BBA 75328

USE OF SMALL ANGLE X-RAY DIFFRACTION TO INVESTIGATE DISORDERING OF MEMBRANES DURING PREPARATION FOR ELECTRON MICROSCOPY

II. ALDEHYDES

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(Received April 8th, 1969)

SUMMARY

1. The influence of some standard electron microscope aldehyde fixatives on the molecular structure of myelin membranes of frog sciatic nerve was studied with a combination of small angle X-ray diffraction, electron microscopy and biochemical techniques.

2. Small angle X-ray diffraction was found to be very useful for the study of aldehyde fixation, since the absence of a heavy metal allows a direct interpretation of diffraction patterns.

3. It was shown that conventional aldehyde fixatives introduced changes in the diffraction pattern of fixed nerve. Conventional acetone and alcohol dehydration introduced large changes in the diffraction patterns of fixed nerve, apparently as a result of significant extraction of both polar and non-polar lipids.

4. Modified fixation, dehydration and embedding techniques are described. The electron microscope appearances are described of myelin embedded with some water-soluble embedding materials in which lipid extraction is significantly reduced.

INTRODUCTION

As discussed in the previous paper[1], there have been increasing indications of artifacts occurring during some conventional fixation, dehydration and embedding procedures used in preparing thin sections of biological specimens. Interpretation of the conventional tri-laminar thin section appearance of membranes is of particular interest. It has been shown[1] that OsO_4 and $KMnO_4$ did not give sufficient cross-linking either of polar or non-polar lipids to stabilize the structure against extraction of lipids during dehydration with organic solvents. It was concluded that OsO_4 acted to evert or exteriorize the polar groups of the phospholipids. $KMnO_4$ acted to oxidize some

of the polar groups of lipid, and also partially denatured some of the protein in the molecular structure of frog sciatic nerve myelin.

In this report, small angle X-ray diffraction is used to investigate the effects of aldehyde fixatives on the molecular structure of frog sciatic nerve. Since no heavy metals were added to the membrane, we were able to see the effects of fixation, dehydration and embedding on the molecular structure directly. Results of electron microscopy of several water-soluble embedding materials used to minimize lipid extraction during dehydration are also presented.

Portions of this report have been published in abstract form[2].

MATERIALS AND METHODS

The sciatic nerve of grass frogs (*Rana pipiens*) was used. X-ray diffraction, biochemical and electron microscope techniques were described in the previous paper[1].

EXPERIMENTS AND RESULTS

Experiments of formalin fixation on X-ray diffraction of myelin

Fig. 1a shows the small angle X-ray diffraction pattern of a fresh frog sciatic nerve immersed in phosphate buffered saline at 4–7°, and is discussed in the previous paper[1]. The X-ray diffraction pattern in Fig. 1b was obtained after fixation for 4 h in 4% formaldehyde (10% Fisher formalin) in phosphate buffered saline, pH 7.2 at 4°. Since no heavy metal stain was added, the pattern shows directly that molecular re-arrangement has occurred. The intensity distribution is modified and the periodicity is reduced from 171 to 162 Å (6%). However, the higher order reflections are not simple orders of 162 Å.

