BBA 75327

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

I. OSMIUM TETROXIDE AND POTASSIUM PERMANGANATE

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(Received April 8th, 1969)

SUMMARY

1. The influence of standard electron microscope OsO_4 and $KMnO_4$ fixatives, and some standard dehydration and embedding procedures, on the molecular structure of the myelin membrane of frog sciatic nerve were studied with a combination of small angle X-ray diffraction, electron microscopy and biochemical techniques.

2. The observed periodicity and intensity changes indicated that re-arrangement of the membrane structure occurred with these fixatives. OsO_4 and $KMnO_4$ fixatives did not sufficiently stabilize the molecular structure, since subsequent acetone and alcohol dehydration agents produced large changes in the X-ray diffraction pattern of fixed nerve. These changes were associated with a considerable degree of extraction of cholesterol and a smaller degree of extraction of polar lipids.

INTRODUCTION

Various fixation, dehydration and embedding procedures[1] have been employed in the thin section technique of preparing biological specimens for viewing in the electron microscope. Recently, there have been increasing indications of artifacts occurring during some standard electron microscope preparation procedures. In particular, the interpretation of the tri-laminar thin section appearance of membranes has been questioned. Unless a large amount of lipid extraction occurs during conventional thin section preparation techniques, it is difficult to understand why cell membranes have the same thin section appearance after thorough lipid extraction. FLEISCHER *et al.*[2] have shown that lipid-depleted mitochondria retained the lamellar membrane structure seen in conventional thin sections of mitochondrial membranes. NAPOLITANO *et al.*[3] have shown that myelin extracted with chloroform–methanol (2:1, v/v) retains a lamellar membrane structure which is similar to the conventional thin section appearance of myelin. For myelin, there is a considerable difference in the periodicity observed by X-ray diffraction of fresh peripheral nerve (171–173 Å for amphibians, 180–185 Å for mammals[4]), and by electron microscopy of thin

section (115–155 Å for most electron microscope fixation and embedding procedures). This may be interpreted as a removal of water layers between membranes, but also as due to a change in thickness of the membranes due to molecular disorder. Recently, several quantitative studies of lipid extraction during processing for electron microscopy have been reported[5–13]. In general, these studies have indicated that glutaraldehyde fixation gives weak cross-linking of fixation of both phospholipid and cholesterol[7]. Strong stabilization of the phospholipid, but very weak stabilization of cholesterol is given by OsO_4 and $KMnO_4$ fixation[7,10–12] and aldehyde *plus* OsO_4 fixation gives relatively strong stabilization of both phospholipids and neutral lipids[8,9]. These studies also show that while a very small amount of lipid is extracted during fixation[11,13], significant lipid extraction occurs during dehydration[7–13] and embedding (refs. 5–7 and 9). These results raise the question of whether the thin section image gives a reliable indication of the fine structure. In addition, the thin section method is known not to show certain membrane-associated structures shown by other electron microscope techniques, *e.g.* mitochondrial 90-Å inner membrane subunits and their associated stems shown by negative staining[14,15]; the polysaccharide coat associated with cell membranes seen in light microscopy[16,17] is only occasionally seen by electron microscopy[17–21]; and the surface projections of cell membranes and organelles shown by freeze etching[22–26]. Other specimen preparation techniques are known to eliminate some alterations common to the thin section technique, *e.g.* elimination of shrinkage of the tri-lamellar periodicity of myelin by freeze etching[27], and the retention of cytochemical and histochemical activity obtained with freeze substitution[28].

In this report highly ordered myelin in peripheral nerve is examined by small angle X-ray diffraction. When correctly phased[29], the X-ray diffraction pattern can supply more detail about molecular structure than is possible to obtain with the current techniques of electron microscopy. Therefore, we have used small angle X-ray diffraction to investigate the effects of electron microscope thin section preparative techniques.

Portions of this report have been published in abstract form[30].

MATERIALS AND METHODS

Grass frogs (*Rana pipiens*) were kept without feeding in an enclosure with a constant flow of cold tap water for an average of from 7 to 30 days. The frogs were pithed and the sciatic nerve immediately removed and carried through the preparation procedure of interest. X-ray diffraction of nerves immersed in phosphate buffered saline[31] was obtained using our described[29] automated Kratky camera arrangement[32].

Individual electron microscope fixation techniques will be described in EXPERIMENTS AND RESULTS. Staining of sections was carried out with lead citrate[33], or a 25% solution of uranyl acetate in methanol (filtered immediately before use) followed by lead citrate, or $KMnO_4$ (ref. 34). Sections were examined in a modified four-stage Siemens Elmiskop Ia (ref. 35) or in a Philips EM 300 electron microscope.

