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Epoxy resin as fixative during freeze-substitution

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Abstract

An alternative protocol for freeze-substitution is described. Araldite/Epon embedding medium (20% in acetone) is first used as a stabilizer (as e.g., OsO_4) and then as embedding medium. The major components of the Araldite/Epon resin formulation react with proteins and lipids and provide for an excellent preservation and reasonable visualisation of the ultrastructure. The ultrastructural appearance can be deliberately influenced with the standard freeze-substitution procedure [Van Harreveld, A., Crowell, J., 1964. Electron microscopy after rapid freezing on a metal surface and substitution fixation. Anat. Rec. 149, 381–386.] using OsO_4 as stabilizing agent by protocols which degrade cytoplasmic and membrane proteins. Epoxy stabilized and embedded samples may become an important tool to get information about the effects of different reagents and protocols used in freeze-substitution. We believe that an in-depth understanding of the procedures is required to correctly interpret images and to complement studies of dynamic processes by light microscopy with reliable, highly detailed ultrastructural information. The block face of epoxy stabilized samples after ultrathin sectioning is highly suited for the analysis of the ultrastructure by AFM.

Keywords: Epoxy resin; Fixative; Freeze-substitution; High-pressure freezing; Protein degradation; Structural preservation; TEM; AFM

1. Introduction

Most of our knowledge about cellular ultra structure has been obtained by transmission electron microscopy of ultrathin sections of biological material, chemically fixed with aldehyde, and OsO_4 followed by dehydration in graded series of organic solvents. The problems of the preparative procedures based on chemical fixation have been described and illustrated in numerous publications (Ameye et al., 2000; Hayat, 2000; Hunziker et al., 1984). They mainly originate from structural alterations of the cellular constituents due to the interaction with fixatives and dehydrating agents (conformational changes of proteins, partial hydrolysis of proteins, shrinkage, and dimensional changes due to the loss of membrane semi permeability, loss of lipids, limited time resolution for dynamic cellular events etc.). Techniques based on rapid cryoimmobilisation have been shown to overcome many of these problems.

Freeze-substitution is now the most widely used procedure to prepare cryoimmobilized biological samples for ultramicrotomy combining advantages of cryofixation with the ease of sectioning at room temperature. Generally, acetone containing 1–2% of OsO_4 is used to dehydrate and stabilize frozen biological materials. Freeze-substituted samples are then embedded into epoxy or acrylic resins. While, the former provide good structural preservation and identification, the latter (Lowicryl, LR-Gold) may be better suited for immunocytochemical experiments, since impregnation and UV-polymerisation can be performed at high subzero temperatures, eliminating the need for OsO_4 (Humbel and Mueller, 1986; Monaghan et al., 1998) and because there is a less intense interaction between proteins and acrylic resins (Kellenberger et al., 1987).

A great variety of substitution protocols are described in the literature. These procedures are difficult to compare as they are usually optimized for the solution of a specific problem and the substitution regimes (time, temperature) are often incompletely reported in the publications. In addition, the quality of freezing is most often unclear and can

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vary from the amorphous (vitrification) to a crystalline state with undetectable or barely visible segregation patterns due to ice crystal growth. Experience suggests that the final structural appearance depends on both freezing quality and substitution protocol. Substantial evidence has been provided e.g. by Studer et al. (1995).

At present it appears nearly impossible to achieve a constant quality of freezing, and vitrification rarely occurs except in very thin superficial layers or of objects that contain significant amounts of natural cryoprotectants. The effects of all steps (freezing quality, dehydration, and recrystallisation during freeze-substitution, stabilisation by fixatives, interaction with embedding resins and heavy metal stains) need to be understood to make full use of the potential of the rapid freezing-freeze-substitution concept and to properly interpret ultrastructural results at a high-resolution level. Descriptions of biological ultrastructure more closely related to the living state are important for complementing studies of dynamic events in living cells by fluorescent light microscopy. In some selected cases, cryosections can serve as a reference by which the influence of the steps of a particular freeze-substitution procedure can be characterized. True cryosections, however, are obtained only from vitrified material (Al-Amoudi et al., 2004; Michel et al., 1992) and cryosectioning of non pre-treated biological samples has remained very demanding despite some significant technical progress (Al-Amoudi et al., 2003). At present, it remains uncertain, whether cytological samples (cells and tissues up to approximately 100 μm thick) can ever be routinely vitrified to permit high-resolution investigation of the mutual interaction of cell organelles, of cell organelles and macromolecular components of the cytoplasm etc. by electron tomography of cryo-sections (Baumeister, 2002). Many relevant questions might be approached by high-pressure freezing, freeze-substitution, and subsequent embedding, if the effects of the steps involved in freeze-substitution and embedding were known more in detail.

In this paper, we want to show that the epoxy and anhydride groups, present in epoxy resin embedding formulations can be used as powerful stabilising agents during freeze-substitution. The chemical interaction of the constituents of the common epoxy resin formulations with biological material has been discussed by Causton (1985). The reactions of the components in a particular epoxy resin formulation with biological material are very complex. The most frequent reactions between epoxy resin and proteins occur at sites rich in nucleophilic substituents and end groups. Table 1 shows the most important reactions of the individual components in an epoxy resin formulation: the reactions of anhydride (cross-linker) and epoxy groups with protein.

We investigated a new procedure for freeze-substitution which is based on acetone and epoxy resin for stabilisation and embedding to design an optimized preparation protocol for cryoimmobilized biological material for the analysis of intracellular structures by AFM and to better understand the effects of the various interactions of epoxy and anhydride groups with cellular components.

Table 1
Reaction between nucleophiles present in proteins and reactive groups of the epoxy resin

Nucleophiles	Occurrence in proteins	Reaction group
Carboxyl	Glutamic acid, aspartic acid, chain ends	Epoxy
Phenol	Tyrosine	Epoxy, anhydrides
Amino	Lysine, chain ends	Epoxy, anhydrides
Imidazole	Histidine	Epoxy, anhydrides
Guanidyl	Arginine	Epoxy, anhydrides
Indole	Tryptophan	Epoxy, anhydrides
Amide	Glutamine, asparagines	Epoxy, anhydrides
Aliphatic hydroxyl	Serin, threonine	Epoxy, anhydrides
Sulphydryl	Cystine	Epoxy

2. Materials and methods

2.1. Chemical fixation

Adult *Caenorhabditis elegans* were prepared by classical fixation. Specimens were fixed in 0.7% glutaraldehyde, 0.7% OsO_4 in 10 mM Hepes buffer for 1 h. After washing in 10 mM Hepes buffer, the tails and the heads of the worms were cut and the material was post-fixed in 2% OsO_4 in 10 mM Hepes buffer for 3 h. Prior to dehydration in graded series of ethanol, the specimens were carefully rinsed with water. The dehydrated specimen were embedded in Araldite/Epon epoxy resin (Jorgensen et al., 1995).

2.2. High- pressure freezing

Adult *C. elegans*, a kind gift of Prof. M. Gotti, ETH Zürich, and Dr. I. Wakker, MPI Heidelberg, were high-pressure frozen in cellulose capillaries as described earlier (Hohenberg et al., 1994).

Antenna of the parasitic wasp *Cotesia glomerata* (Hymenoptera: Braconidae), a kind gift of Prof. Dorn, ETH Zurich, was isolated from the life wasp at 4 °C and than immediately frozen.

Life cat's mite *Otodectes cynotis* was mounted in aluminium platelets filled with hexadecane and immediately frozen.

The human lung fibroblast tissue was frozen using carbon coated sapphire discs as described earlier (Monaghan et al., 2003).

All freezing procedures were performed by a HPM 010 high-pressure freezer (Bal-Tec, Principality of Lichtenstein).