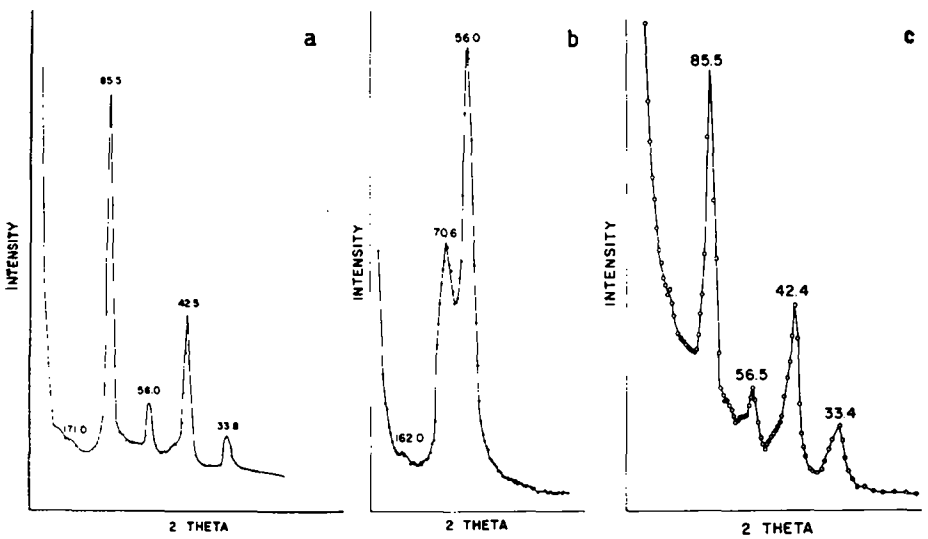

Fig. 1. Small angle X-ray diffraction of frog sciatic nerve myelin: (a) Fresh nerve immersed in phosphate buffered saline. (b) After formalin fixation. (c) After fixation with formaldehyde prepared from paraformaldehyde.

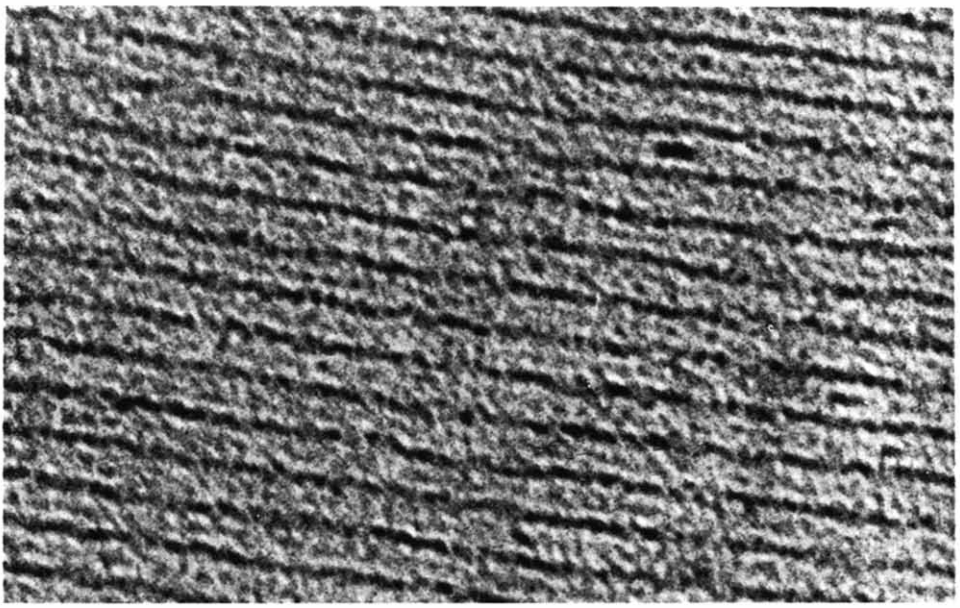

Fig. 2. Electron micrograph of sciatic nerve myelin fixed with formaldehyde (as Fig. 1c), post-fixed with PALADE's OsO_4, and embedded in Epon. 437 200 ×.

Effects of paraformaldehyde fixation of myelin

A 4% solution of paraformaldehyde in phosphate buffered saline was dissociated by heating to 60° (see ref. 3) to give a 4% formaldehyde solution in phosphate buffered saline, pH 7.2. When a sciatic nerve was fixed 4 h at 4° in formaldehyde prepared in this manner, we obtained a considerable improvement. The stiffened nerve showed a completely normal periodicity and X-ray intensity distribution (Fig. 1c). It was disappointing to find that normal dehydration in acetone and Epon embedding[1] caused severe changes (presumably due to loss of lipids) unless the nerve was post-fixed with OsO_4 fixative of PALADE[4] (Fig. 2).

Effect of glutaraldehyde solution on frog sciatic nerve myelin

Figs. 3a–3c shows the X-ray diffraction patterns of frog nerve myelin after fixation with various purified and non-purified stocks of glutaraldehyde. Fixation was in a 3% solution in phosphate buffered saline, pH 7.2 for 1.5 h at 4°. All solutions caused a significant molecular re-arrangement as indicated by a relative increase in the third order reflection and a depression of the fourth order. However, there was no large alteration of the membrane repeat unit size. Attempts were made to purify the glutaraldehyde by combinations of re-distillation and charcoal filtration[5] to remove the disordering effect, but all such attempts were unsuccessful. We also found that the addition of sucrose to glutaraldehyde caused the appearance of additional peaks (Fig. 3d), perhaps suggesting a cross-linking of the sucrose to the membrane.

