, specimens were transferred into small metal containers (diameter 4 mm) placed on the object table. After loading, the object table was transferred into the CFD. FD was carried out at a pressure of about 10^{-4} mbar; periods of freeze-drying temperatures are given in the figure legends.

2.3. Embedding

Embedding in Lowicryl was carried out as described by Edelmann & Ruf (1996). Freeze-dried specimens were embedded in Spurr's resin either after exposure for 1 h to osmium vapour or without osmication. In the first case, the CFD was vented with pure nitrogen gas, the object table with the dried specimens was tightly sealed at atmospheric pressure with a glass plate inside the FD unit (see Edelmann, 1994b) and heated to 27 °C (a few degrees above room temperature). The object table was removed from the CFD, transferred to a fume hood and opened again. One small OsO_4 crystal was placed on the object table, which was closed afterwards for 1 h. After removal of the crystal, drops of pure Spurr's resin were filled into the small containers (see above). After 6 h of infiltration of the specimens with Spurr's resin, they were transferred from the small containers into fresh resin in embedding moulds and polymerized for 1 day at 60 °C. Embedding of dried specimens in Spurr's resin (without osmication) was carried out as follows. At the end of the FD procedure, the temperature of the object table was lowered to −20 °C and the CFD was vented with pure nitrogen gas. The small containers with the specimens were filled at atmospheric pressure with drops of a 1 : 1 DER-ERL mixture (two low viscosity components of Spurr's resin) inside the CFD. After 6 h the object table was warmed up to room temperature and removed from the CFD. The specimens were transferred to pure Spurr's resin and after 2–4 h placed in fresh resin in embedding moulds and polymerized for 1 day at 60 °C. When using Epon the first step of infiltration at −20 °C was carried out with a 1 : 1 propylenoxid–Epon mixture.

2.4. Electron microscopy

Ultrathin sections (60–70 nm thick) were obtained by using a Diatome diamond knife (Diatome, Biel, Switzerland). The

sections were stained with uranyl acetate and lead citrate for 1 min each and were photographed in a Zeiss EM 902 (LEO Elektronenmikroskopie, Oberkochen, Germany).

2.5. Immunolabelling

Ultrathin sections were washed in PBS containing 5% BSA and 5% goat serum for 30 min, incubated for 60 min at room temperature with the primary antibody in incubation buffer (PBS, 0.1% BSAc from Aurion, Wageningen, The Netherlands) and incubated with the secondary antibody for 60 min. After fixation for 1 min (0.5 glutaraldehyde in PBS) the sections were stained with uranyl acetate (1 min) and lead citrate (1 min). Liver sections were labelled by using either rabbit anti-bovine catalase or rabbit anti-rat CuZn superoxide dismutase (SOD) and protein A-gold (10 nm) (the antibodies were kindly donated by G. Posthuma, Utrecht, The Netherlands). The primary antibody used for labelling of CLP-36 (a protein associated with actinin) of human platelets was kindly donated by W. Siess, Münich, Germany (see Bauer *et al.*, 2000). The secondary antibody was goat anti-rabbit IgG conjugated to 10 nm gold (Aurion).

3. Results

3.1. FD and embedding in epoxy resins without chemical fixation

A good preservation of structure could be obtained after FD and embedding in Spurr's resin or Epon without using any fixative. The following examples are shown: rat kidney Fig. 1(A); human muscle Fig. 2(A), (B); rat liver Figs 3(C), 4(A)–(D), 5(A), (B); human blood platelet Fig. 6(E), human RBC Fig. 7(E) and Jurkat cell Fig. 8(A). Halos around mitochondria are visible in Figs 1(A), 2(B) and 3(C), around RBCs in Fig. 7(C). In the area where the best cryofixation occurred a new state of preservation is visible, as shown in Figs 4, 5(A), (B) and 6(E). The cytoplasm and the mitochondrial matrix appear as heavily stained phases with a very fine granularity not seen before in freeze-substituted and embedded preparations (compare Fig. 5(B) with Fig. 5(C)). Membranes appear in sharp negative contrast and differential shrinkage artefacts are not visible in this area (no halos around mitochondria). A high number of vesicles or cross-sections of tubular systems are seen in liver preparations (Figs 4(B) and 5(A)).

Examples of immunogold labelled sections are given in Fig. 4(C) (SOD in rat liver lysosomes), Fig. 4(D) (catalase in rat liver peroxisomes) and Fig. 6(E) (CLP-36 in human platelet).

3.2. FD and osmium vapour treatment before embedding in Spurr's resin

The preservation of structure and the appearance of biological material after FD and OsO_4 vapour treatment and subsequent embedding in Spurr's resin approached the results that may be obtained after FS in acetone supplemented with OsO_4 (kidney Fig. 1(B), liver Fig. 3(D), leucocyte Fig. 7(E)). Membranes display a positive contrast. However, in contrast to results obtained by FS, certain cells or subcellular organelles show differential shrinkage: halos around mitochondria (Fig. 3(D)) and around RBCs (Fig. 7(D) and (E)) are visible. A comparison of Fig. 7(D) with Fig. 7(E) shows that shrinkage is less pronounced in RBCs with ice crystal damage. Loss of cellular material is demonstrated in Fig. 7(D); the RBC, well cryofixed and exposed to OsO_4 vapour after FD is very labile, most likely due to insufficient dehydration.

3.3. FD and LTE in Lowicryl without chemical fixation

When freeze-dried biological material was embedded at low temperature in Lowicryl without using a chemical fixative, the area of best cryofixation was very labile (liver Fig. 3(A), blood platelet Fig. 6(A), RBC Fig. 7(A)). Loss of cellular material and/or deformation of cells could be observed after wet sectioning. This phenomenon has been described in detail elsewhere (Edelmann & Ruf, 1996).

3.4. FD and chemical fixation during or after LTE

Stable wet-cut sections of low temperature embedded specimens could be obtained by using different methods: (a) mild chemical fixation before cryofixation (blood platelet, Fig. 6(B)); (b) embedding in a Lowicryl K11M-HM20 mixture supplemented with 0.3% uranyl acetate (muscle Fig. 2(C), blood platelet Fig. 6(C), Jurkat cells Fig. 8(B) and (C)); (c) osmication of polymerized Lowicryl blocks (liver Fig. 3(B), RBCs Fig. 7(B)). For methods (b) and (c) see Edelmann & Ruf (1996).

3.5. FD of gelatine gels

Extraction and deformation phenomena obtained after wet cutting of resin-embedded freeze-dried gels of gelatine are shown in Fig. 9(A) and (C). Stable sections were obtained when the gels were mildly fixed before cryofixation (Fig. 9(B) and (D)).

4. Discussion

4.1. The preservation of cellular ultrastructure: conclusions from presented and earlier published FD results

Freeze drying (FD) is the dehydration of a frozen aqueous material through sublimation of ice. The theoretical virtue of this dehydration method is the maintenance of the distribution of the dry material as it is captured in the frozen specimen; of course this holds strictly true only if all components of the dry material are somehow mutually linked before and/or after dehydration. With the development of cryofixation techniques

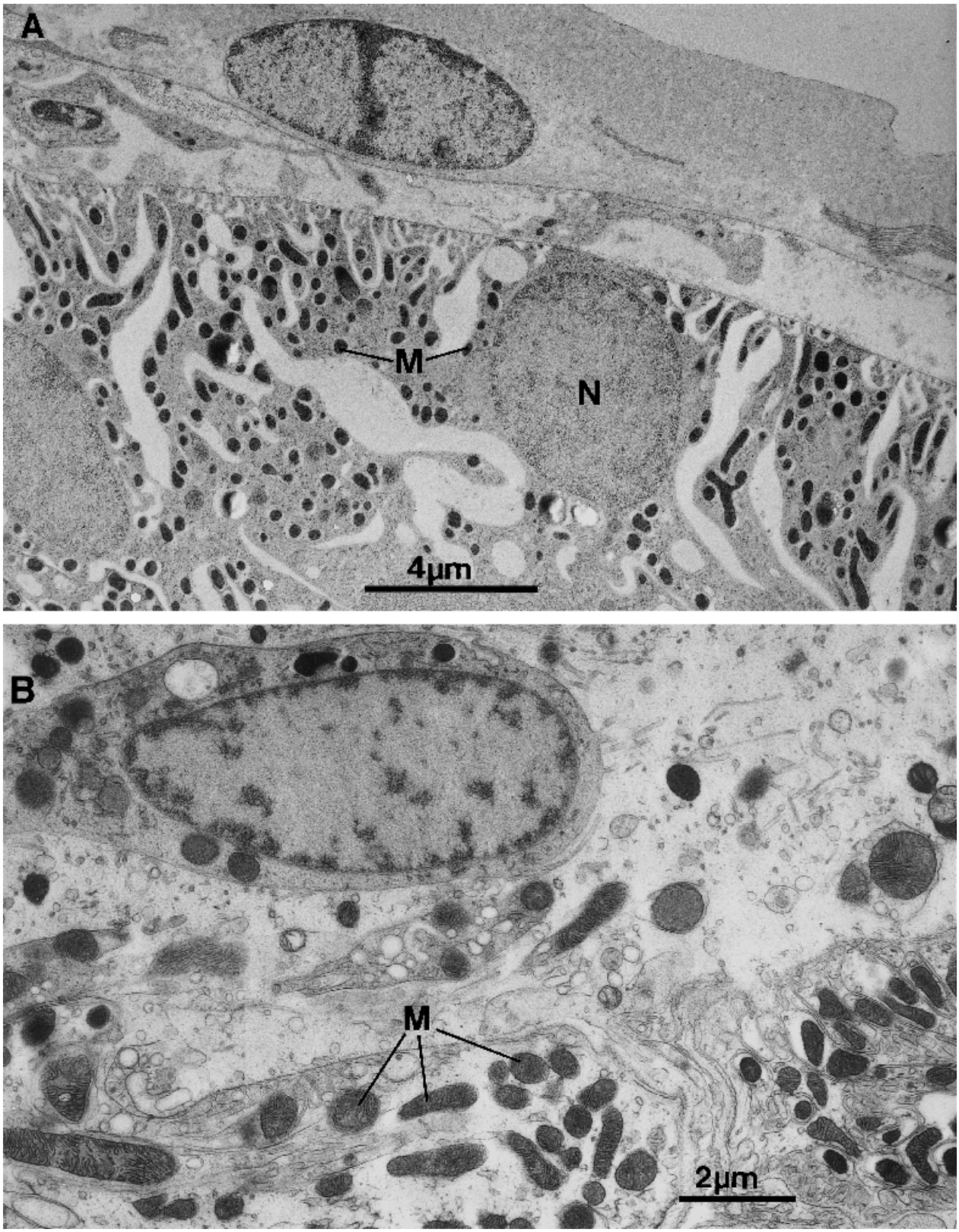

Fig. 1. Rat kidney, cryofixed (MM80), freeze-dried and embedded in Spurr's resin. FD: temperature increase 1 °C h^{-1} from −125 °C to −50 °C (75 h), 90 h at −50 °C, temperature increase 1 °C h^{-1} from −50 °C to + 25 °C (25 h), 24 h at +25 °C (total time: 214 h ≈ 9 days). (A) without chemical fixation, N: nucleus; (B) with OsO_4 vapour fixation after drying, M: mitochondria.