2.3. Freeze-substitution and embedding

Standard freeze-substitution was performed in acetone containing 2% OsO_4, The samples were kept at −90 °C, −60 °C and −30 °C for 8 h at each temperature, and finally warmed to 0 °C. The substitution medium was replaced by pure anhydrous acetone immediately after having reached 0 °C. Between the steps, the temperature was raised by 1 °C/min. After warming to room temperature, the samples were embedded in Araldite/Epon embedding mixture which was composed of 49% w/w Araldite/Epon stock solution, 49%

w/w Hardener DDSA (Fluka), and 2% w/w Accelerator DMP-30 (Fluka). The Araldite/Epon stock solution consisted of 41% w/w Epoxy-Einbettungsmittel (Fluka), 54% w/w Durcupan ACM (Fluka), and 5% w/w Dibutylphthalate (Fluka). Infiltration was performed stepwise (33% resin in water-free acetone for 4 h, 66% resin in acetone for 4 h, 100% resin overnight in a desiccator, evacuated with a membrane pump to 10 mbar to maintain dry conditions). All samples were polymerized at 60 °C for 72 h. Block faces for AFM analysis were produced within 1 day after polymerization.

To study the effects of OsO_4, the above regime was altered by warming the sample to 25 °C and keeping it for 2 h prior to washing with pure acetone and embedding. Alternatively, samples were exposed to 1% OsO_4 in acetone for 8 h after having reached 0 °C (standard procedure, 2% OsO_4 in acetone) then washed with pure acetone and brought to room temperature for embedding as described above.

The stabilizing properties of the complete Araldite/Epon embedding formulation as well as of the Epoxy component (stock solution) and of the cross-linker component alone, were examined by the standard freeze-substitution regime. Instead of the OsO_4, however, either 20% Araldite/Epon stock solution, or 20% Dodecenyl Succinic Anhydride (DDSA, cross-linker) or 20% of the complete Araldite/Epon formulation was used as a stabilizing agent.

2.4. Ultrathin sections and block-face preparation

The resin embedded specimens were mounted in special holders which at the same time fit the microtome and are suitable for the examination of the block face by AFM. Ultrathin sections (10–50 nm) were obtained using a Leica Ultracut E microtome (Leica, Austria) equipped with a diamond knife (Diatome, Switzerland). Sections for TEM analysis were collected on carbon coated formvar grids, stained with uranyl acetate, and lead citrate (Reynolds, 1963) and examined in a EM 912 Omega (Zeiss, Oberkochen BRD) electron microscope equipped with a ProScan 1 × 1 k slow scan CCD camera (Proscan, Munich, BRD).

The block faces of specimens were treated with pure Ethanol for varying time prior to AFM examination in order to remove free components of the resin mixture (Matsko and Mueller, 2004).

2.5. Atomic force microscopy

Atomic force microscopy (AFM) images were collected in tapping mode using Digital Instruments NanoScope III equipped with a J scanner (scanning range 150 μm) and silicone nitride cantilevers with natural frequencies in the 300 kHz range (force constant 20 N/m, tip radius 10 nm (NT-MDT, Russia)) as described earlier (Matsko and Mueller, 2004). All images were collected in a "hard-tapping" mode (Magonov and Reneker, 1997).

2.6. Immunocytochemistry on ultrathin resin sections

Epoxy sections (10–30 nm) were transferred on formvar/carbon coated copper grids, and then washed 1–2 min with pure ethanol to remove unpolymerased plastic.

Subsequently, the sections were incubated with blocking buffer (PBG) for 10 min, immersed in the primary antibody (monoclonal Anti-a Tubulin (mouse IgG1 isotope), Sigma) and incubated for 30 min at room temperature. After being rinsed in PBG five times, they were immersed in the second antibody solution (anti mouse IgG gold conjugate 10 nm, Sigma) for 30 min at room temperature. Finally, sections were rinsed four times in PBG, two times in PBS and two times with water, stained with uranil acetate during 5 min and investigated by EM.

3. Results and discussion

We provide evidence, that differences in the appearance of the ultra structure are due to a more complete preservation of the cellular proteins by comparison of chemically fixed and acetone/OsO_4 freeze-substituted nematodes (*C. elegans*). We also show that the ultrastructural appearance can be deliberately influenced with the standard freeze-substitution protocol (Van Harreveld and Crowell, 1964) using OsO_4 as stabilizing agent by regimes, which degrade cytoplasmic and membrane proteins.

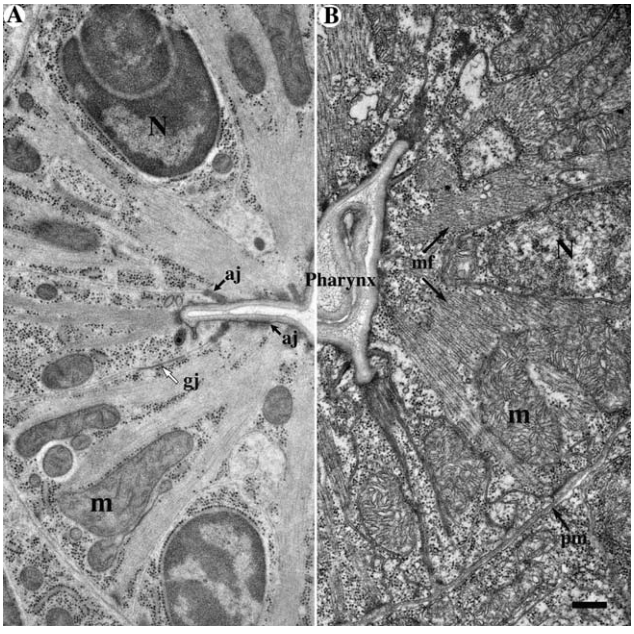

Fig. 1. TEM images of a similar area of the nematode *C. elegans*. (A) High-pressure frozen and freeze-substituted in acetone containing 2% OsO_4. The substitution medium was replaced by pure acetone at 0 °C. (B) Chemically fixed. Scale bar equals 500 nm. N, nuclei; fm, pharyngeal muscle filaments; m, mitochondria; aj, adherens junction; gj, gap junction; and pm, pharyngeal membrane.

3.1. Chemical fixation and acetone/OsO_4 freeze-substitution of a nematodes (*C. elegans*)

Fig. 1 compares cross-sections of chemically fixed (1B) and high-pressure frozen (HPF) and freeze-substituted (FS) nematodes (*C. elegans*) (1A) prepared as described in Section 2 (standard procedure). The comparison shows the well-established differences between the two methods. After chemical fixation (Fig. 1B) membranes are more clearly seen but appear undulated and the cytoplasm as well as the organelles (mitochondria, nuclei) exhibits much less denser matrices. The overall organisation seems to be distorted in addition. The reason for the latter may be shrinkage due to dehydration, while strong contrast and membrane visibility are attributed to the partial hydrolysis of the proteins by OsO_4 (Hayat, 2000).

The nematode in Fig. 1A was freeze-substituted in acetone containing 2% of OsO_4. After a temperature of 0 °C was reached, the substitution medium was replaced by pure solvent and embedding was started. The much-reduced contrast of the membranes as well as the higher density of the organelle and of the cytoplasm is evident.

3.2. Effect of OsO_4 treatment on the ultrastructural appearance of biological sample

Both soluble and insoluble proteins undergo alterations in their secondary structure when treated with aqueous OsO_4 (Hayat, 2000).