Effect of glutaraldehyde vapor fixation of frog sciatic nerve myelin

Although attempts to purify the glutaraldehyde so as to remove the disordering effect were unsuccessful, it was found that more extensive cross-linking (greater

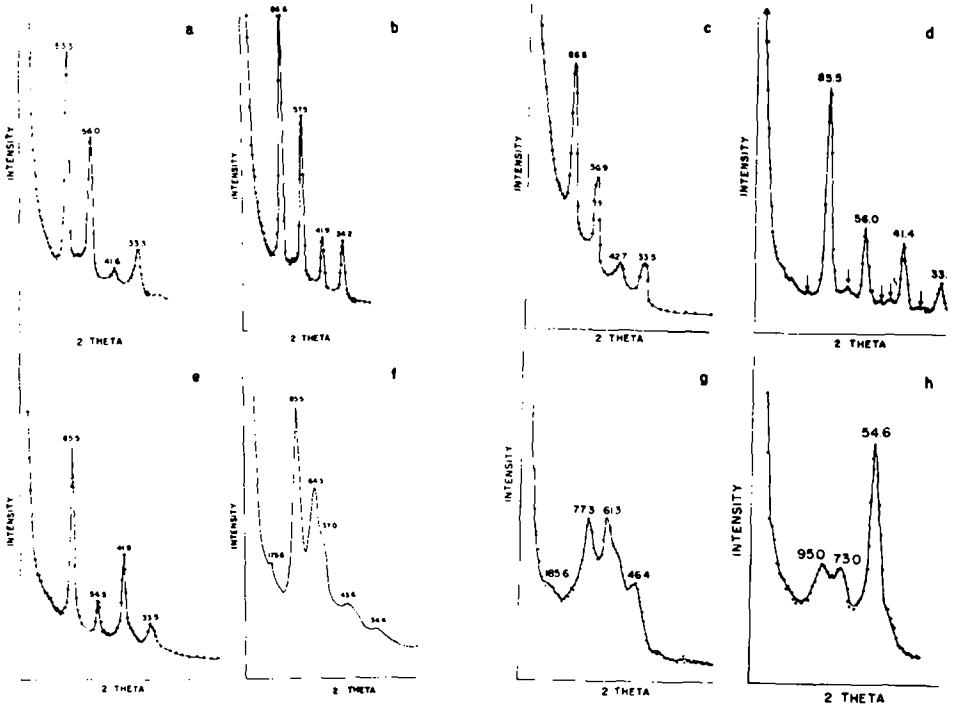

Fig. 3. Small angle X-ray diffraction of frog sciatic nerve myelin. (a) Eastman Organic glutaraldehyde. (b) Fisher 50% glutaraldehyde, Biological Grade. (c) Fisher glutaraldehyde, purified and re-distilled. (d) Fisher glutaraldehyde with sucrose. (e) Glutaraldehyde vapor, phosphate buffered saline immersion. (f) Glutaraldehyde vapor, no phosphate buffered saline. (g) Glutaraldehyde vapor, acetone dehydration. (h) Glutaraldehyde solution, acetone dehydration.

stiffening of the nerve) could be obtained by using glutaraldehyde vapor. This vapor fixation (with a di-aldehyde) gave the nerve a stiffening similar to that obtained by a solution of paraformaldehyde (a mono-aldehyde). Fisher 50% glutaraldehyde stock was placed in a sealed container (just enough to cover the bottom, *plus* filter paper soaked in the stock solution placed around the sides) and allowed to stand 24 h at 4° to create a saturated atmosphere. The nerve was suspended in this saturated atmosphere for 4 h at 4°. The X-ray diffraction pattern obtained when the fixed nerve was immersed in phosphate buffered saline during the scan was remarkably similar to that of fresh nerve in both periodicity and intensity distribution (Fig. 3e). The nerve was initially as stiff as a piece of catgut, but on removal from the X-ray cell after 2 h immersion in phosphate buffered saline, the nerve had lost a good deal of its stiffness. Since this suggested that the fixative was washing out during the X-ray scan while immersed, a nerve fixed for 4 h in glutaraldehyde vapor was scanned when no phosphate buffered saline was present in the X-ray cell. The X-ray diffraction pattern (Fig. 3f) shows that drastic changes have occurred during fixation. To determine that no significant dehydration occurred during the X-ray scan (2 h) a fresh nerve was placed in the X-ray cell with no phosphate buffered saline present. The X-ray diffraction pattern obtained was identical to that of the fresh nerve immersed in phosphate buffered saline (Fig. 1a). The fixed nerve was dehydrated in acetone