The solubility of cholesterol (Eastman Organic Chemicals, m.p. 148–150°) and egg lecithin (Nutritional Biochemicals Co.) in a range of dehydration and embedding solvents was examined (work in collaboration with D. Wong). The firmness of lipid cross-linking after different fixation techniques was estimated by extraction

by chloroform–methanol (2:1, v/v) and various dehydration and embedding reagents (work with Dr. J. G. Robertson, using thin-layer chromatography).

EXPERIMENTS AND RESULTS

Effect of OsO_4 on the myelin structure

Fig. 1a shows the X-ray diffraction pattern (full line) of fresh frog sciatic nerve immersed in phosphate buffered saline at 4–7°. The four main reflections are higher orders of the fundamental of 171–173 Å due to a double membrane repeat unit.

Very small amounts (less than one-tenth normal electron microscope fixation amounts) of OsO_4 were added to fresh nerve by exposure to the vapor of a 1% aqueous solution for 10 min at 4°. The nerve was suspended in a water saturated atmosphere in a sealed container to preclude dehydration. Analysis of the X-ray pattern so obtained (dashed curve, Fig. 1a) suggests that the osmium goes mainly to a single site. There is no significant molecular disorder at this concentration of osmium since the X-ray periodicity remains normal and the intensity distribution is only affected by the small expected amount for one main site of labeling[29]. The

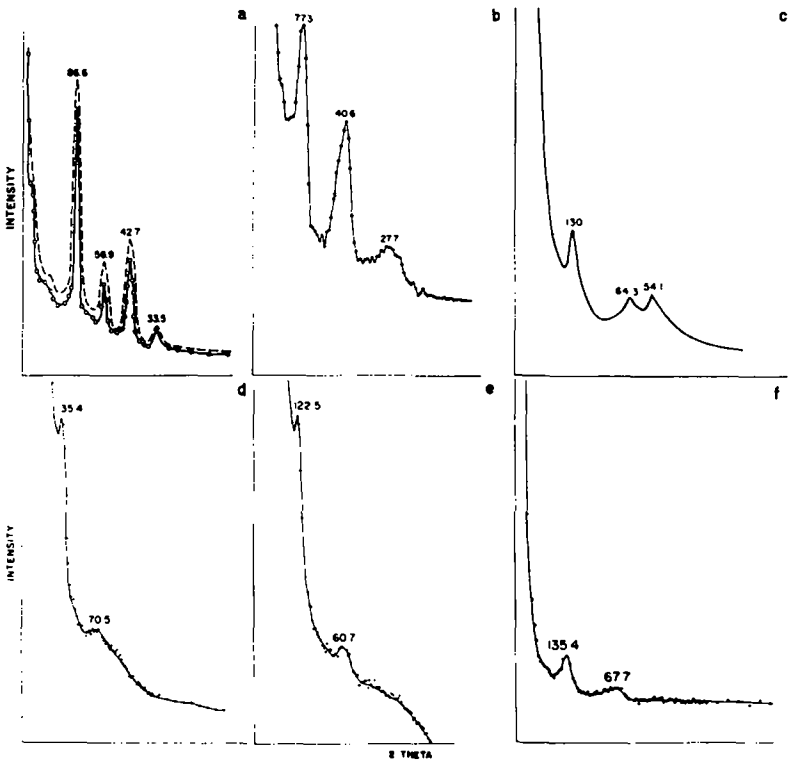

Fig. 1. Small angle X-ray diffraction of frog sciatic nerve. (a) (Full line) fresh sciatic nerve immersed in phosphate buffered saline. (Dashed line) fresh sciatic nerve stained with OsO_4 vapor for 10 min. (b) Sciatic nerve fixed 1.5 h in PALADE's OsO_4. (c) Acetone dehydration following PALADE's OsO_4. (d) Ethanol dehydration following PALADE's OsO_4. (e) Methanol dehydration following PALADE's OsO_4. (f) Sciatic nerve fixed in PALADE's OsO_4, dehydrated in acetone, embedded in Epon and polymerized.

electron microscope appearance of such light concentrations of osmium is shown in Fig. 2. Following exposure to the osmium vapor, the nerve was fixed for 4 h in glutaraldehyde vapor, dehydrated in acetone and embedded in Epon according to LUFT[36] (see below for procedures used).