The organelles (mitochondria, amphid neurons, and muscles cells) of freeze-substituted samples kept exposed

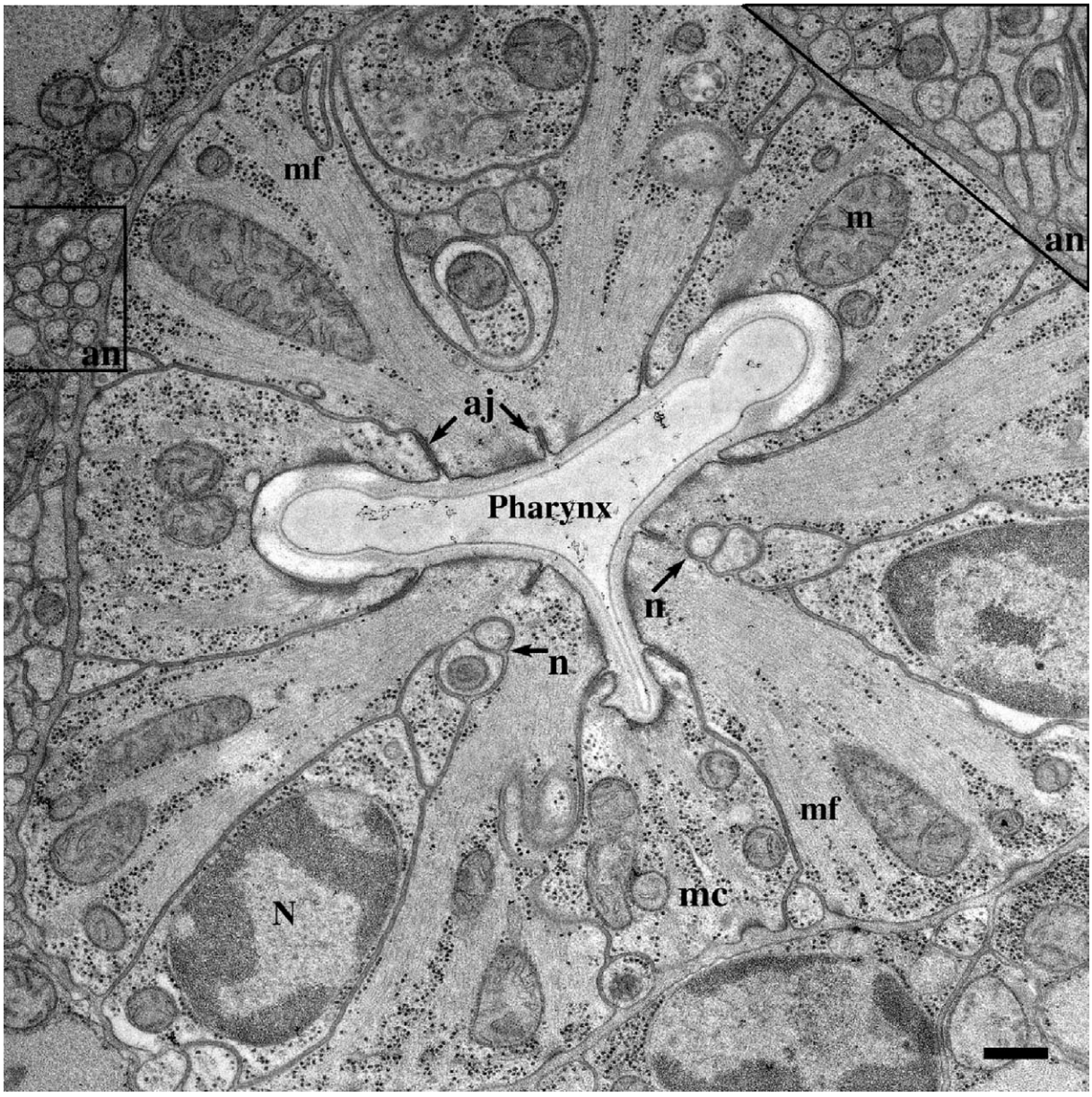

Fig. 2. TEM image of the pharyngeal area of *C. elegans*. The samples were high-pressure frozen, freeze-substituted in acetone containing 2% OsO_4. The substitution medium was replaced by pure acetone after having kept the sample at 25 °C during 2 h. Scale bar equals 300 nm. N, nucleus; fm, pharyngeal muscle filaments; m, mitochondria; aj, adherens junction; an, amphid neurons; and n, neurons.

to osmium tetroxide in acetone for a longer period of time show less contrast after post-staining, as demonstrated by Fig. 2 (overview) and Fig. 3. And at the same time, membranes become more clearly visible. The sample which was kept in OsO_4 during 2 h at room temperature (Figs. 2 and 3B) shows the amphid axons in close apposition (Fig. 3B), similar to the standard procedure (Fig. 3A). After overnight treatment with 1% OsO_4 (Fig. 3C) the axonal membranes changed shape and appeared undulated with sharp angles. We believe, that this is due to distortion and removal of proteins necessary to stabilise the membrane structures.

The intensity of the post-staining is governed by two major aspects, the penetration of the heavy metal salts, and their affinity to the cellular components. The penetration of the heavy metal salts into the sample depends on the density of the sample, which is a combined result of the amount and preservation of mainly the proteins and of the degree of polymerisation of the resin. The second aspect is the high affinity of the staining agents to some of the proteins, lipids, nucleic acids, and carbohydrates. From TEM images, it is therefore difficult to directly correlate contrast with amount and state of e.g., proteins. It may be important to investigate the same sample by alternative methods of microscopy (e.g., AFM shows that amphid neurons are filled with protein (Fig. 7)).

3.3. Epoxy resin as alternative fixative during freeze-substitution

The problem of protein degradation during freeze-substitution can be avoided if epoxy resin in organic solvent instead of osmium tetroxide is used. Figs. 4 and 5A provide evidence that the known structural elements of the cells have been preserved during freeze-substitution in acetone containing 20% of Araldite/Epon embedding mixture as a stabilizing agent. If we compare epoxy fixed (Figs. 4 and 5A) and osmium tetroxide fixed material (Figs. 2 and 5B) we find the following differences:

3.3.1. Membrane shape and visibility

The membranes in sections of epoxy stabilized samples (Figs. 4 and 5A) could not be post-stained with heavy metal salts and therefore remain light greyish in appearance in contrast to samples freeze-substituted in the presence of OsO_4 (Figs. 2 and 5B). We believe that the membrane associated proteins remain intact and in place, and may hamper access for the heavy metal salts (uranyl acetate, lead citrate) to stain the lipid bilayer in thin sections. Similar appearance of the membranes can be obtained after freeze-drying (Edelmann, 2002) after freeze-substitution in a pure solvent followed by low temperature embedding into Lowicryl (Humbel and Mueller, 1986). In contrast to the membrane proteins, the proteins of the microtubules seem to have high affinity for the post-staining reagents and appear as dark rings in both samples (Figs. 5A and B (inset)).

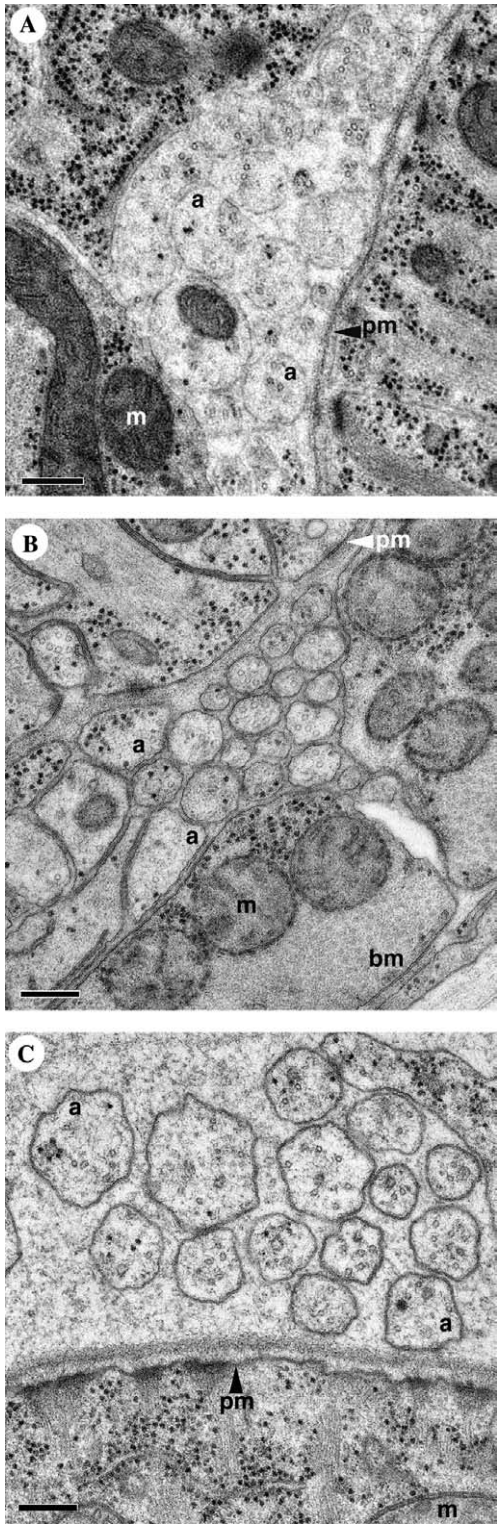

Fig. 3. TEM images of the amphid neuronal area of the nematode. The samples were high-pressure frozen, freeze-substituted in acetone containing 2% OsO_4. The substitution medium was replaced by pure acetone immediately after having reached 0 °C (A), after having kept the sample at 25 °C for 2 h (B). The substitution medium was replaced at 0 °C by 1% OsO_4 in acetone and kept at 4 °C overnight (C). Scale bar equals 200 nm. m, mitochondria; a, amphid axons; pm, pharyngeal membrane; and bm, bodywall muscles.