and embedded in Epon[1]. Nothing was done to minimize lipid loss during dehydration, so it was not surprising that the general level of preservation was poor (since lipid extraction is greater after aldehyde than after OsO_4 fixation[6]).

Water-soluble embedding media

In view of the results of experiments *in vitro* on lipid solubility in organic dehydrating reagents[1], we have investigated a number of water-soluble embedding materials which were expected to minimize lipid extraction, especially of glutaraldehyde vapor fixed myelin. The first material used was the glycol methacrylate–butyl methacrylate embedding procedure proposed by LEDUC AND BERNHARD[7]. We modified the procedure (Scheme 1) so that sectioning difficulties following glutar-

1. Water was eliminated from the procedure and both monomers were dried at least 24 h in contact with $CaSO_4$, then filtered before use.
2. The catalyst used was 2% (w/v) benzoyl peroxide, and was added to both monomers before mixing together.
3. The final embedding mixture was made with 17 parts catalysed glycol methacrylate *plus* 3 parts catalysed butyl methacrylate.
4. The prepolymer was made by placing the final mixture in lightly stoppered test tubes and heated to about 90° in a water bath. After about 5 min the color began to change, and a slight thickening of the mixture occurred. At this point, the test tubes were plunged into an ice water bath and agitated until they reached the temperature of the bath. This operation was carried out in a fumehood.

Scheme 1. Glycol methacrylate–butyl methacrylate embedding. Done at 4°.

aldehyde fixation[7] were minimized, but were unable to obtain acceptable large scale preservation. However, experiments *in vitro* which were subsequently conducted, showed that both cholesterol and egg lecithin are very soluble in the "final mixture" of glycol methacrylate and butyl methacrylate (solubility is low in the glycol metha-

1. A bacteriological grade of gelatin was used.
2. The tissue was dehydrated through 10-, 30-, 50- and 70% solution of the gelatin (2% glycerin was added to the 70% solution) for 30 min each at 37°.
3. The tissue was placed in fresh 70% solution and allowed to dry at 37° (ref. 26) for 4–6 h, then brought to room temperature. Pieces 4 or 5 mm on a side were cut out and placed in a vacuum desiccator attached to a mechanical pump for 2 h.
4. The dried blocks were affixed to wooden dowels, with epoxy glue, allowed to dry, trimmed to 0.1 mm on a side and sectioned.
5. Dehydration schedule:
   a. Glycol methacrylate monomer *plus* catalyst, 20 min.
   b. Final mixture, 2 times each, 20 min.
   c. 1:1 mixture of the prepolymer and final mixture, 20 min.
6. Infiltration was overnight in the prepolymer.
7. Embed in gelatin capsule.
8. Polymerize with ultraviolet. We used Norelco 14 in ultraviolet lamps with type 05 phosphor. Two lamps were used; one directly above and one directly under the capsule. A fan circulated cool air around the capsules and minimized heating during polymerization. The blocks were hard after 9 h, but the time could be varied somewhat to give a slight variation in hardness.
9. Sectioning was done with the knife at a small clearance angle, with a low meniscus of fluid in the boat. The fluids used were pure water, 10% and 20% acetone in water, 20% glycerin in water, or 0.5 M $BaCl_2$ in water.

Scheme 2. Modified gelatin embedding procedure.

crylate alone). Qualitative results indicate that appreciably more than 1 mg of lipid (both cholesterol and lecithin) is dissolved in 1 ml of the final mixture in less than 12 h at 4°.