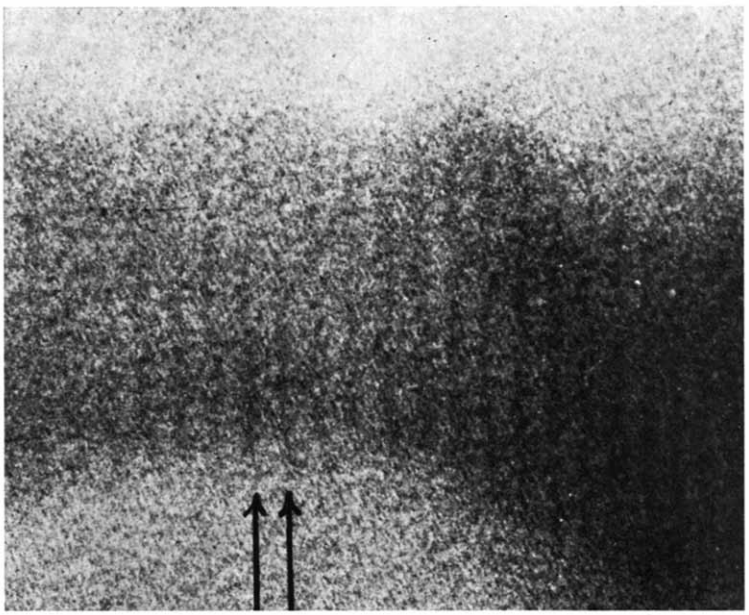

Fig. 2. Electron micrograph of sciatic nerve stained with OsO_4 for 10 min, fixed in glutaraldehyde vapor 4 h, dehydrated in acetone, embedded in Epon. 177960 ×.

Addition of further osmium (either as vapor or in the fixative solution of PALADE[37], 1.5 h at 4°) caused a transition from the X-ray pattern of Fig. 1a to the X-ray diffraction pattern of Fig. 1b. The first main site of osmium deposition quickly saturated and a significant staining of a second site occurred. The main observed abnormality is the shrinkage of the periodicity from 171 to 155 Å, suggesting molecular re-arrangement of the membrane. Disordering is also suggested by Fourier synthesis calculations of the expected intensity distribution based on averaged densitometer tracings of electron micrographs (Fig. 3). The densitometer trace records the

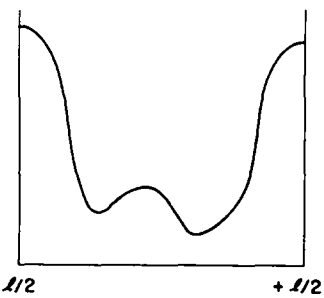

Fig. 3. Step function of OsO_4-fixed, Epon-embedded frog sciatic nerve myelin obtained from optical densitometry of electron micrographs.

variation in amount of osmium deposited, the rest of the unstained structure having too low a density to be recorded. Because of the linear relationship between optical density and mass thickness of the section, the densitometer trace can be taken as a direct indication of the linear distribution of osmium, and hence, of electron density. The electron density (in arbitrary units) was used with a Fourier synthesis to calculate a set of observed X-ray intensities. The calculated data were matched with the observed intensities by normalizing the (200) peaks. The degree of fit obtained between calculated and observed intensities is indicated in Table I.

TABLE I

COMPARISON OF OBSERVED X-RAY INTENSITIES OF OSMIUM-FIXED NERVE WITH EXPECTED INTENSITIES CALCULATED FROM DENSITOMETRY OF ELECTRON MICROGRAPHS (FIG. 3)

hlk	*Observed Embedded nerve*	*Calculated**
100	800.0	1982.0
200	100.0	100.0
300	32.0	90.0
400	Not observed	57.6
500	Not observed	0.77
600	Not observed	0.81

(200) used as reference point

* Assuming phases of reflections all positive.

TABLE II

ACETONE DEHYDRATION FOR EPON EMBEDDING

The tissue was brought from 4° to room temperature following the change into 70% acetone. The 100% acetone was made anhydrous by contact drying with $CaSO_4$ (no indicator). Analytical reagent acetone was used, as lower grades gave irreproducible results. The vial in which the tissue was dehydrated was capped throughout the dehydration procedure.

30% acetone in water	— 2 min at 4°
70% acetone in water	— 2 min at 4°
90% acetone in water	— 2 min at room temperature
100% acetone	— 2 changes of 3 min at room temperature

The effects of acetone, ethanol and methanol dehydration of OsO_4 fixed nerve were investigated. The time of dehydration (Table II) was adjusted to minimize exposure of the nerve to the dehydrating reagent. Following dehydration the fixed nerve was placed in the sealed cell without any dehydrating reagent, and examined by the small angle X-ray diffraction camera. (Parallel experiments demonstrated that presence or absence of the dehydrating reagent had no significant effect on the X-ray diffraction pattern.) The resulting X-ray diffraction patterns showed a further larger change (Figs. 1c–1e). The myelin structure now appears to be considerably disordered as indicated by the weak reflections obtained. The reduction in coherent scattering is not due to the change of solvent since the myelin has an electron density close to that of water, while the solvent is less electron dense (*e.g.* acetone is 0.23 $e/Å^3$) than water (0.33 $e/Å^3$). The observed reflections must now be interpreted as orders