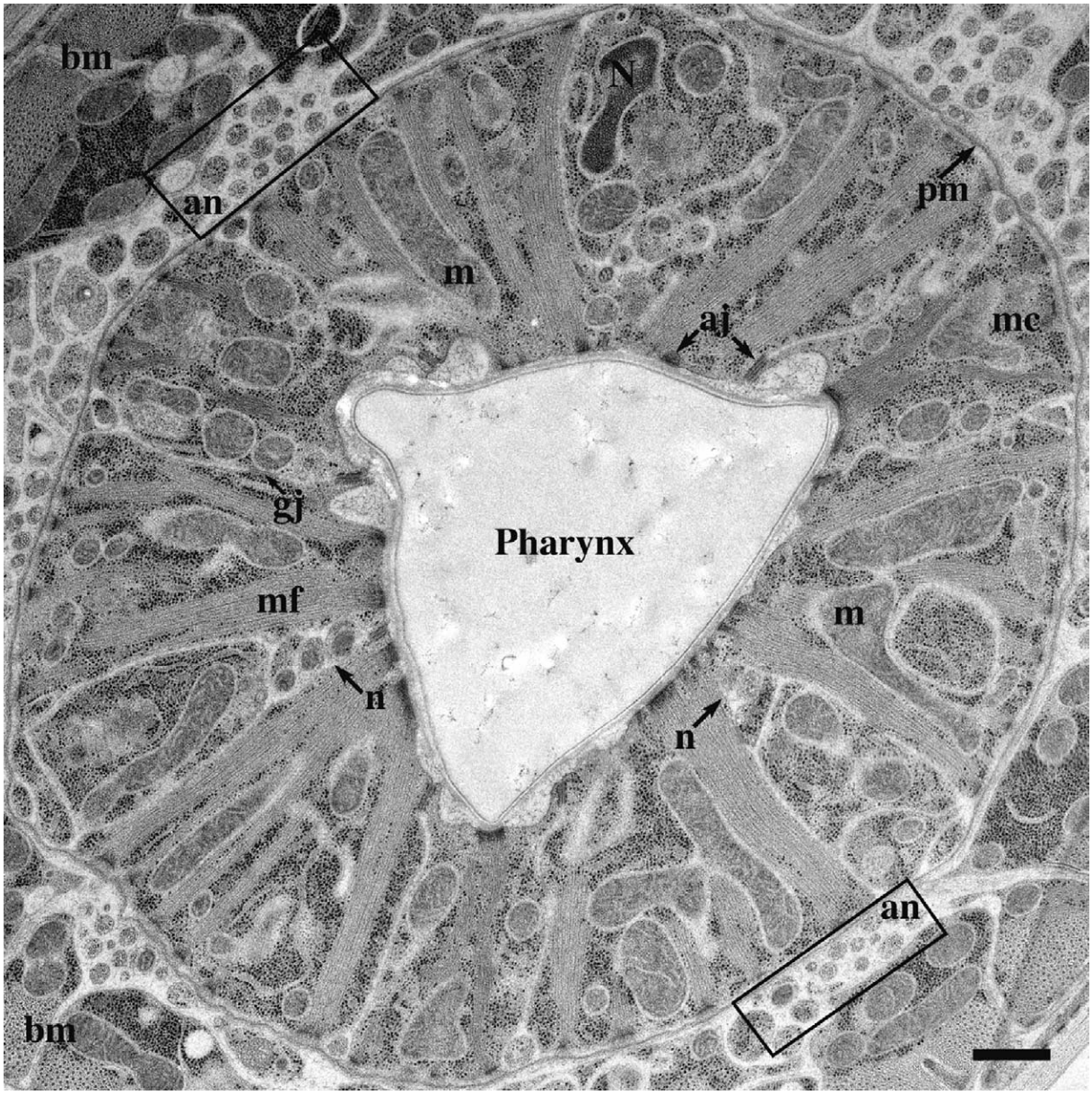

Fig. 4. TEM micrograph of a pharyngeal area of *C. elegans*. The samples were high-pressure frozen, freeze-substituted in acetone containing 20% Epon/Araldite mixture (see Section 2.3). Scale bar equals 500 nm. N, nuclei; fm, pharyngeal muscle filaments; m, mitochondria; aj, adherens junction; gj, gap junction; an, amphid neurons; bm, bodywall muscles; pm, pharyngeal membrane; and mc, marginal cells.

The membranes appear much more undulated in Fig.5B (OsO_4 exposed for 2 h at room temperature), than in Fig. 3A (OsO_4 removed at 0 °C) and, no undulation can be deduced in Fig. 5A (no OsO_4). We can conclude from this that degradation of the membrane proteins destabilizes the lipid bilayer resulting in undulated membranes. This finding is particularly pronounced in chemically fixed material (Fig. 1B, compare also Fig. 3).

3.3.2. Staining density of the epoxy and osmium tetroxide fixed material

The amphid neuron matrix appears very dense (Fig. 4, frame) in the epoxy stabilized sample in contrast to the sample substituted in aceton/OsO_4 (Figs. 2, 3B, and C) where no difference between outside and inside of the axons can be observed. A pronounced difference in staining intensity between the pharyngeal area and the intercellular space between pharyngeal membrane and wallmuscle (e.g., frame in Fig. 4) cells is evident in the epoxy fixed sample (Fig. 4) and almost negligible in the osmium tetroxide fixed sample (Fig. 2). These findings are in good agreement with our AFM data, which indicate that the intercellular space is less densely packed with proteins than the pharyngeal area (Fig. 7).

The structure of junctions (gap junction—gj, adherens junction—aj), of actin/myosin in the radial muscle filaments (Figs. 2 and 4), and in the transverse section of the longitudinal muscles (Fig. 5) as well as of cross-sections of microtubules appears to be better preserved, and a more