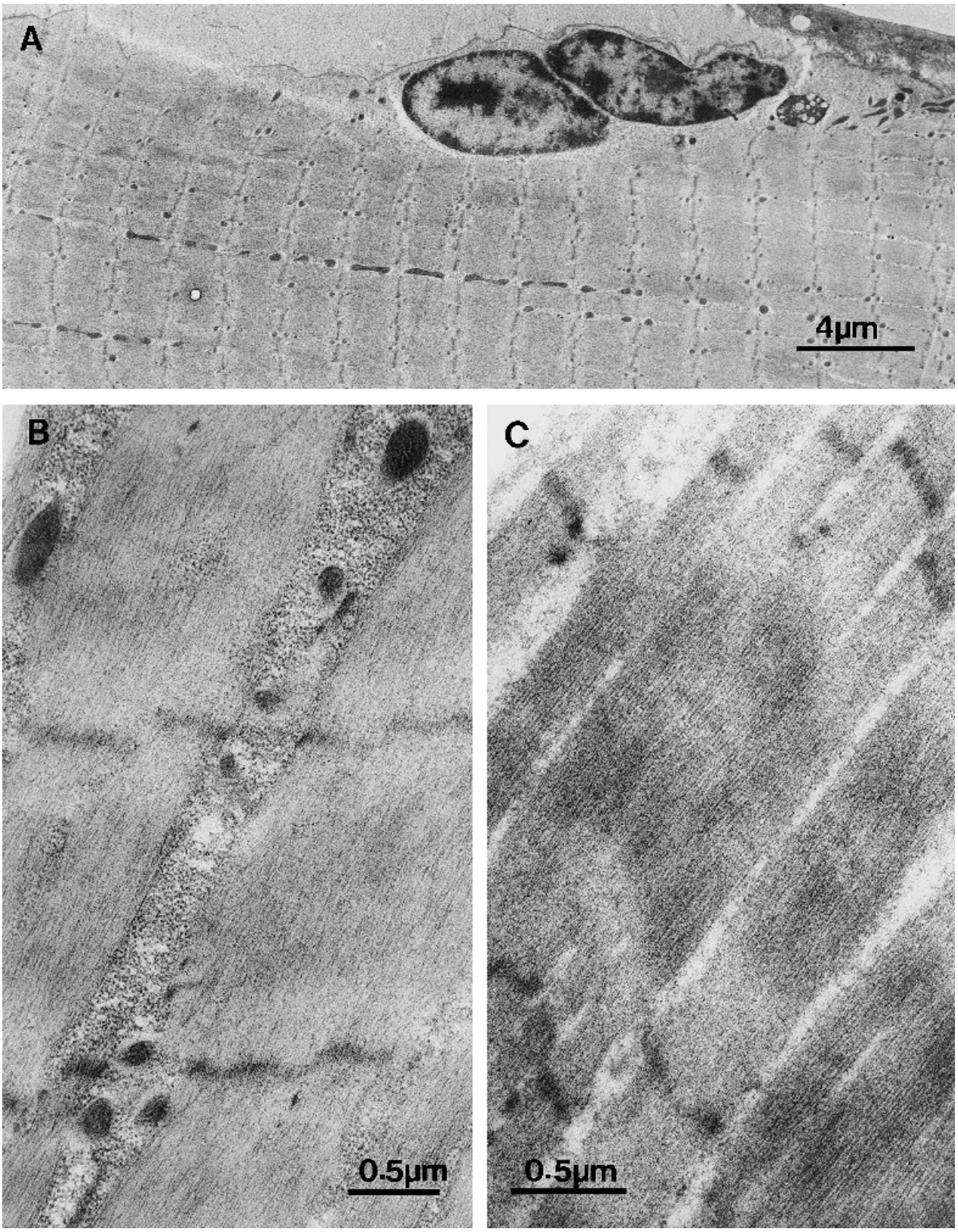

Fig. 2. Human muscle (*M. vastus lateralis*), fine needle biopsy, high pressure frozen (HPM 010, courtesy of H. Hohenberg), freeze-dried and resin embedded. FD (A, B): temperature increase 10 °C h^{-1} from –160 °C to –90 °C (7 h), 32 h–90 °C, temperature increase 0.3 °C h^{-1} from –90 °C to 0 °C (300 h), 48 h 0 °C (total time: 387 h ≈ 16 days). (A, B) embedded in Spurr's resin, (C) embedded in Lowicryl HM20/K11M supplemented with 0.3% uranyl acetate.

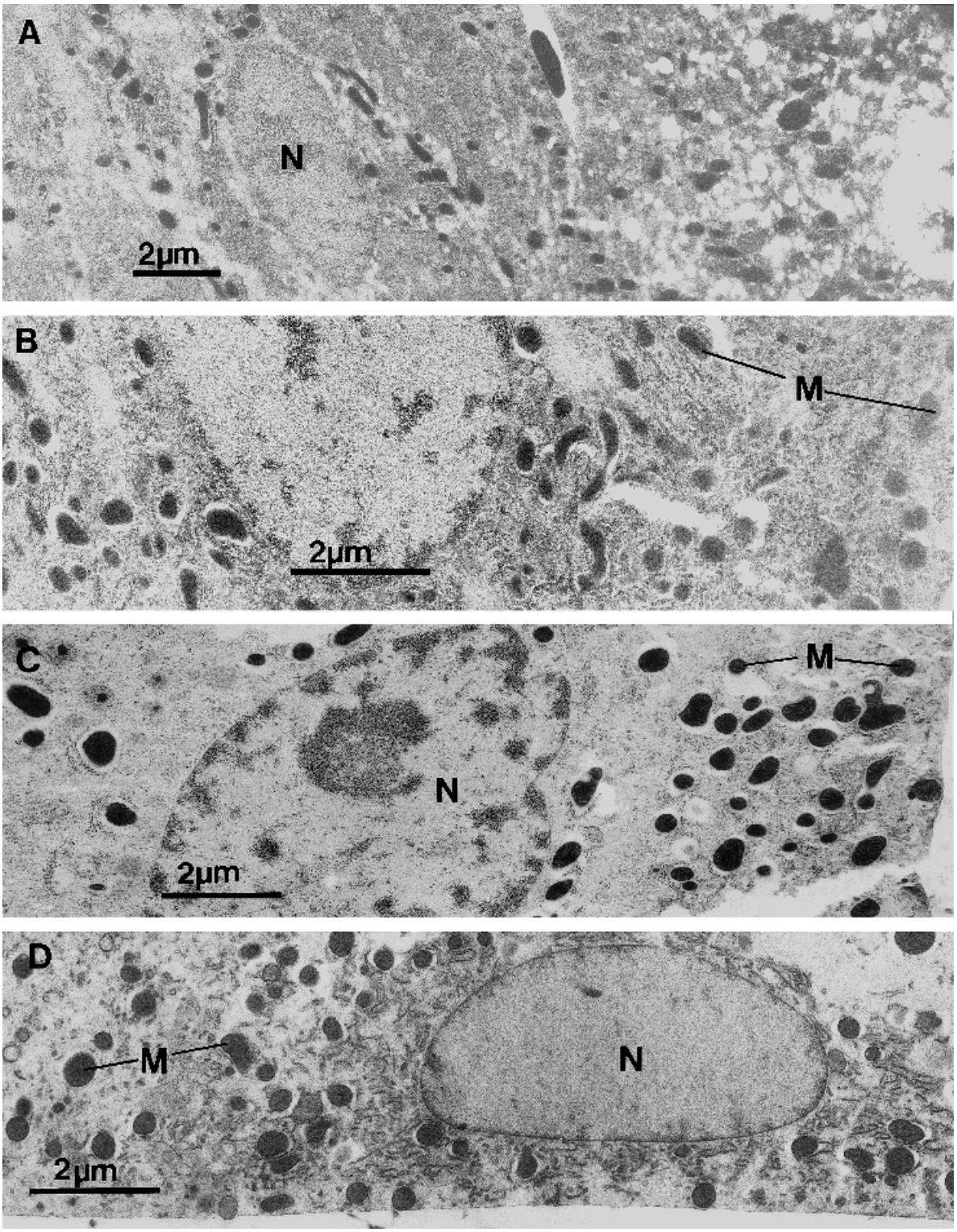

Fig. 3. Rat liver, cryofixed (MM80), freeze-dried and resin embedded. FD (A, B): temperature increase 0.33 °C h^{-1} from −90 °C to −10 °C (240 h), 24 h at −10 °C (total time: 264 h = 11 days). (A) Embedded in Lowicryl HM20; (B) embedded in Lowicryl HM20, osmication of the polymerized block. Note the differences in structure preservation (see text) on the right sides (near the freezing planes) of (A) and (B). FD (C, D): temperature increase 0.2 °C h^{-1} from −90 °C to −30 °C (300 h), temperature increase 1 °C h^{-1} from −30 °C to −10 °C (20 h), 10 h at −10 °C (total time: 330 h ≈ 14 days). (C) No chemical fixation, embedded in Spurr's resin. (D) Osmium vapour fixation after drying, embedded in Spurr's resin. Freezing plane of (C) right side, (D) bottom line. N: nucleus, M: mitochondria.