Using similar tests *in vitro*, we examined several water-soluble embedding media in which there was no significant solubility of cholesterol and lecithin (qualitative results indicate minimal solubility over periods of up to 36 h). We first used gelatin as described by FERNANDEZ-MORAN AND FINEAN[8] and GILEV[9], *plus* a modification of our own (Scheme 2). Following OsO_4 fixation of frog sciatic nerve, all three methods produced good sections with little difficulty during sectioning. However, it was extremely difficult to obtain sections from glutaraldehyde vapor fixed sciatic nerve, even following attempts to further cross-link the gelatin with aldehydes after embedding. The best sections were obtained by trimming the block to 0.1 mm on a side and floating the sections on a 10 % solution of glycerin in water. General preservation was poor, but ordered areas of the myelin were found (Fig. 4).

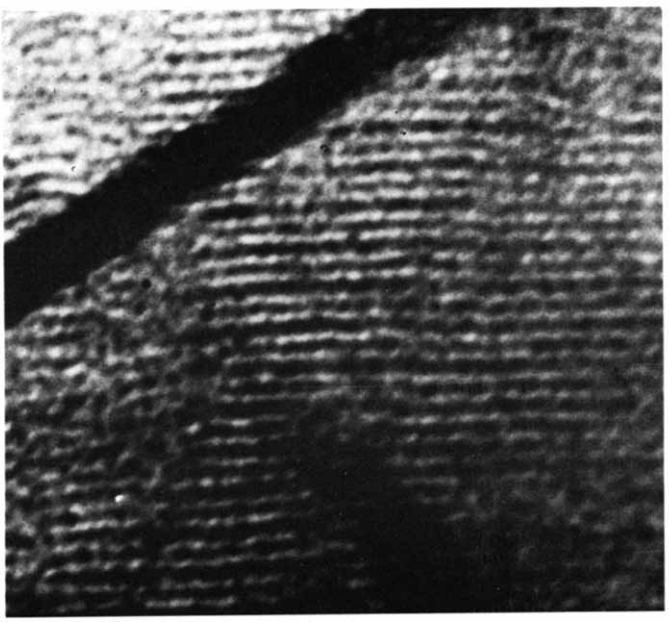

Fig. 4. Electron micrograph of myelin after glutaraldehyde vapor fixation, gelatin embedding stained with $KMnO_4$. 480450 ×.

Next, we investigated agar as an embedding medium (Scheme 3), with additional cross-linking of the block pieces by formaldehyde solution. Again, the cross-linking was ineffective, and we encountered sectioning difficulties. Also, similar to gelatin, general preservation was poor. Due to the necessity of doing dehydration and infiltration at the relatively high temperature of 45°, we concluded that there were no advantages to the agar over gelatin.

Glutaraldehyde vapor fixed frog sciatic nerve was embedded in the water-soluble epoxy resin Aquon[10] according to GIBBONS[10, 11], except that DMP-30 (used with Epon) was substituted for benzoyl dimethylamine as the catalyst with equally good

1. All solutions were made in boiling water and had to be maintained at 45°.
2. Dehydration and infiltration were done with the following solutions: 0.5-, 1-, 2- and 5% for 30 min each at 45°.
3. Change into fresh 5% for 30 min at 45°.
4. The 5% solution with the pieces of tissue was poured into a shallow dish and allowed to set at room temperature.
5. Pieces were cut out and dried in a vacuum desiccator attached to a small mechanical pump for 4–6 h.
6. Dried pieces were mounted on pegs with epoxy glue, and set till dry.
7. Sectioning was with a diamond knife, and sections were floated onto 10% glycerin in water.

Scheme 3. Agar embedding procedure.

results. Sections cut best on a 0.5% solution of chrom-alum ($K_2SO_4 \cdot Cr_2(SO_4)_3 \cdot 24 H_2O$, ref. 9), but there was a tendency for the sections to break up despite all precautions. $KMnO_4$ gave better section contrast than the uranyl acetate and lead citrate, but this was still very low. A combination of all three stains gave improved contrast, but still much lower than with conventional embedding materials. General preservation of the larger section fragments was fair, but non-uniform.

Durcupan[12] water-soluble epoxy resin was also used. Blocks polymerized at 37° for 24 h sectioned easily on double glass-distilled water, although some breaking up of the sections occurred. Similar to the Aquon embedded glutaraldehyde vapor fixed sciatic nerve, the best section contrast was obtained using the triple-stain. However, use of 100 kV accelerating voltage with a 30 μ objective aperture still gave a low contrast.