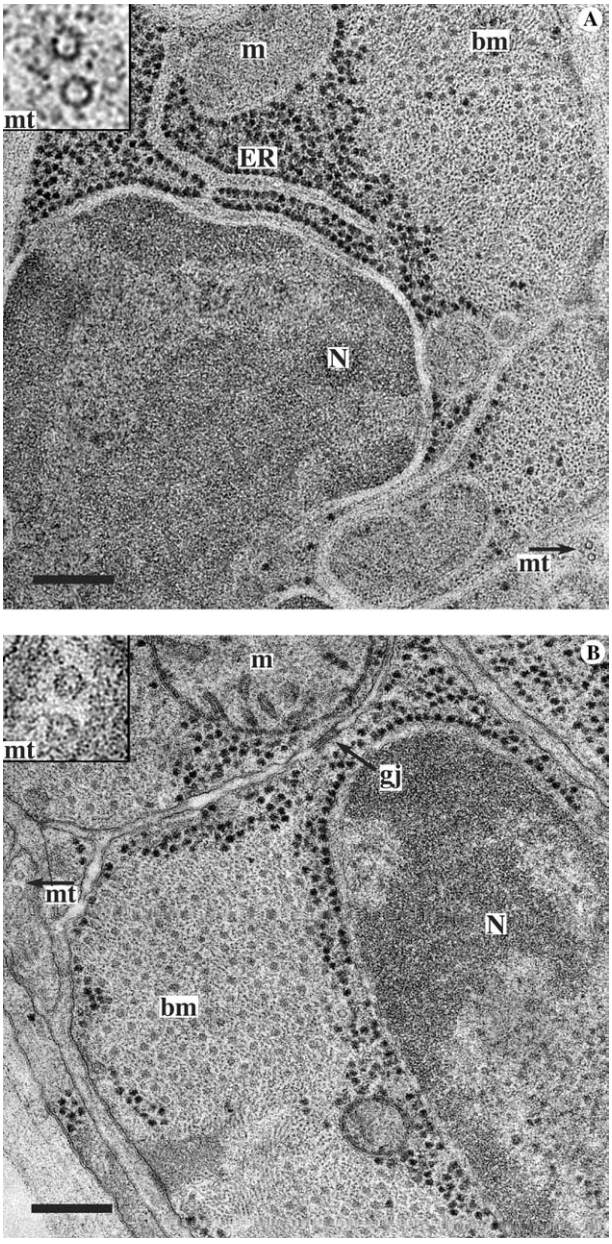

Fig. 5. TEM micrographs of a similar area of *C. elegans*, high-pressure frozen, freeze-substituted in acetone containing 20% Epon/Araldite mixture (A), and inaceton containing 2% OsO_4 as in Fig. 2 (B). Scale bars equal 200 nm. N, nucleus; m, mitochondria; gj, gap junction; bm, body-wall muscles; mt, microtubules, and ER endoplasmic reticulum.

crisp image is obtained after post-staining. This is attributed to more completely preserved proteins.

3.4. Mechanism of freeze-substitution with epoxy resin as a stabilizing agent

We believe that initially the ice of the frozen material is dissolved and replaced by acetone. The epoxy and anhydride groups start to react with the corresponding groups of the biological material upon complete removal of the bound water at approximately −50 °C to −30 °C. This assumption is derived from earlier experiments to understand protein cross-linking by glutaraldehyde at low temperatures (Humbel and Mueller, 1986). By applying the epoxy resin already at −90 °C, the reactive molecules will be evenly distributed and can react immediately when the water is gone. The biological structures may still be stabilised at this temperature.

3.5. Fixative properties of the main components of the epoxy resin mixture

High-pressure frozen nematodes were freeze-substituted in water free acetone containing 20% dodecenyl

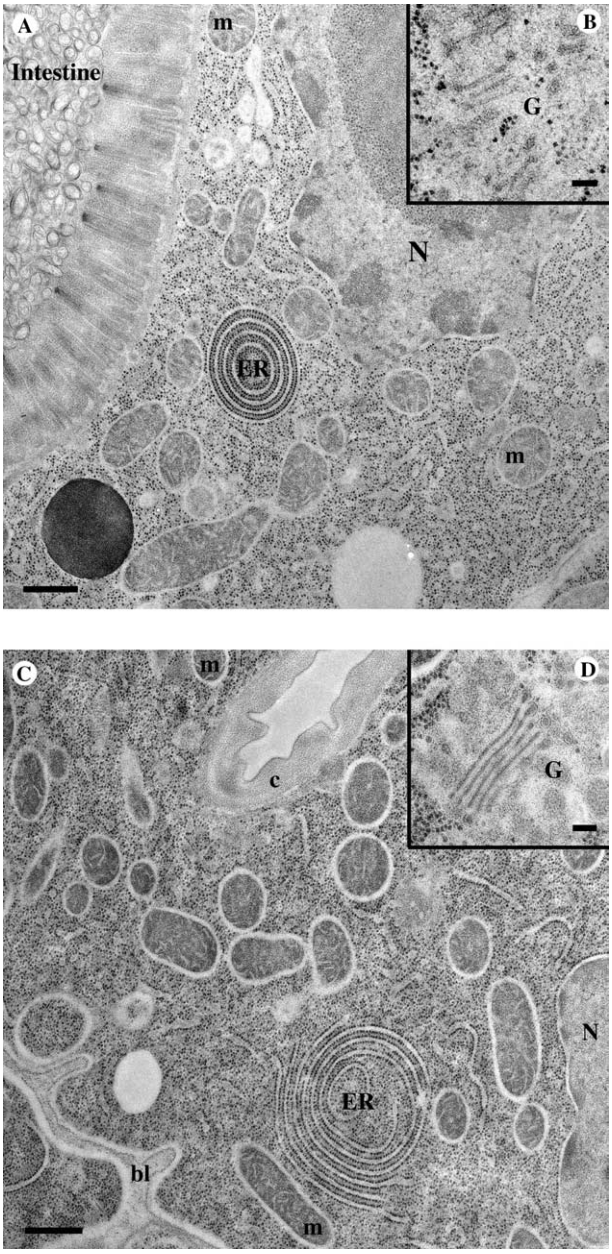

Fig. 6. TEM micrographs of nematodes high-pressure frozen, freeze-substituted in acetone containing 20% Epon/Araldite stock solution (A) or acetone containig 20% DDSA (C). In the inserts (B and D) Golgi apparatuses are displayed at higher magnification. Scale bars correspond to 500 nm in (A and C) and 100 nm in (B and D). N, nucleus; m, mitochondria; ER, endoplasmic reticulum, G, Golgi apparatus, and c, cuticle.

succnic anhydride (Figs. 6C and D) or 20% Araldite/Epon stock solution (Figs. 6A and B) to find out whether or to what extent the main components (epoxy and anhydride) exhibit fixative properties. Freeze-substitution was performed as described above. Fig. 6 shows that both the anhydride (Fig. 6C) and epoxides (Fig. 6A) have

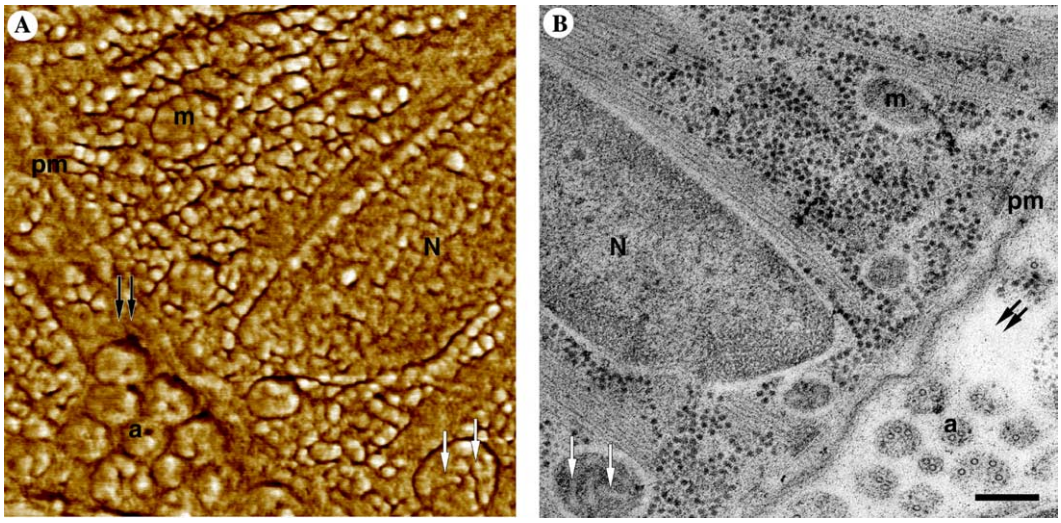

Fig. 7. Corresponding AFM (block face) and TEM (section) images of *C. elegans*, high-pressure frozen and freeze-substituted in acetone containing 20% Epon/Araldite mixture. Black arrows point to intercellular space, white arrows to mitochondrial cristae. Bar equals 200 nm. N, nucleus; m, mitochondria; a, axon; and pm, pharyngeal membrane