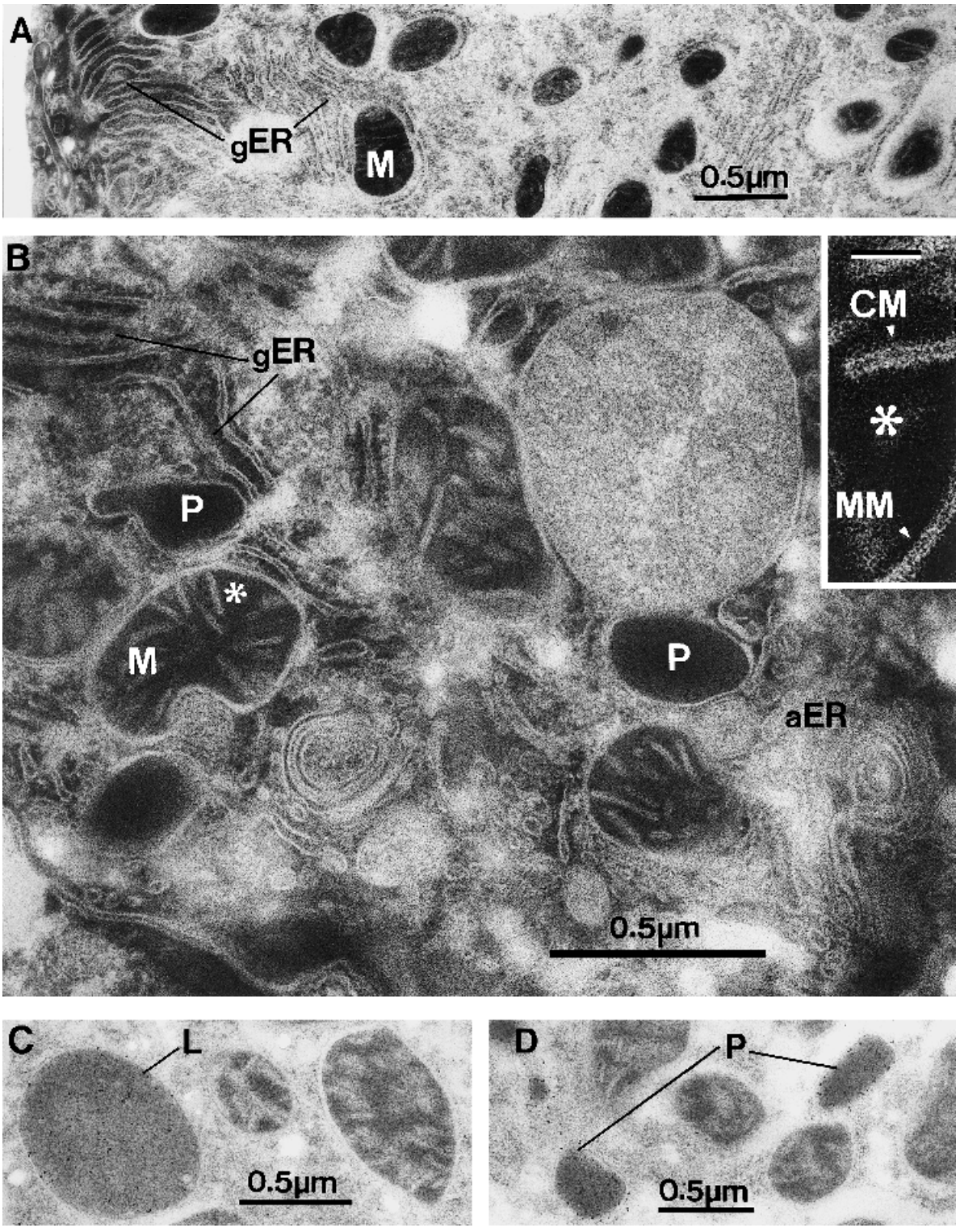

Fig. 4. Rat liver, cryofixed (MM80), freeze-dried and embedded in Spurr's resin (no chemical fixative). FD as in Fig. 3(C) and (D). Left side of (A) is near the freezing plane (area of best cryofixation), (B) is taken from the area of best cryofixation. aER: agranular endoplasmic reticulum, gER: granular endoplasmic reticulum, M: mitochondrion, P: peroxisome. The area around the asterisk (*) in the mitochondrion (M) is shown at higher magnification in the inset: membranes are orientated perpendicular to the plane of the section. The two crista membranes (CM) are thicker than the outer and the inner membrane (MM) of the mitochondrion. Scale bar: 50 nm. (C) Immunogold labelling of SOD in a lysosome (L) (see Liou *et al.*, 1993). (D) Immunogold labelling of catalase in peroxisomes (P).

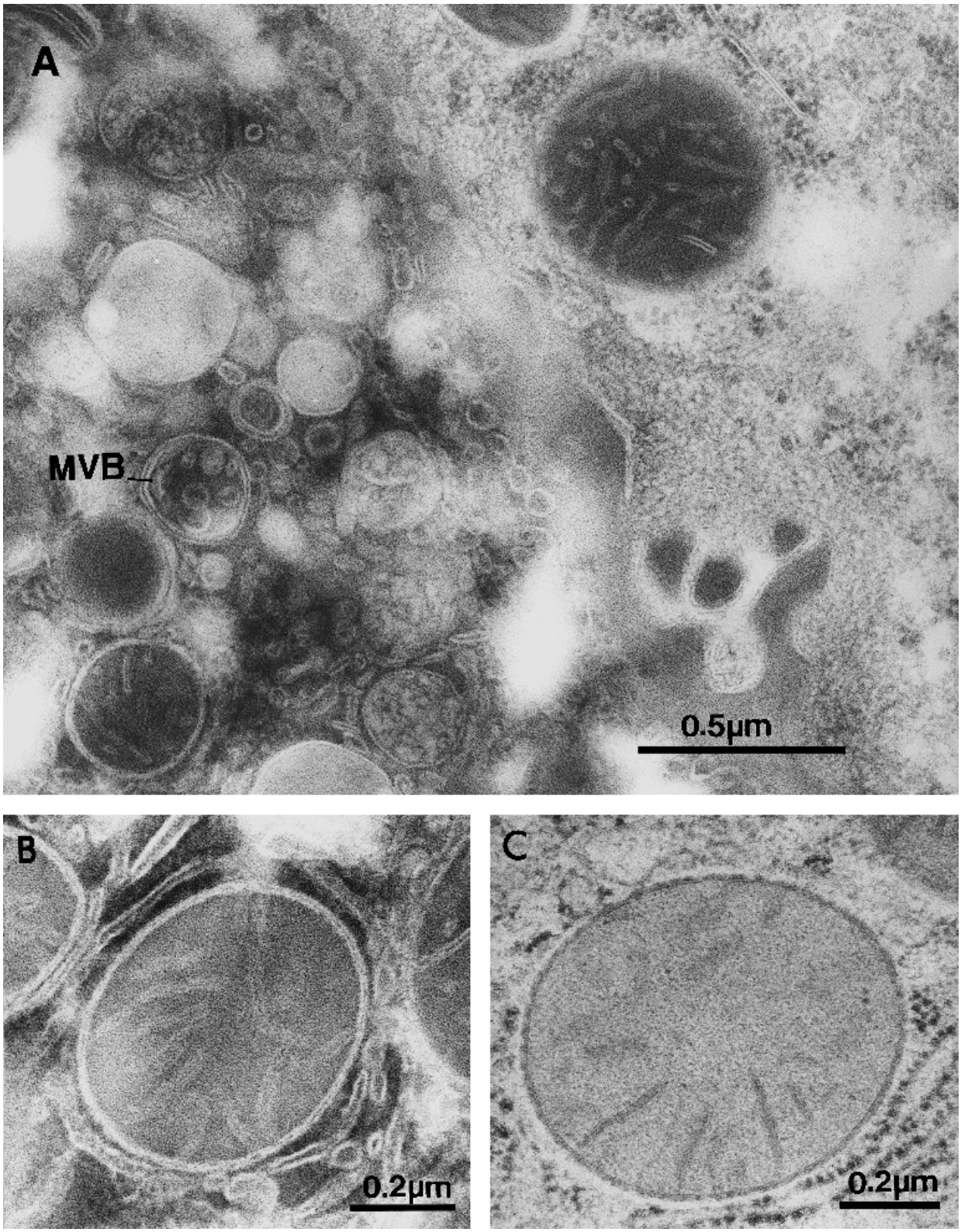

Fig. 5. Rat liver, high pressure frozen (EMPACT, courtesy of D. Studer) (A, B) freeze-dried and embedded in EPON. FD: 24 h at −90 °C, temperature increase 0.4 °C h^{-1} from −90 °C to −50 °C (100 h), temperature increase 1 °C h^{-1} from −50 °C to −10 °C (40 h), 48 h at −10 °C (total time: 212 h ≈ 9 days). (C) Freeze substituted (acetone + OsO_4), and embedded in EPON as described by Studer *et al.*, 2001, specimen courtesy of D. Studer. Note the different aspects of mitochondria and surrounding structures obtained with the different dehydration techniques FD (B) and FS (C). MVB: multivesicular body.