DISCUSSION

Fixative action

Formaldehyde. Since formaldehyde is only monofunctional, its cross-linking capabilities are less than those of the bifunctional glutaraldehyde. Formaldehyde is known to react with unsaturated bonds of phospholipids[13], and so may cause eversion of the hydrocarbon chain from a hydrophobic region to the aqueous polar region, as appears to be the case for OsO_4[1]. Formalin fixatives used in light microscopy can cause an "unmasking" of lipids with respect to lipid stains[14]. The X-ray data show a shrinkage in periodicity (Fig. 1b) together with a large redistribution of intensity indicating molecular re-arrangement, perhaps due to denaturation of membrane protein by the impurities present (methanol, formic acid, *etc.*).

Freshly prepared formaldehyde solutions made by dissociation of paraformaldehyde suspensions in phosphate buffered saline produced a cross-linked (stiffened) nerve which gave an X-ray pattern with normal periodicity and intensity distribution. However, the degree of cross-linking seemed inadequate since immersion of the fixed nerve in phosphate buffered saline for several h led to loss of most of its rigidity. More extensive cross-linking was expected using the bifunctional glutaraldehyde.

Glutaraldehyde. Allowance also has to be made for the fact that the available glutaraldehyde preparations are very impure (containing among other things large amounts of phosphoric acid to reduce polymerization) and are intended for use in tanning. Alterations in the X-ray diffraction intensity distribution following glutaraldehyde fixation could result from acrolein and acids[13] that are present in most

available commercial glutaraldehyde preparations. These products could cause a number of alterations in the molecular structure of proteins similar to the changes apparent after formalin fixation. However, similar intensity changes are seen after fixation with re-distilled and purified glutaraldehyde (Fig. 3c).

Several possibilities have been investigated to determine the nature of the effects of glutaraldehyde on cellular structures. The osmolality of the fixative solution is considered a factor by some investigators (*e.g.* see MASER *et al.*[15]). The effects of sucrose added to purified glutaraldehyde solution in phosphate buffered saline (Fig. 3d) suggest that the tonicity or osmolality may be significant factors in obtaining a normal X-ray pattern with glutaraldehyde solution. Consideration has also been given to the results of LENARD AND SINGER[16], who showed by optical rotatory dispersion studies that glutaraldehyde causes significant structural modifications in protein helices. On the other hand, glutaraldehyde has been used in X-ray crystallography to stabilize protein crystals without severe conformational changes in the protein structure[17].

A third possibility that is currently being investigated in our laboratory is the nature and extent of the effects of impurities present in glutaraldehyde (J. C. ROBERTSON, personal communication) as determined by the 350–220 mμ region of the ultraviolet spectrum (see ANDERSON[5]). A stock solution from a previously unopened bottle of Fisher 50% Biological Grade glutaraldehyde gave a spectrum with peaks at 280 mμ and 235 mμ. Purification of the stock with Norit EXW charcoal yielded a spectrum with a peak at 280 mμ, but an absence of a peak at 235 mμ. We concluded that the peak at 235 mμ is due to impurities present in the glutaraldehyde (polymers, oxidation product impurities, acrolein, acids, *etc.*) (see also ANDERSON[5]). Current work indicates that the concentration of the impurity (235 mμ peak) increases on storage. The feasibility of obtaining a pure glutaraldehyde reagent and of stabilizing it for routine use in fixation is being investigated in our laboratory.

It is possible that the X-ray diffraction pattern changes seen after fixation of the nerve with glutaraldehyde solutions are due to conformation changes of the membrane protein. It is also possible that the amount of glutaraldehyde taken up by the membrane protein is so large that the glutaraldehyde residues themselves modify the electron density of regions of the membrane. This possibility was investigated by calculating the electron density maps for the glutaraldehyde fixed membranes. The phase of all reflections was taken as positive[18]. The results are summarized in Fig. 5. They indicate a re-arrangement of the structure of one portion of the membrane rather than a piling up of electron dense groups.

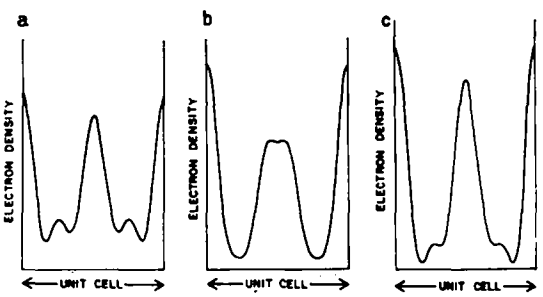

Fig. 5. Electron density maps. (a) Normal. (b) Glutaraldehyde solution. (c) Glutaraldehyde vapor.