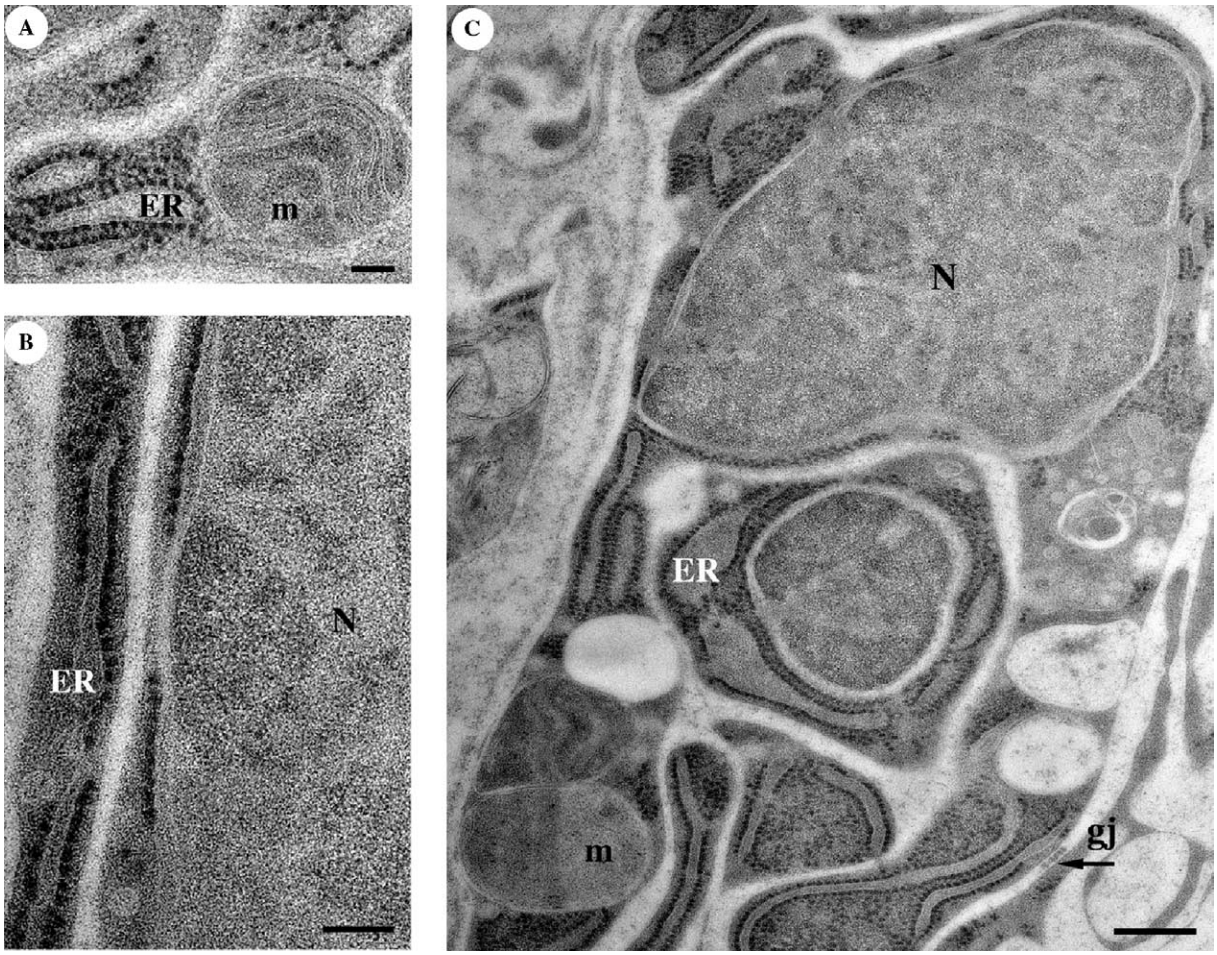

Fig. 8. TEM images of the longitudinal section of the cat's mite *O. cynotis*. The organism was high-pressure frozen, freeze-substituted in acetone containing 20% Epon/Araldite mixture (see Section 2.3). Scale bars equal 100 nm in (A and B), and 300 nm in (C). N, nuclei; m, mitochondria; gj, gap junction; and ER, endoplasmatic reticulum.

stabilising properties during freeze-substitution as suggested by Table 1. The Golgi apparatuses, displayed in the inserts (Figs. 6B and D), however, indicate that the structures are significantly better stained in the anhydride than in the epoxy-stabilized sample. In contrast to the DDSA, which is a relatively small molecule, the long chained epoxy molecules can bind to other epoxy molecules in addition to the biological structures. A very dense network is thus created upon Araldite/Epon embedding and polymerisation, which worsens the interaction with the heavy metal stains. This network is much looser in the case of freeze-substitution in the presence of anhydride because the DDSA molecules, irreversibly bound to the biological structures, occupy reactive groups usually used by the epoxy molecules.

The most comprehensive structural preservation is, however, obtained by freeze-substitution in acetone containing 20% of the full Araldite/Epon mixture including accelerator (DMP-30) and plasticizer (dibutylphatalate) (Figs. 4 and 5A). In this way stabilisation is done with a balanced ratio of epoxide to anhydride molecules, yielding adequate preservation, stainability, and section properties.

3.6. AFM and TEM of epoxy fixed biological samples

AFM can visualize the biological ultrastructure of epoxy embedded biological material similar to TEM. It mainly provides information on distribution and shape of proteins (Matsko and Mueller, 2004). The blockface of epoxy fixed and embedded nematodes was prepared for AFM (Fig. 7A) while the last ultrathin section was collected, post-stained with uranyl acetate and lead citrate and used for TEM (Fig. 7B). AFM and TEM provide similar images and the cell organelles in both images are readily identified. Brighter signals in the TEM image (Fig. 7B, nuclear and mitochondrial membranes, intercellular space) correspond to either protein rich structures that are not or only faintly stained (e.g., membranes) or to areas with low protein density. The AFM image, which shows the proteins as bright signals (Matsko and Mueller, 2004) can unambiguously demonstrate that the intercellular space (black arrow Figs. 7A and B) contains very little