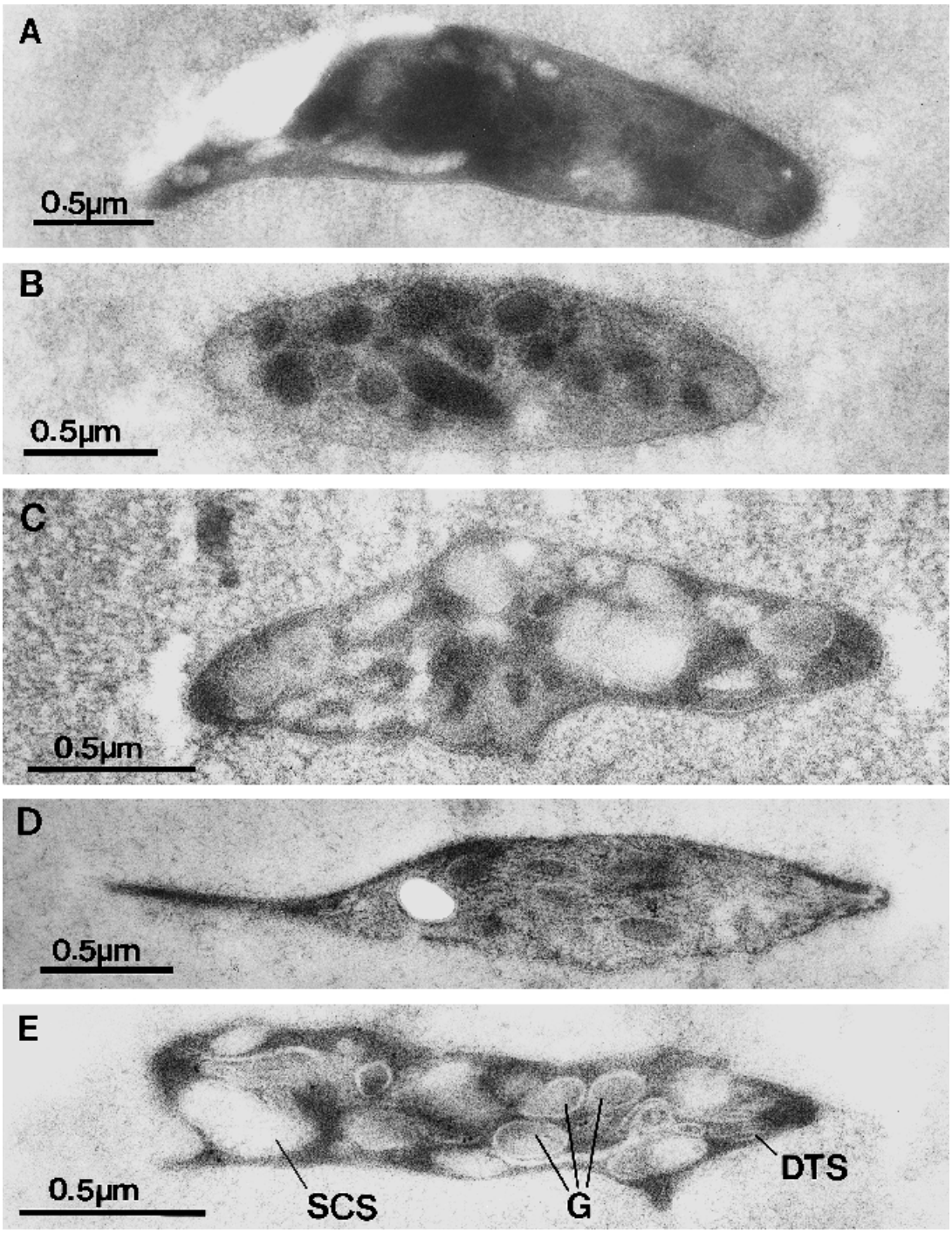

Fig. 6. Human platelets, cryofixed (MM80), freeze-dried and resin embedded. FD (A, D, E): temperature increase 50 °C h^{-1} from –140 °C to –90 °C (1 h), temperature increase 0.27 °C from –90 °C to –50 °C (150 h), temperature increase 1 °C h^{-1} from –50 °C to 0 °C (50 h), 24 h at 0 °C (total time: 225 h ≈ 9.5 days). FD (B): temperature increase 10 °C h^{-1} from –160 °C to –100 °C (6 h), temperature increase 0.33 °C h^{-1} from –100 °C to 0 °C (300 h), 24 h 0 °C (total time: 330 h ≈ 14 days). FD (C): temperature increase 10 °C h^{-1} from –160 °C to –90 °C (7 h), 32 h at –90 °C, temperature increase 0.3 °C h^{-1} from –90 °C to 0 °C (300 h), 48 h at 0 °C (total time: 387 h ≈ 16 days). (A, B) Embedded in Lowicryl HM20, (A) without chemical fixation, (B) after mild chemical fixation (3% paraformaldehyde, 0.2% glutaraldehyde in 0.1 M cacodylate buffer for 1 h) before cryofixation. (C) Embedded in Lowicryl HM20/K11M supplemented with 0.3% uranyl acetate. (D, E) Embedded in Spurr's resin. The distance of the platelet shown in (D) from the freezing plane is about 10 µm, whereas all other platelets stem from areas distant from the freezing plane less than 5 µm. (E) Immunolabelled (black dots: 10 nm gold) for CLP-36, a protein associated with actinin (Bauer *et al.*, 2000). SCS: surface connected membrane system, G: secretory storage organelles, DTS: dense tubular system = agranular endoplasmic reticulum.

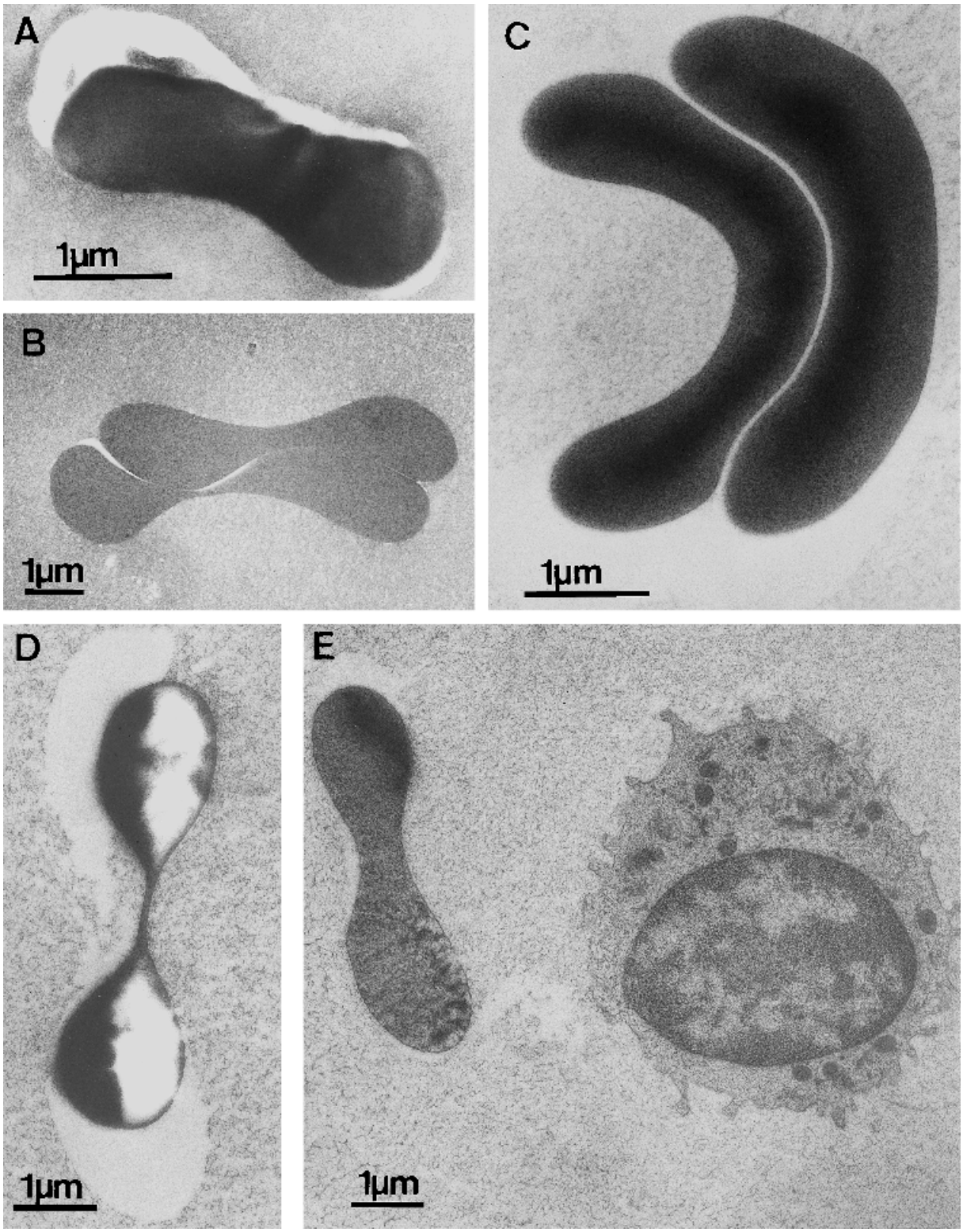

Fig. 7. Human red blood cells, cryofixed (MM80), freeze-dried and resin embedded. FD (A, B, C): temperature increase 10 °C h^{-1} from −150 °C to −100 °C (5 h), 0.33 °C h^{-1} from −100 °C to 0 °C (300 h), 48 h at 0 °C (total time: 353 h ≈ 15 days). (A, B) Embedded in Lowicryl HM20, (B) after osmication of the polymerized block. (C) Embedded in Spurr's resin. Note the differential shrinkage of the cells in (C) that have been freeze-dried as the cells in (B). Only the cells in (C) are shrunken relative to the surrounding plasma. FD (D, E): temperature increase 1 °C h^{-1} from −140 °C to −70 °C (70 h), temperature increase 10 °C h^{-1} from −70 °C to 25 °C (9.5 h), 48 h at +25 °C (total time: 127.5 h ≈ 5.3 d). Note the quality of preservation of the leucocyte in (E).