Normal myelin structure was initially considered preserved when glutaraldehyde vapor fixation was used for a limited time (4 h), but the structure appeared to deteriorate after further fixation (16 h or more). However, it was subsequently determined that the glutaraldehyde was washed out by the phosphate buffered saline during the X-ray scan of the 4-h fixed nerve. After longer periods of fixation, there apparently was sufficient glutaraldehyde present in the myelin so that the fixative was not washed out by the phosphate buffered saline in the short time (2 h) required for a scan. The electron density map for glutaraldehyde vapor fixed material (Fig. 5c) appears similar to that for purified glutaraldehyde solution (Fig. 5b) indicating that the structural changes are probably similar for vapor and solution, and occur when the nerve accumulates more than a certain concentration of aldehyde. Presumably, these changes occur when cross-linking to form inter- and intramolecular protein bridges[13,19,20] becomes extensive.

The reaction of aldehydes with lipids has not been very well studied[13], but since lipid can be readily extracted from aldehyde fixed tissue[6], it appears that lipid is weakly cross-linked by aldehydes. It is evident from our studies that a more systematic search needs to be made for suitable bifunctional cross-linking reagents that will produce intermolecular cross-links between like molecules of polar and non-polar lipids, and between these molecules and proteins.

Dehydration and embedding

Although the normal myelin membrane periodicity is retained following fixation with formaldehyde prepared from paraformaldehyde, post-fixation with OsO_4, acetone dehydration and Epon embedding causes a final shrinkage of the electron microscope periodicity from 171 to 144 Å (Fig. 2). The myelin membrane structure is very regular, similar to the appearance of lipid depleted myelin[21,22]. The fine structure also shows a marked globular or beaded appearance similar to that reported for other fixation, dehydration and embedding procedures[23–25].

Acetone dehydration of frog sciatic nerve fixed by glutaraldehyde in either solution or vapor phase resulted in an expansion of the myelin membrane periodicity from 171 to 185–190 Å (Fig. 3g). The absence of a shrinkage on dehydration suggests that myelin does not contain significant water layers between membranes. Removal of the membrane lipid does not cause a shrinkage of the myelin membrane periodicity, although all the cholesterol (20 % of the membrane lipid) and about 80 % of the polar lipids (nearly 70 % of the membrane lipid) were assumed to be extracted during dehydration (see also KORN AND WEISMAN[6]). We assume that the glutaraldehyde has more firmly stabilized the protein structure of the myelin against dehydration.

Modification of the fixation and embedding techniques

It was suggested[1] that an improved fixation procedure might use an initial exposure of the nerve for a short period (5–10 min) to 1 % OsO_4 vapor, since this amount of heavy metal fixative does not result in any molecular re-arrangement of the membrane structure. Also, it was proposed that the second step should be multiple steps of aldehyde fixation. However, preliminary results indicate that even at low concentrations of aldehyde, the combined fixative procedure can produce significant molecular alterations. It is necessary to find a reagent which will give sufficient cross-linking of lipids (particularly the non-polar lipids), and yet not cause re-arrangement

of the molecular structure. Another requirement is the development of a water-soluble embedding material which will not cause extraction of lipids during dehydration and polymerization.

The improvement of existing thin section preparation techniques is only a partial solution to the visualization of biological specimens in the electron microscope. The ultimate most promising approaches to these problems appear to be the use of extra high voltage (1 MeV or higher) because the reduced ratio of inelastic to elastic scattering cross sections leads to reduced electron beam specimen damage. In addition, the development of wet specimen chambers for maintaining membrane specimens in fully hydrated conditions for electron diffraction and electron microscopy are indicated. The development of methods (other than heavy metal staining) for contrast enhancement (*e.g.* phase contrast, strioscopy) are also necessary. We are presently pursuing these long-term objectives in our laboratory. In the meantime, we wish to improve the present techniques to make them more reliable, and a more realistic representation of intact biological systems.

ACKNOWLEDGMENTS

The authors wish to thank A. Hamad and S. Young for their assistance. This work was supported by American Cancer Society grant, E-457, and National Science Foundation grant, GB-7130.

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