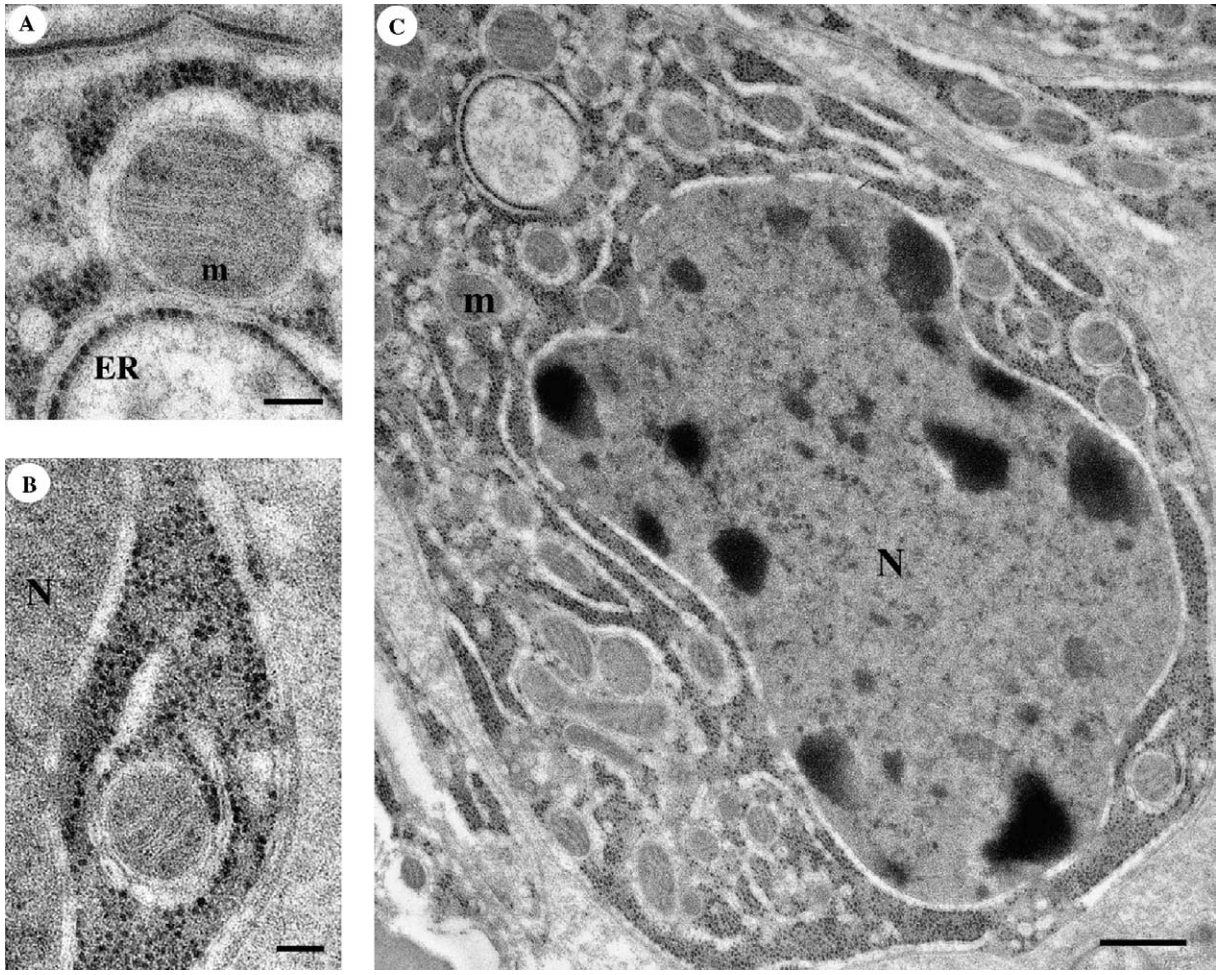

Fig. 9. TEM micrographs of the cross-section of the antenna of the parasitic wasp *C. glomerata* (Hymenoptera: Braconidae). The organism was high-pressure frozen, freeze-substituted in acetone containing 20% Epon/Araldite mixture (see Section 2.3). Scale bars equal 100 nm in (A and B), and 500 nm in (C). N, nuclei; m, mitochondria; and ER, endoplasmatic reticulum.

proteins and that the structures inside the mitochondria (white arrow in Figs. 7A and B) are rich in proteins. The AFM images also support the assumption that in biological material, freeze-substituted in acetone/epoxy, the membrane associated proteins are well preserved so that the heavy metal stains cannot access the lipid bilayer to render it visible.

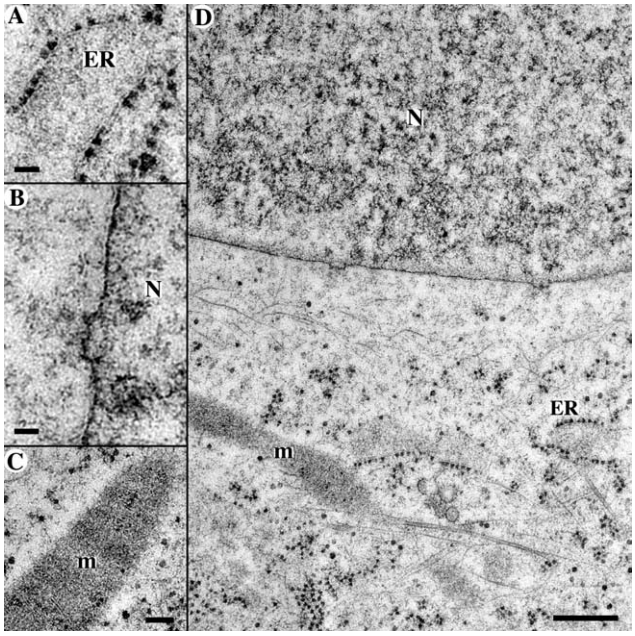

Fig. 10. TEM images of the human lung fibroblast tissue. The cells were high-pressure frozen, freeze-substituted in acetone containing 20% Epon/Araldite mixture (see Section 2.3). Scale bars equal 50 nm in (A and B), 100 nm in (C), and 300 nm in (D). N, nuclei; m, mitochondria; and ER, endoplasmatic reticulum.

3.7. Interrelation between the structural preservation and membrane visibility of the high-pressure frozen and epoxy freeze substituted biological objects

The visibility of the plasma membranes after high-pressure freezing/freeze-substitution procedure is one of the central problems related to the HPF method, which appears since the latter was invented. Now it is clear that membrane appearance depends on many factors: quality of the freezing [vitrification or semi good freezing quality, when the effect of the ice crystals can be observed in the nuclear envelope, the most sensitive for the freezing quality organelle (Hunziker et al., 1984; Studer et al., 1995)]; applied freeze-substitution protocols [different kind of chemical fixatives (Giddings, 2003; Wild et al., 2001)]; state of the biological tissue before freezing (was bioorganism alive before freezing or native proteolyses process was already started).

This problem will be discussed in more details elsewhere (Matsko and Mueller, to be published). Here, we want to show that epoxy freeze-substitution protocol can provide a good membrane visibility only in case when internal protein was not damaged during the sample preparation before freezing and/or during the high-pressure freezing process.

Figs. 8–10 present three different organisms, which were high-pressure frozen and then epoxy freeze-substitution protocols was applied. Fig. 8 shows cross-section of the mite *O. cynotis*, Fig. 9 shows cross-section of the antenna of the parasitic wasp *C. glomerata* (Hymenoptera: Braconidae), and Fig. 10 the human lung fibroblast tissue. All organisms were in the life state before the process of freezing was started, so the problem with the native proteolysis can be avoided. The quality of the freezing is

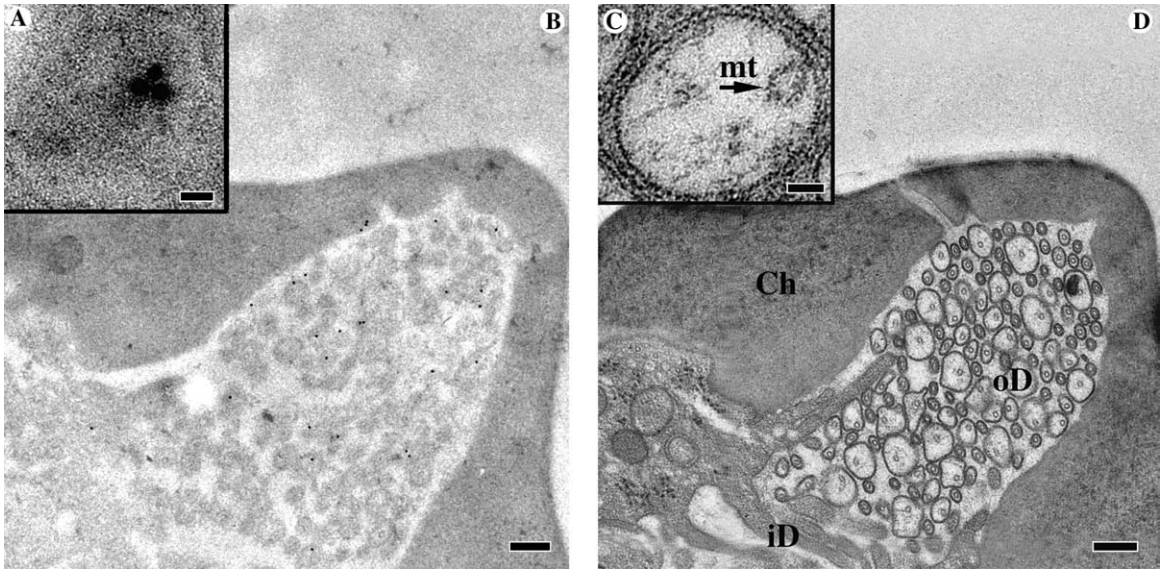

Fig. 11. TEM micrographs of a similar area of the antennal sensilla *placodea* of the parasitic wasp *C. glomerata*, high- pressure frozen, freeze-substituted in acetone containing 20% Epon/Araldite mixture (A and B), and in acetone containing 2% OsO_4 (C and D). Fig. 11 (A and B) shows immunocytochemical analysis of the location of tubulin (the major building block of microtubules). Scale bars equal 200 nm in (B and D) and 20 nm in (A and C). Ch, chitin; iD, inner dendrites; oD, outer dendrites bear microtubules (mt), reaching cuticle pores.