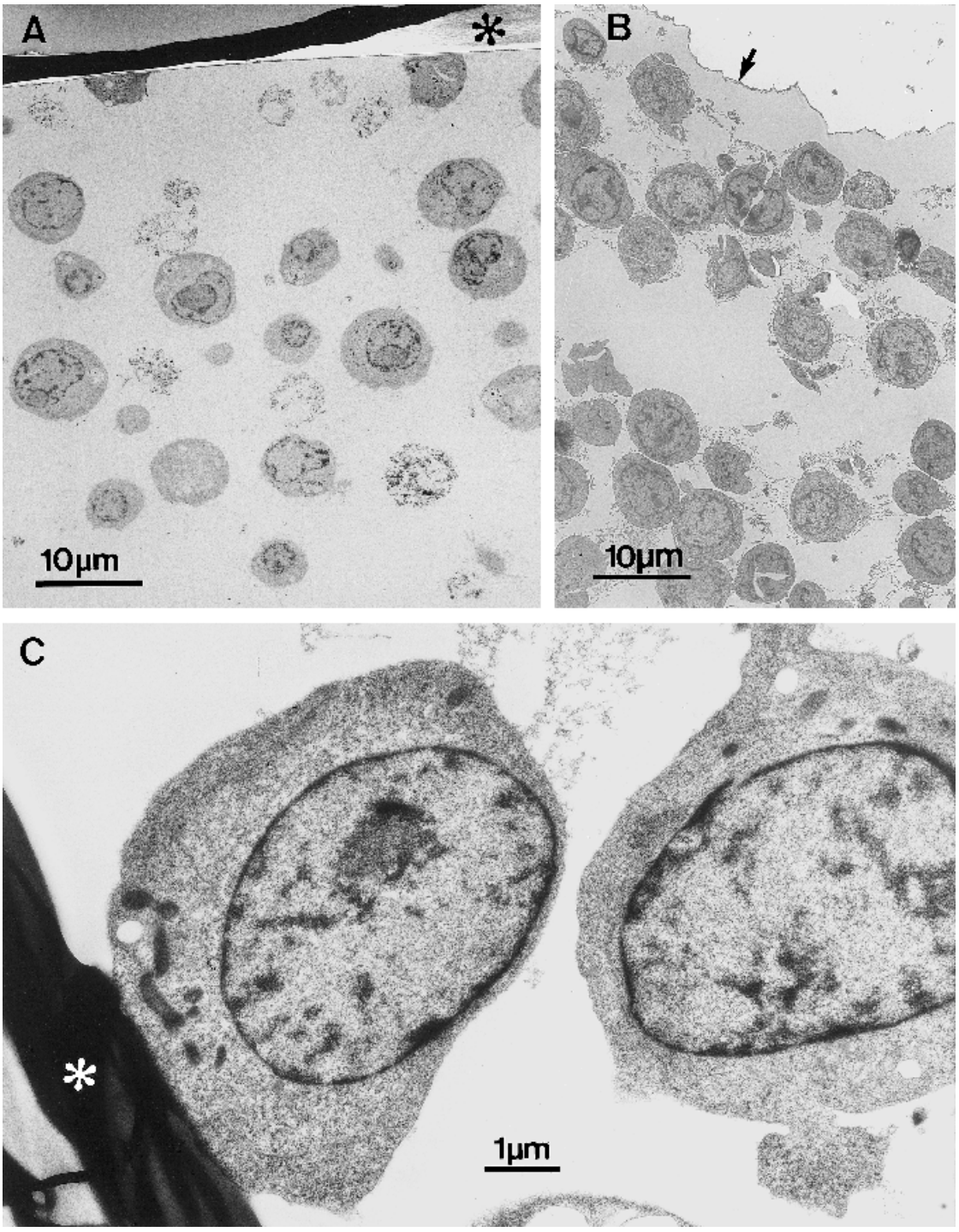

Fig. 8. Jurkat cells high pressure frozen (HPF), freeze-dried and resin embedded. (A, C) Frozen in cellulose microcapillaries (HPM 010). (B) Frozen in copper tubes (EMPACT). (A) HPF courtesy of P. Monaghan, (B) HPF courtesy of D. Studer, (C) HPF courtesy of H. Hohenberg. FD (A) temperature increase 25 °C h^{-1} from −140 °C to −90 °C (2 h), temperature increase 0.2 °C h^{-1} from −90 °C to −65 °C (125 h), temperature increase 0.5 °C h^{-1} from −65 °C to 0 °C (32.5 h), 24 h at 0 °C (total time: 183.5 h ≈ 7.7 days). FD (B) 24 h at −90 °C, temperature increase 0.4 °C h^{-1} from −90 °C to −50 °C (100 h), temperature increase 1 °C h^{-1} from −50 °C to −10 °C (40 h), 48 h at −10 °C (total time: 212 h ≈ 9 days). FD (C): temperature increase 10 °C h^{-1} from −160 °C to −90 °C (7 h), 32 h at −90 °C, temperature increase 0.3 °C h^{-1} from −90 °C to 0 °C (300 h), 48 h at 0 °C (total time: 387 h ≈ 16 d). (A) embedded in Spurr's resin, (B, C) embedded in Lowicryl HM20/K11M supplemented with 0.3% uranyl acetate. The asterisks (*) in (A) and (C) represent the cellulose of the used microcapillaries. The arrow in (B) points to the inner surface profile of the used copper tube. The Jurkat cells shown in (B) and (C) were kindly provided by Professor M. Hoth, Homburg/Saar.

it became evident that biological material can be frozen in such a way that the cellular structure is well preserved. In the best case, the cellular water can be vitrified, which means that it is solidified without allowing it to crystallise before cryofixation is completed (Dubochet *et al.*, 1987). As a result, structural and redistribution artefacts are minimized in the vitrified area. Frozen-hydrated cryosections of vitrified biological material reveal a very uniform, homogeneous distribution of cellular and extracellular macromolecules under the cryo TEM. The central question is now: what conditions are necessary to dehydrate the frozen material by FD in such a way that artificial changes are minimized?

During FD a process may occur that is similar to the one proposed by Kellenberger *et al.* (1986; p. 11) for the stabilization of cryofixed biological material during freeze-substitution (FS). Upon slow removal of water molecules at low temperature, certain sites of macromolecules are liberated first, which may combine with sites of opposite polarity thereby stabilizing the system. Further slow dehydration causes more cross-links step by step, hence more stability. Compared to FS, the FD process has an important advantage: during dehydration there is no interaction between cellular components and a liquid organic solvent that may cause dissolution or extraction of certain components or a change in the physicochemical properties of macromolecules. Structural changes and redistribution artefacts are only dependent on the removal of water molecules. Therefore, one may expect less redistribution of mobile macromolecules and ions which bind at low temperature to liberated free sites of neighbouring macromolecules or whose former weak binding (mobile adsorption) is increasingly strengthened upon stepwise dehydration at low temperature. Whether this optimistic view is compatible with presented and earlier published FD results is answered in the discussion of the following interconnected problems: the problem of water removal from cryofixed biological material by FD and problems of shrinkage and redistribution of cellular components.

4.1.1. Dehydration by FD and morphological results

The problem of water removal from biological material by FD has been reviewed and discussed by several authors (e.g. Robards & Sleytr, 1985; Edelmann, 1986b; Steinbrecht & Müller, 1987; Echlin, 1992). Assuming optimum FD conditions (vacuum and cold trap position, Umrath, 1977), the sublimation rate from cryofixed biological material is dependent on the temperature of the specimen, the structure of the frozen cellular water and the structure of mechanical barriers (e.g. cell walls, membranes, subcellular compartments). In the literature both long and short FD times have been proposed to yield good morphological results at electron microscope magnifications. Linner *et al.* (1986) obtained well preserved preparations with the following FD procedure, named molecular distillation drying (MDD). FD is carried out between −140 °C and −70 °C with a temperature increase of 1 °C h^{-1}. After heating the specimens to room temperature they are exposed to OsO_4 vapour and embedded in Spurr's resin. It was argued that short FD is possible because the vitrified water of well cryofixed biological material is probably sublimated much faster than predicted for hexagonal ice (for critique see Elder *et al.*, 1992). On the other hand, our own experiments carried out with the striated muscle of the frog led to the following conclusion (Edelmann, 1978, 1986b). The best structural preservation of chemically unfixed muscle, freeze-dried and embedded in Spurr's resin, is obtained by long FD at low temperature (e.g. 3 days at −80 °C, 6 days at −60 °C). By comparing these drying times of the 0.3 mm thick muscle specimens used with drying times of pure ice (see Umrath, 1983; Edelmann, 1986b) a prolongation factor (sublimation rate of the cellular ice divided by the sublimation rate of pure ice) of about 1000 can be estimated. In addition, it was found that shrinkage during FD of 100–200 nm thick frozen-hydrated cryosections can be minimized and the best structure preservation is obtained after similarly prolonged FD at low temperature either in a cryo TEM or in the CFD (Sitte *et al.*, 1994; Edelmann, 1994a). From these results, one may conclude that the 'safe' removal of the water molecules by FD is mainly dependent on the water structure as it is found in the frozen specimen. Cellular and subcellular compartments are cut open, hence mechanical barriers should not play a major role when drying ultrathin frozen-hydrated sections.

In the present study, FD procedures of different lengths have been used, including the short MDD procedure. The main conclusion derived from the results of these widely different experiments is surprising: water removal from frozen native biological material by FD depends on the quality of cryofixation; the dehydration process is slowed down as the cryofixation is improved. The results pointing to this phenomenon are discussed in the following:

1 As a rule, biological tissue that was not submitted to chemical fixation was well preserved after rather long FD at low temperature and embedding in Spurr's resin. However, qualitatively different aspects have been obtained (see, e.g. right and left sides of Figs 4(A) and 5(A)). The new state of preservation that is obtained only in the areas of best cryofixation (most likely: vitrified areas) suggests an unusual high retention of hydration water around macromolecules – despite prolonged FD at rather high temperature (Figs 4(A), (B), 5(A), (B) and 6(E)). It appears that the original architecture of homogeneous plasma phases and of membrane systems is stabilized without visible collapse or aggregation artefacts by the retention of a considerable amount of water molecules.

A phenomenon of insufficient drying – especially pronounced with RBCs – is exemplified in Fig. 7(C). The cryofixed cells have been freeze-dried for a rather long time even at a rather high temperature (similar to those shown in Fig. 7(B)). The shrinkage relative to the surrounding extracellular plasma

(not yet visible in Fig. 7(B)) must have occurred during infiltration and polymerization in Spurr's resin. Apparently, the water remaining after prolonged FD in the RBC – preventing good polymerization in Lowicryl – was at least partly removed from the cellular macromolecules during embedding in the epoxy resin, resulting in a (not necessarily uniform) shrinkage of the RBC. The differential shrinkage of mitochondria in well cryofixed liver areas (Fig. 3C) may be interpreted the same way. Mitochondria were less dry than the surrounding cytoplasm; during infiltration and polymerization in Spurr's resin the mitochondria were further dehydrated and shrunk more, the more water was left after FD.

2 The phenomenon of reduced dehydration of cells in well cryofixed areas can also be deduced from results obtained with the MDD procedure (including Os vapour stabilization). Figure 7(D) shows a considerable shrinkage of a well cryofixed RBC. In addition, cellular material was extracted from this cell during wet cutting, indicating insufficient dehydration before resin embedding. In contrast, a RBC damaged by ice crystal formation was apparently well dehydrated. It showed much less differential shrinkage relative to the surrounding plasma and was well embedded in the resin (Fig. 7E). Furthermore, the phenomenon of differential shrinkage of mitochondria (halo formation) in well cryofixed areas is a common feature of the MDD procedure (Linner *et al*., 1986, e.g. Fig. 11). Because – after FS – such a differential shrinkage rarely occurs (e.g. Sitte *et al*., 1987; Fig. 6) it was concluded that halos around well cryofixed mitochondria in freeze-dried preparations may reflect incomplete drying of the mitochondria at low temperature (see Edelmann, 1994a; discussion with reviewers). Prolonged FD at low temperature should reduce this artefact. Figures 1(B) and 3(D) show preparations obtained after rather long FD followed by OsO_4 vapour fixation and subsequent embedding in Spurr's resin: as expected, no halos or only small ones around mitochondria are visible.

3 Freeze-dried biological material not treated with a chemical fixative was not uniformly stabilized after LTE in Lowicryl. Wet-cut sections of freeze-dried liver showed severe extraction artefacts in the area of best cryofixation (Fig. 3(A)). Such a phenomenon has been observed earlier with freeze-dried leucocytes and it is also shown here with freeze-dried blood platelets (Fig. 6A) and RBCs (Fig. 7A). The well cryofixed cells were not stabilized enough in the polymerized blocks and therefore it was very difficult to obtain even the sections for the figures shown. It was necessary to produce rather thick (150 nm) wet-cut sections at high cutting speed (10 mm s^{-1}); at a section thickness of about 60 nm most of the cellular material was lost from the section. Apparently, remaining hydration shells around macromolecules or organelles prevented perfect cross-linking during embedding (Kellenberger, 1987). It is noteworthy that mildly chemically fixed and freeze-dried cells were well cross-linked in the low temperature embedded Lowicryl block (Fig. 6(B)). It is concluded that fewer hydration shells are found in a chemically fixed preparation than in a native one.

4 Results of freeze-dried and embedded 20% gelatine gels – either not treated with fixatives or mildly fixed – resemble those of freeze-dried embedded biological material: in the area of best cryofixation the untreated gel was labile after LTE in Lowicryl and after embedding in Spurr's resin. Extraction artefacts shown in the wet-cut Lowicryl section (Fig. 9(A)) resemble those of sections of Lowicryl embedded liver (Fig. 3(A)) or leucocytes (Edelmann & Ruf, 1996). By contrast, the chemically fixed gels were apparently evenly freeze-dried and stabilized in the polymerized blocks (Fig. 9(B) and (D)). It is concluded that chemically fixed gels were dehydrated to a higher degree than gels not treated with a fixative.

The findings obtained with biological material and gelatine gels may be interpreted as follows. In living cells and in the chemically unfixed gel, considerable amounts of water dipoles are influenced and ordered by certain macromolecules maintained in specific conformations (Kellenberger, 1987: hydration shells; Ling, 1965, 1992; Ling *et al*., 1993: polarized multilayers of water). In vitrified parts this order of water molecules may be preserved to a higher degree than in areas that contain ice crystals (cubic or hexagonal ice). In chemically fixed preparations the physicochemical properties of the macromolecules are modified in such a way that the order of hydration shells is greatly distorted and a greater quantity of less organized water is present in the fixed preparation. By assuming lower sublimation rates of vitrified more ordered water compared to those of vitrified less ordered water and also compared to those of crystalline ice (cubic or hexagonal), the above discussed results can be explained in a consistent manner. In order to test this interpretation the gelatine experiments have been performed. It is well known that gelatine macromolecules in aqueous solutions exhibit an unusually intense interaction with the surrounding water molecules (Kunitz, 1927). An exhaustively dialysed 20% gelatine solution with a protein concentration of a few mM exhibits an osmotic activity of about 200 mOsm, which increases to about 2240 mOsm in a 50% gelatine solution (Ling, 1983). The high osmolality (or low water activity) must be due to the water ordering effect of the gelatine macromolecules. If this water ordering effect persists during and after vitrification by means of cryofixation, the sublimation rates of the 'structured' water should be reduced compared to that of less ordered water. The experiments confirm this expectation. Extraction artefacts seen in sections of freeze-dried and embedded gelatine gels (Fig. 9(A) and (C)) point to water retention in the best cryofixed parts of the specimens (osmolality of the gels: 200 mOsm, measured with a Wescor vapor pressure osmometer, model 5500). However, the chemically fixed gels (osmolality of the gels not

exceeding those of the used buffer solution: 145 mOsm) are sufficiently dried – even near the freezing plane – and stabilized after FD and embedding (Fig. 7(B) and (D)). By comparing these results with those obtained with cell suspensions in blood plasma (e.g. Figs 6(A) and 7(A)) it can be noted that the extracellular areas are well stabilized despite an osmolality of about 300 mOsm of the plasma. Therefore, the osmolality *per se* is not the reason for the incomplete dehydration of the freeze-dried gelatine preparations shown in Fig. 9(A) and (C).

4.1.2. Redistribution of mobile macromolecules and mobile ions during FD

It is well known that the living cell is a highly dynamic aqueous system in which not only the water molecules but also many macromolecules and, in particular, most cellular ions (e.g. K^+) are highly mobile. As a rule, however, the macromolecules are densely packed, i.e. each macromolecule is surrounded by other macromolecules. Once the whole system is immobilized by cryofixation and slowly dehydrated at low temperature in such a way that an almost uniform dense network of 'dry' macromolecules is left, it appears unlikely that macromolecules move out of their original place – even at higher temperatures and even during resin-embedding. The experiments of Gingras & Bendayan (1994) support this view. In a detailed study they evaluated the distribution of amylase in zymogen granules of pancreas after different preparation procedures. Immunogold-labelled sections of well preserved freeze-dried and embedded tissue (according to the MDD protocol) showed that many secretory granules displayed a peculiar pattern: the antigenic sites were concentrated in some areas, leaving others devoid of labelling. Serial sections and double labelling experiments were performed for further evaluation of the results and for assessing artificial displacement of proteins during tissue preparation. It was concluded that 'the secretory proteins are segregated within the zymogen granule – which appears thus a well organized structure'. This finding was expected from earlier independent studies but it could only be detected in freeze-dried embedded preparations. All other procedures tested by Gingras & Bendayan (1994), and cryofixation followed by freeze-substitution (Ichikawa *et al.*, 1987), yielded preparations in which the amylase was homogeneously distributed in the granules.

A most difficult problem in ultrastructure research is the correct evaluation of the intracellular distribution of the main cellular cation K^+. According to the current view, most of the mobile cellular K^+ ions are freely dissolved in free cellular water – hence they should follow the cellular water distribution. On the other hand, it has been postulated (Ling & Ochsenfeld, 1966) that the bulk of cellular K^+ is weakly adsorbed (not completely immobilized) to β- and γ-carboxyl groups of cellular proteins; the K^+ distribution should follow the distribution of these side chains. The divergent views have been tested with normal K^+-containing frog muscles and living muscles in which most of the cellular K^+ was (reversibly) replaced in a mole-for-mole fashion with the K^+ surrogates Rb^+, Cs^+ or Tl^+ (Ling, 1977). It could be shown by FD and embedding and by investigating frozen-hydrated preparations that the ions are preferentially localized at proteins rich in β- and γ-carboxyl groups (e.g. myosin) and that they do not follow the water distribution (Edelmann, 1977, 1980a, 1984, 1986a, 1988, 2001). This phenomenon could be demonstrated most clearly by TEM. Dry-cut sections of freeze-dried and resin-embedded muscles (preserved like those shown in Fig. 2) and frozen-hydrated cryosections showed similar distribution patterns of the electron-dense Cs^+ and/or Tl^+ ions (Edelmann, 1988).

In summarizing this section it is concluded that cellular mobile macromolecules and mobile ions can be stabilized by FD and embedding close to that subcellular place they occupy in the living cell. A precondition is an optimized structure preservation, which can be obtained by prolonged FD at low temperature (see Results). It must be emphasized that insufficient drying at low temperature may result in great shrinkage and subcellular redistribution of macromolecules and ions (Edelmann, 1994a).

4.1.3. The problem of seeing the structure of living cells by TEM

With the development of cryofixation and cryosectioning techniques, and of cryo-electron microscopy it became possible to visualize the ice-embedded structure of living cells in frozen-hydrated cryosections by TEM. Micrographs of *Zea mays* meristem cells prepared by: (1) conventional resin-embedding and sectioning; (2) LTE and sectioning of freeze-substituted samples; and (3) cryosectioning of vitrified samples have been compared by Dubochet & Sartori Blanc (2001). The most interesting finding was that both methods (1) and (2) produced aggregation and redistribution artefacts not observed in the frozen-hydrated cryosection. In the latter method the major cellular and extracellular phases appeared with a very fine granularity like thick soups of homogeneously distributed particles occupying all the available space. In addition, a surprisingly rich variety of vesicles of various size and content were identified in the frozen-hydrated cryosection but not in the two other preparations.

This paper shows for the first time dehydrated and embedded biological material (Figs 4B, 5A,B and 6E) – not treated with any chemical fixative – that exhibits cellular plasma phases with very fine granularities and a rich variety of intracellular membrane systems. It is assumed that the original structure of membranes and the original structure of homogeneous plasma phases are preserved because the water molecules necessary to stabilize the native structure of certain macromolecules were not removed. It appears that FD and embedding of native biological material can be carried out in such a way that its structure resembles that of vitreous preparations or its living state, respectively. A similar conclusion follows from the similarity between the visualized pattern of ion distribution in

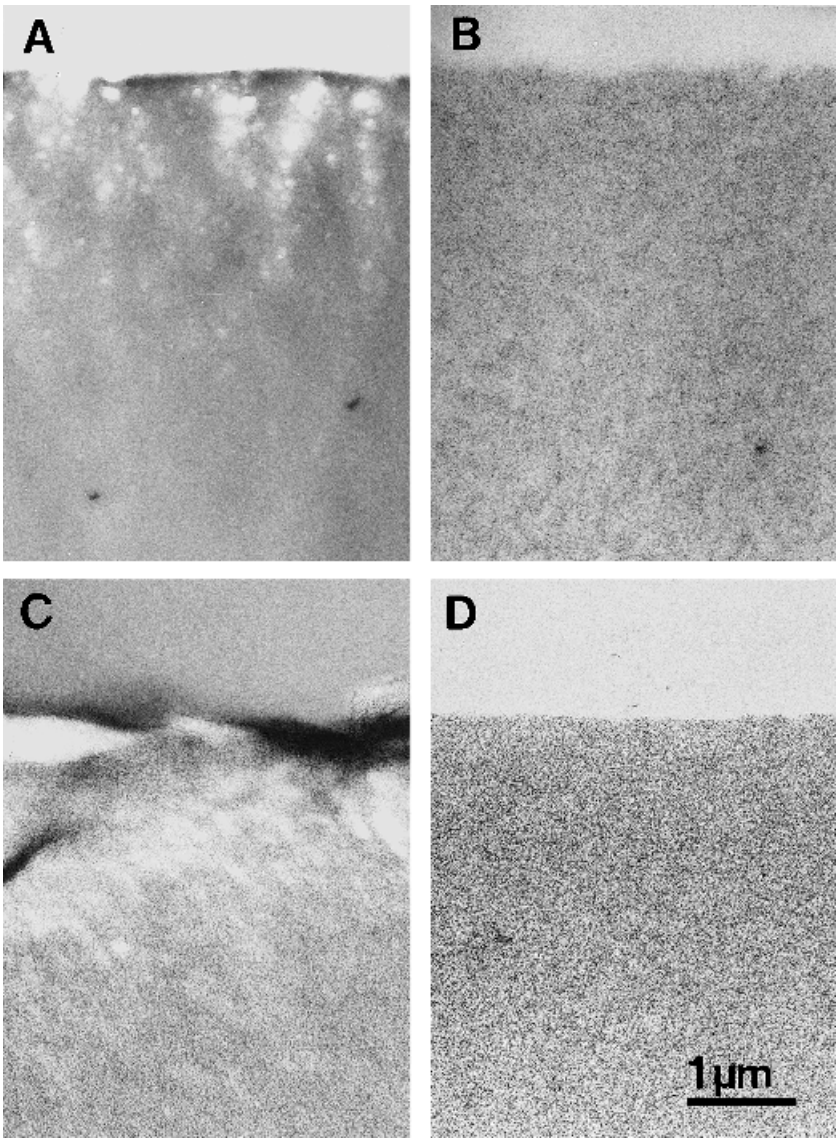

Fig. 9. Gelatine gels (20% gelatine, 80% H_2O) cryofixed (MM80), freeze-dried and resin embedded; the shown borders represent the freezing planes. FD: temperature increase 10 °C h^{-1} from −160 °C to −100 °C (6 h), temperature increase 0.33 °C from −100 °C to 0 °C (300 h), 24 h at 0 °C (total time: 330 h ≈ 14 days). (A) Without chemical fixation, embedded in Lowicryl HM20 (no staining) the gelatine is not well stabilized in the resin. The upper part of the section containing only Lowicryl has been separated from the gelatine; extraction artefacts are visible in the area of best cryofixation. (B) Mild chemical fixation before cryofixation, embedded in Lowicryl HM20. (C) Without chemical fixation, embedded in Spurr's resin; distortions are visible in the area of best cryofixation. (D) Mild chemical fixation before cryofixation, embedding in Spurr's resin. (B–D) Stained with uranyl acetate and lead citrate.

frozen-hydrated cryosections and in sections of freeze-dried embedded material (Edelmann, 1988).