the only difference between these three organisms. As it can be clearly seen, the mite (Fig. 8) was frozen almost in the vitrified state [we can recognise it from the homogeneous nuclear state, without any segregation pattern (Fig. 8B)]. The contrast of the membranes, which appear as unstained lines, is significant. Also cytoplasm shows very dense matrixes. Endoplasmatic reticulum looks like highly ordered organelles. Each ribosome appears almost identical in shape and equidistant from each other. Fig. 9 shows the cross-section of the antenna of wasp, which was good frozen but not vitrified since very small ice segregations can be detected in the nuclear envelop (Figs. 9B and C). The membrane contrast becomes worse in comparison with Fig. 8 but still very strong compared to Fig. 10, where almost no membrane contrast can be observed. From the other hand, the quality of the freezing of the fibroblast obviously is insufficient (ice segregation almost everywhere in the cytoplasm). Nuclear membrane appears in traditional way as two black lines. It is clear that heavy metal stains have access to lipids belayers and can intensively stain them.

Thus, the epoxy freeze-substitution protocol cannot guarantee high membrane visibility of the sample, protein content of which was damaged due to the ice crystal growth during the freezing process, or when the native proteolyses of the sample occurs before freezing. For such samples OsO_4 freeze-substitution protocols can be more efficient since it provides good staining quality when the protein state is already damaged anyway. But when the goal is to obtain an excellent structural preservation and the TEM contrast simultaneously, the epoxy freeze-substitution protocol applied for the vitrified biosample appears to be the most suitable sample preparation procedure.

Epoxy freeze-substitution protocol can be also useful when immunocytochemical analysis is needed. Fig. 11 shows the cross-section of the antennal sensilla, labelled against tubulin, the major building block of microtubules. Positive reaction as indicated by the presence of gold particle is present inside the outer dendrites. No specific signal is observed over the tissue section. The immunocytochemical responds is quite strong in spite of the fact that epoxy resin was used as embedding medium instead of acrylic resin.

Thus, the epoxy freeze-substitution method, followed by epoxy embedding, generates samples suitable for both high-resolution structural studies by AFM and TEM, and immunocytochemical analysis.

4. Conclusion

A more complete preservation of cellular proteins is achieved by freeze-substitution using epoxy resin instead of OsO_4 only as a stabilizing agent. The anhydride component (cross-linker) also exhibits stabilizing properties in addition to the epoxy components of the embedding mixture. By varying of the stabilizing and embedding protocols one can alter the density of the polymerized resin, thus opening new possibilities for immunocytochemistry as well as for the detection of intracellular structures by AFM (Matsko and Mueller, 2004).

Epoxy stabilized and embedded samples permit study of the effects of various substitution protocols using OsO_4 as stabilizer and, therefore, may help to improve the interpretation of ultrastructural findings.

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References

Al-Amoudi, A., Chang, J., Leforestier, A., McDowall, A., Salamin, L., Norlen, L., Richter, K., Blanc, N., Studer, D., Dubochet, J., 2004. Cryo-electron microscopy of vitreous sections. EMBO J. 23 (18), 3583–3588.

Al-Amoudi, A., Dubochet, J., Gnaegi, H., Luthi, W., Studer, D., 2003. An oscillating cryoknife reduces cutting-induced deformation of vitreous ultrathin sections. J. Microsc. 212, 26–33.

Ameye, L., Hermann, R., DuBois, P., Flammang, P., 2000. Ultrastructure of the echinoderm cuticle after fast-freezing/freeze-substitution and conventional chemical fixations. Microsc. Res. Tech. 48 (6), 385–393.

Baumeister, W., 2002. Electron tomography: towards visualizing the molecular organization of the cytoplasm. Curr. Opin. Struct. Biol. 12, 679–684.

Causton, B.E., 1985. Does the embedding chemistry interact with tissue? In: Mueller M., Becker, R.P., Boyde, A., Wolosewick, J.J. (Eds.), The Science of Biological Specimen Preparation for Microscopy and Microanalysis. Scanning Electron Microscopy, AMF O'Hare 1985, pp. 209–214.

Edelmann, L., 2002. Freeze-dried and resin-embedded biological material is well suited for ultrastructure research. J. Microsc. 207, 5–26.

Giddings, T., 2003. Freeze-substitution protocols for improved visualization of membranes in high-pressure frozen samples. J. Microsc. 212, 53–61.

Hayat, M.A., 2000. Principles and Techniques of Electron Microscopy: Biological Applications, fourth ed. Cambridge University Press, Cambridge.

Hohenberg, H., Mannweiler, K., Mueller, M., 1994. High-pressure freezing of cell suspensions in cellulose capillary tubes. J.Microsc. 175 (1), 34–43.

Humbel, B., Mueller M., 1986. Freeze-substitution and low temperature embedding, In: Mueller, M., Becker, R.P., Boyde, A., Wolosewick, J.J. (Eds.), The Science of Biological Specimen Preparation for Microscopy and Microanalysis. Scanning Electron Microscopy, AMF O'Hare 1985, pp. 175–183.

Hunziker, E., Herrmann, W., Schenk, R., Mueller, M., Moor, H., 1984. Cartilage ultrastructure after high-pressure freezing, freeze-substitution, and low temperature embedding. I. Chondrocyte ultrastructure-implications for the theories of mineralization and vascular invasion. J. Cell Biol. 98 (1), 267–276.

Jorgensen, E., Hartwieg, E., Schuske, K., Nonet, M., Jin, Y., Horvitz, H., 1995. Defective recycling of synaptic vesicles in synaptotagmin mutants of *Caenorhabditis elegans*. Nature 378, 196–199.

Kellenberger, E., Dürrenberger, M., Villiger, W., Carlemalm, E., Wurtz, M., 1987. The efficiency of immunolabel on Lowicryl sections compared to theoretical predictions. J. Histochem. Cytochem. 35, 959–969.

Magonov, S., Reneker, D., 1997. Characterization of polymer surfaces with atomic force microscopy. Annu. Rev. Mater. Sci. 27, 175–222.

Matsko, N., Mueller, M., 2004. AFM of biological material embedded in epoxy resin. J. Struct. Biol. 146, 334–343.

Michel, M., Gnagi, H., Muller, M., 1992. Diamonds are a cryosectioner's best friend. J. Microsc. 166 (1), 43–56.

Monaghan, P., Perushige, N., Müller, M., 1998. High-pressure freezing for immunocytochemistry. J. Microsc. 192 (3), 248–258.

Monaghan, P., Cook, H., Hawes, P., Simpson, J., Tomley, F., 2003. High-pressure freezing in the study of animal pathogens. J. Microsc. 212 (1), 62–70.

Reynolds, E., 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. 17, 208–212.

Studer, D., Michel, M., Wohlwend, M., Hunziker, E., Buschmann, M., 1995. Vitrification of articular cartilage by high-pressure freezing. J Microsc. 179 (3), 321–332.

Van Harreveld, A., Crowell, J., 1964. Electron microscopy after rapid freezing on a metal surface and substitution fixation. Anat. Rec. 149, 381–386.

Wild, P., Schraner, E., Adler, H., Humbel, B., 2001. Enhanced resolution of membranes in cultured cells by cryoimmobilization and freeze-substitution. Microsc. Res. Tech. 53 (4), 313–321.