4.2. FD for future investigation of biological problems

4.2.1. The technique of freeze-drying and embedding

Compared to the FS and embedding procedure, requiring several changes of dehydrating and embedding liquids, the FD and embedding technique is very simple and requires only minute amounts of a resin. When using the CFD the most difficult step is to place small well cryofixed specimens into the moulds of an object table. Afterwards the object table is transferred into the FD chamber. The program for FD is chosen and commenced after closing and evacuating the chamber. At the end of FD the chamber is opened and the moulds are filled with a resin. After allowing some time for infiltration of the specimen with resin, polymerization at low or high temperature may follow. The more complicated procedure, described in Materials and methods, for embedding the specimens in acrylic resins was chosen because specimens were freeze-dried simultaneously for embedding in Lowicryl and Spurr's resin. In this case only one type of resin can be polymerized in the moulds on the object table of the CFD. When embedding the freeze-dried specimens inside the CFD in an acrylic resin the described change of resin may simply be carried out by removing and adding the liquid with a syringe from and into the moulds. Most biological material does not require vacuum infiltration (Edelmann, 1994a) but in case of infiltration problems (e.g. plant material), a simple technique of vacuum-pressure infiltration described by Fritz (1989) may be used (see Bücking & Heyser, 2001). Loss of single cells, which may happen after FS of cell suspensions with low concentrations of extracellular

macromolecules, is usually not observed after FD and embedding (see Fig. 8).

A universal FD program suitable for all different kinds of biological material cannot yet be given because of the complex problems associated with the sublimation of differently cryofixed cellular water. During the last 5 years many FD procedures have been tried and a selection of good morphological results are presented in this article together with the used FD protocols. Instead of repeating and rating these results in a table with a subjective judgement on the different qualities of structure preservation, it appears to be more helpful to present a summary of important technical hints and typical findings. This summary should be kept in mind when using and further improving the FD technique for electron microscopic studies.

- At least one side of an optimally cryofixed specimen should be as small as possible (e.g. 0.3 mm, see Edelmann, 1994b, discussion with reviewers).
- Long FD times between −90 °C and −50 °C are essential. Prolonged drying below −90 °C does not seem to improve the results. As a rule, culture cells with their high water content are well preserved after rather short (about 1 week) FD procedures (Jurkat cells, Fig. 8(A), see also dendritic cells, Spehner *et al.*, 2002). Tissues such as kidney and liver require longer FD (about 2 weeks) at low temperature for good structure preservation (Figs 1(A) and 4(A)–(D)).
- When using an epoxy resin it is recommended to start infiltration at about −20 °C at atmospheric pressure with a low viscosity component and to replace this liquid with pure resin at room temperature (see Materials and methods).
- Extremely well cryofixed areas of chemically unfixed biological material are less stable in the polymerized block when compared to areas of less well cryofixed areas. Whereas the stability of well cryofixed specimens embedded in Spurr's resin is sufficient for ultrathin sectioning, extraction artefacts are observed after wet cutting of Lowicryl-embedded material. Stabilization of these Lowicryl-embedded specimens is possible by using uranyl acetate in the embedding medium (Fig. 6C) or by osmication of the polymerized block (Fig. 7(B), see also Edelmann & Ruf, 1996). Biological material that has been chemically fixed before cryofixation is well stabilized after freeze-drying and embedding in Spurr's resin or in Lowicryl (Fig. 6B).
- Sections of freeze-dried specimens embedded in Spurr's resin are well suited for immunocytochemical and for microanalytical studies (see sections 4.2.3 and 4.2.4).

The usefulness of freeze-dried and embedded biological material for studies in different fields of ultrastructure research will be discussed next.

4.2.2. Morphological studies

It is now generally accepted that the size of cells and subcellular organelles of chemically unfixed biological material may be well preserved after cryofixation, subsequent FS and resin embedding. Therefore, morphological studies aimed at determining the volume and shape of different compartments – as found in the living cell – may be carried out successfully by using this cryo procedure. However, at the molecular level, results obtained with the FS technique probably represent severe artefacts. Sjöstrand (1990) has convincingly shown that proteins are denatured by substitution media and in particular by the strong denaturation action of OsO_4 when used in a substitution medium. The extensive denaturation of the protein molecules explains why the structure of membranes appears basically the same after FS (e.g. in acetone supplemented with OsO_4) and after the conventional fixation procedure. Sjöstrand (1997) has developed a fixation and embedding procedure leading to resin-embedded biological material with minimized protein denaturation. When using this low denaturation embedding technique the membranes appear in negative contrast, the cytoplasm and the plasma of mitochondria show a very fine granularity. Whereas after conventional preparation and after FS in acetone supplemented with OsO_4 the different mitochondrial membranes all are similarly positively stained showing a thickness of about 5 nm, these membranes appear quite different after low denaturation embedding. The thickness of the outer membrane is about 5 nm, the inner membrane is 7 nm and the cristae membranes are 12.5 nm – a dimension that is required for the membrane to accommodate respiratory chain and citric acid cycle enzyme molecules (Sjöstrand, 1997; p. 162). These membranes are negatively stained because their main components, namely globular proteins, remain unstained in their interior when preserved in their original globular conformation. Note that the positive contrast of membranes seen in conventional and in freeze-substituted (acetone supplemented with OsO_4) preparations does not necessarily reflect the localization of lipids. Fleischer *et al.* (1967) have shown that mitochondria with their normal lipid content and lipid-depleted mitochondria reveal similarly positively stained triple-layered mitochondrial membranes after conventional preparation with OsO_4 treatment.

If one compares the aspect of mitochondria obtained after the low denaturation embedding technique (Sjöstrand, 1997) with that obtained in the best cryofixed area after FD and embedding in Spurr's resin's or Epon, the similarity is striking. The negatively stained membranes of cristae are thicker than the negatively stained outer and inner mitochondrial membranes (see inset of Fig. 4(B)). It is concluded that chemically unfixed biological material may be freeze-dried and resin-embedded with minimized protein denaturation allowing morphological studies similar to those described by Sjöstrand (1997). It should be noted that the procedure proposed by Sjöstrand starts with a short glutaraldehyde fixation – inevitably leading to cellular and subcellular redistribution of ions, water and macromolecules (see, e.g. Morgenstern, 1991) – which has been avoided in the FD embedding

technique described here. Whether further improvement of this technique is possible by choosing other embedding media and other polymerization temperatures remains to be investigated.

4.2.3. Immunocytochemical studies

The purpose of immunocytochemistry is accurately to localize cellular and extracellular antigens with labelled antibodies. Essential preconditions for meaningful immunocytochemical studies are the preservation of antigenicity at the correct place of the tissue ultrastructure and the accessibility of the antigenic site for the applied antibody.

Studies at the light microscopic level demonstrate convincingly that immunocytochemical labelling of fixation sensitive antigens is excellent after cryofixation, freeze-drying and paraffin embedding of tissue that has not been chemically fixed. The morphology is comparable or better than after conventional formalin fixation and paraffin embedding; the antigenicity is similar to that found in cryostat sections, which give poor morphological details (Stein *et al.*, 1984; Onetti-Muda *et al.*, 1991; Onetti-Muda *et al.*, 1992; Onodera *et al.*, 1992).

Numerous immunocytochemical strategies for electron microscopy are well documented in the literature (Skepper, 2000). Fixation-sensitive antigens of biological material may be detected after cryofixation of native material, subsequent FS or FD, resin-embedding and sectioning. Although the possibility of using freeze-dried embedded biological material for immunocytochemical studies is acknowledged in recent review papers (Polak & van Noorden, 1997; Newman & Hobot, 1999; Skepper, 2000) it is not immediately evident that freeze-dried embedded material may be particularly well suited for immunocytochemical studies. Polak & van Noorden (1997) wrote (p. 15): 'The morphological preservation (after FD) is never good enough for electron microscopy. Improvements in antibodies and method sensitivity have meant that routinely fixed tissue has become suitable for most immunocytochemical reactions and FD is not much employed now.' Arguments against using freeze-dried material embedded in epoxides (Araldite, Epon, Spurr's resin) may be deduced from past experience. 'The epoxides' tough, hydrophobic, 3-D cross linking has impeded sensitivity in many modern postembedding applications and made subjective measures such as etching with sodium ethioxide or hydrogen peroxide necessary to reduce the resins' hydrophobia and, for example, expose surface epitopes' (Newman & Hobot, 1999). The reservation against using freeze-dried material in acrylic resins (Lowicryl, LR White/Gold, Unicryl), which are as a rule well suited for immunocytochemical studies, is probably due to the finding that 'the level of structural preservation is not as good as that following cryosubstitution' (Newman & Hobot, 1999).

With the results documented here, the scepticism against using freeze-dried embedded material is no longer justified. Provided that the tissue is well cryofixed, excellent preservation of structure may be obtained after prolonged freeze-drying at low temperature and subsequent resin embedding. Stable well preserved specimens may be obtained after embedding freeze-dried material in Spurr's resin without using any fixative. Effective immunogold labelling of antigens is possible without taking special measures to uncover hidden antigenic sites (Figs 4(C), (D) and 6(E), see also Spehner *et al.*, 2002). Immunolabelling of serial sections of blood platelets prepared as shown in Fig. 6(E), poses no difficulties. This method is currently used for reconstruction of whole platelets and the three-dimensional localization of antigenic sites, as has been done with freeze-substituted platelets (Morgenstern *et al.*, 2001).

A reason for effective labelling could be that the freeze-dried material is not as intensely cross-linked in the used Spurr's resin, as is usually the case after the conventional procedure or even after FS. The remaining water molecules that cannot be removed by FD – even at rather high temperatures – may be responsible for such a phenomenon. Whether Epon or Araldite is equally well suited as Spurr's resin for immunogold labelling of freeze-dried material remains to be investigated.

The problem of stabilizing extremely well frozen material after FD in acrylic resins without using chemical fixatives has not yet been solved (see discussion above). However, resin-embedded material stabilized with low concentrations of fixatives before cryofixation or after freeze-drying may be used for successful immunolabelling. The latter method is most likely better suited to stabilize macromolecules at the original subcellular place, as even the 'mildest' chemical fixation of fresh material causes ion and water redistribution combined with a redistribution of certain macromolecules, in particular of those that are involved in ongoing fusion processes (Morgenstern, 1991).

Immunocytochemical studies with sections of freeze-dried resin-embedded material are highly recommended if heterogeneous distributions of mobile antigens are expected to exist in living cells or subcellular compartments. As shown by Gingras & Bendayan (1994), FD and resin embedding may provide the most reliable results. The findings of Hisano *et al.* (1986) should also be noted, who observed the phenomenon of redistributed antigens in freeze-substituted preparations but not in freeze-dried specimens ('the FD procedure was considered the best').

4.2.4. Microanalytical studies

Detection of chemical elements at the correct subcellular place is a prerequisite for understanding most or probably all cellular functions. In the past, most microanalytical studies have been carried out by X-ray microanalysis of freeze-dried cryosections (Gupta, 1991). The arguments in favour of this method in relation to using sections of resin-embedded freeze-substituted or freeze-dried material have been reviewed by Zierold & Steinbrecht (1987). However, the proof was missing

that the cited dehydration and embedding techniques have been optimized for subcellular element retention (Elder *et al.*, 1992). The experiments with the striated muscle discussed above clearly showed that freeze drying and embedding can be optimized in such a way that even mobile ions (such as K^+, Rb^+, Cs^+, Tl^+) maintain that heterogeneous distribution in a subcellular compartment as is found in frozen-hydrated preparations (Edelmann, 1988). Once a freeze-drying and embedding procedure for a specific biological material is established, reproducible stable preparations may be obtained whose advantages cannot be overestimated. Using the serial sectioning technique, the stable preparation may be used simultaneously for morphological, immunocytochemical and microanalytical investigations.

Besides detecting ions accumulated in the living cells, one may investigate the interaction of physiological ions with the proteins captured in the freeze-dried embedded specimen. For example, it has been shown by 'staining' of resin-embedded sections with solutions of alkali-metal ions that myosin in the muscle cell is able to adsorb large amounts of these ions with a high selectivity (Edelmann, 1980b). Isolated myosin has lost this capability (Carr, 1956; Lewis & Saroff, 1957). Myosin in glutaraldehyde-fixed cells has also lost this capability (Edelmann, 1986a). These experiments are important because they show that the ultrastructure of cellular proteins must be different from that found *in vitro* after biochemical isolation procedures or after chemical fixation of the biological material.

In addition, microanalytical techniques may be used that require ultrathin sections of even thickness (electron energy loss spectroscopy, EELS) or sections with a smooth surface (secondary ion mass spectroscopy, SIMS, see, e.g. Grignon *et al.*, 1997). Recent promising results obtained with freeze-dried embedded material and the different available micro-analytical techniques have been published (e.g. Burns & File, 1986; Grohovaz *et al.*, 1996; Pezzati *et al.*, 1997; Bücking *et al.*, 1998, 2000; Neelissen *et al.*, 1999; Bücking & Heyser, 2000, 2001). In the future, SIMS will become probably the most powerful technique for the microanalytical investigation of freeze-dried embedded material. Localization of the various isotopes of virtually all elements is possible. With the SIMS instrument now available (Hillion *et al.*, 1999) one may record simultaneously several atomic mass images with a high spatial resolution of 50 nm, which is by far superior to laser confocal optical microscopy used with fluorescent markers. By means of multiple isotope mass spectometry one may study intra- and transcellular metabolic pathways, signal transduction, cytoplasmic and nucleoplasmic translocations, RNA and DNA expression and distribution, etc.

4.2.5. Investigation of the water problem

It is well known that specific structures of macromolecules can exist only by interaction with water molecules and it is also accepted that water in the hydrated structures has different physical and chemical properties than bulk water (Bachmann & Mayer, 1987; Kellenberger, 1987). However, how much cellular water is organized differently from extracellular free water is not yet known in a generally accepted manner. The findings described here support the idea that in a living cell more cellular water than usually assumed is influenced by certain proteins maintained in a life-specific conformation. In the literature many experiments can be found that lead to a similar conclusion (for reviews see Clegg, 1979; Negendank, 1986; Ling *et al.*, 1993; Ling, 2001: chapter 11). Two different kinds of studies inevitably pointing to the water problem should be mentioned:

1 2 mm long transversally cut muscle cells – whose cytoplasm is directly exposed to the surrounding Ringer solution (Cameron, 1988) – and intact muscle cells swell in the same manner in hypotonic and in concentrated KCl solutions (Ling & Walton, 1976). Apparently the volume regulation of the muscle preparations is mainly due to different degrees of hydration of certain cellular macromolecules and is not dependent on intact cell membranes. Whereas all extracellular water of intact and cut muscle cells can be removed by centrifuging the specimen at 1000 **g** for 4 min, the cellular water of both types of muscle preparations cannot be removed by this kind of centrifugation. However, in response to different metabolic poisons a high percentage of cellular water can be extracted by the centrifugation method used. These experiments support the view that metabolic poisons may influence the structure of proteins, thereby modifying the degree of interaction between certain cellular proteins and surrounding water molecules.
2 The studies with the alkali-metal ions K^+, Rb^+, and Cs^+ and Tl^+ mentioned above show that the bulk of the main cellular cation K^+ (or Rb^+, Cs^+, Tl^+) is preferentially localized (weakly adsorbed) at certain proteins. It follows that the water activity (or the osmolality) in the cell is not mainly determined by freely dissolved particles in free cellular water (Hill & Kupalow, 1930; Hill, 1930) but it must be determined somehow by the water ordering effect of cellular macromolecules (Ling, 1984, p. 271; Edelmann, 1988, 2001).

Bearing in mind the FD experiments presented, new ways of investigating the most important unsolved water problem are suggested in the following. Systematic FD studies may be used to test the above deduced conclusion (see section 4.1.1) that the observed phenomenon of reduced sublimation of water in the area of best cryofixation is due to a stabilized cell-specific (or hydration) water structure. A systematic study has already been published by Wildhaber *et al.* (1982) and Gross (1987), who measured the temperature-dependent sublimation rates of water from cryofixed HPI layers (hexagonally packed intermediate protein layer). They found that a substantial amount of hydration water is not subliming at temperatures around – 80 °C but only at temperatures above –50 °C. Unfortunately their results cannot be generalized because macromolecules are hydrated to different degrees. Model systems containing

different amounts of hydration water have been systematically studied (Ling & Hu, 1987; Ling, 2001, chapter 11) and are well suited for future FD studies. It is worth noting the finding that native proteins such as haemoglobin, bovine serum albumin and γ-globulin (extracellular globular proteins) are poorly hydrated, whereas gelatine and polymers such as polyethylene oxide, polyethylene glycol, polyvinylpyrrolidone and polyvinylmethyl ether are highly hydrated after exposure to a high water vapour pressure. Systematic FD studies with cryofixed native biological material are of course much more difficult than studies with solutions of proteins and other macromolecules because living cells contain a collection of differently hydrated macromolecules. Nevertheless, specifically designed experiments are imaginable. It is well known that specific movements of macromolecules, ions and water take place during the activation of excitable cells. Structure changes of proteins may cause changes of local water structure and of ion binding properties, leading to fast ion diffusion (e.g. Ca^{++}, K^+) and redistribution of water and mobile macromolecules (see, e.g. Ling, 1984; chapter 16: Muscle contraction and related phenomena). Under the assumption that the cellular water structure is modulated by conformational changes of proteins, one should observe different sublimation rates when comparing the FD process of excitable cells cryofixed either at rest or during excitation (Edelmann, 1989).

5. Concluding remarks

According to Schrödinger (1948), living cells are systems in a state of low entropy (high order). Hence, the death of a living cell means transition to a high entropy state (low order). A living cell is primarily an assembly of water, proteins and K^+ ions; In number, the most abundant component of the living cell is water, the next most abundant is K^+ (Ling, 1984; p. 147). When assuming that most of cell K^+ and cell water are free in the living cell, the entropy gain on cell death could only result from less ordered proteins. But neither the proteins of a 'naturally died' cell (Ling *et al*., 1993) nor those of a cell killed by a chemical fixative become freer and more randomly distributed. Only when considering the reported increasing experimental and theoretical evidence that K^+ ions and water molecules are in an associated state in the living cell due to life–specific interactions with proteins – maintained in life-specific conformations – does Schrödinger's view make sense (Ling, 2001; chapter 14). In a dead cell most cellular K^+ ions and water molecules are free, hence the entropy gain is readily explained. When judging methods for stabilizing biological material for electron microscopy a criterion should be the degree of preservation of the low entropy biological system that can be achieved. Stabilization procedures that involve chemical fixation and treatment of hydrated specimens with organic solvents (at room temperature or at low temperature during freeze-substitution) lead to biological material in which the associated state of water and ions is almost lost. It appears that the associated state of ions and (remaining) water is best preserved in properly freeze-dried preparations. The findings described and discussed here show that systematic freeze-drying studies may be well suited for the investigation of interactions between proteins and mobile cellular ions and between proteins and the 'forgotten' water ('Biology has forgotten water or never discovered it', Szent-Györgyi, 1971, p. 239). If life-specific interactions between proteins, K^+ ions and water molecules persist in freeze-dried preparations one may also conclude that the structure of cellular proteins may be best preserved by using the freeze-drying technique.

Acknowledgements

I wish to thank Mrs Tanja Buhles for excellent technical assistance and Dr H. Hohenberg, Hamburg, Professor E. Morgenstern, Homburg/Saar, Dr K. Neumann, Homburg/Saar, and Dr R. Drillien, Strasbourg for valuable discussions during the preparation of the manuscript. I am grateful to Drs H. Hohenberg, P. Monaghan and D. Studer for high pressure freezing of cells and tissues.

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