TABLE III

EPON EMBEDDING FOLLOWING ACETONE DEHYDRATION

We found that the Epon–DDSA–NMA mixture[36] should be made up fresh daily for best results. Once catalysed, the Epon mixture was not used after standing over 0.5 h. When embedding for electron microscopy, the tissue was chopped into small pieces at the beginning of fixation (this did not alter X-ray results since the nerves used were less than 1.0 mm thick, allowing use of whole nerve) and placed in Beem capsules for embedding.

Step 1	1 : 1 (v/v) mixture of 100% acetone and catalysed Epon (Luft No. 2 (ref. 24)) for 20 min at room temperature (vial remained capped)
Step 2	Pure catalysed Epon for 1 h at 37° (to remove remaining acetone, so the vial was uncapped)
Step 3	Placed in fresh catalysed Epon and polymerized at 70° for 16 h

of a double membrane repeat unit of only 116–135 Å. We interpret these changes as due to combined molecular re-arrangement following removal of water from protein and immersion in a non-polar solvent and due to extraction of the membrane lipids by the dehydrating reagent. Chemical determinations by Dr. John Robertson in our laboratory indicate 55% of the cholesterol, but little of the phospholipid, is extracted during acetone dehydration. A frog sciatic nerve was fixed whole in Palade's OsO_4 fixative, dehydrated in acetone (Table II) and embedded in Epon (Table III). The X-ray diffraction pattern obtained from both non-polymerized and polymerized embedded nerve (Fig. 1f) indicates that little change occurs between dehydration and epoxy resin embedding. The observed reflections remain weak, and the double membrane repeat unit remains at about 116–135 Å.

Effect of $KMnO_4$ fixation of myelin

The small angle X-ray diffraction pattern in Fig. 4a was obtained after fixation of frog sciatic nerve in 1% $KMnO_4$ in phosphate buffered saline or veronal acetate buffer, pH 7.2 at 4° for 1.5 h. The action of $KMnO_4$, unlike that for Palade's OsO_4,

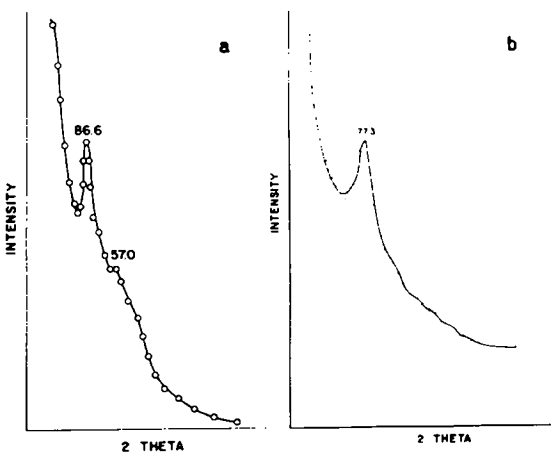

Fig. 4. Small angle X-ray diffraction of frog sciatic nerve. (a) Fixed in $KMnO_4$. (b) Fixed in $KMnO_4$, dehydrated with acetone.

showed no shrinkage of the fundamental double membrane periodicity at this stage. Some molecular rearrangement is indicated as seen in the suppression of the higher order reflections. Acetone dehydration (Table II) of the $KMnO_4$-fixed sciatic nerve resulted in an X-ray diffraction pattern (Fig. 4b) that showed an intensity distribution similar to that obtained following fixation alone. However, the dehydrated nerve now showed a shrinkage of the fundamental periodicity from 173 to 155 Å, and a total absence of all reflections beyond the second order.

DISCUSSION

Fixative action

OsO_4

Alterations of both the relative intensity distribution and the periodicity of the small angle X-ray diffraction patterns of frog sciatic nerves were evident following fixation by the conventional heavy metal techniques investigated.

The chemical and physical actions of OsO_4 have been the subjects of several reports[10,38–48]. OsO_4 is known to react with a variety of protein groups and at the double bonds and polar groups of unsaturated lipids[38–41,43–46]. HAKE[40] has shown that protein–OsO_4 reactions resulted in partial denaturation of the proteins, but that some intermolecular stabilization had also occurred. OsO_4–lipid reactions occur initially at the double bonds of the unsaturated fatty acids to form osmate esters[41,42,44,46,47] and then may move to the polar groups[42,47]. The formation of the esters or polar group bridges may also be between adjacent lipid molecules, and provide the intermolecular links necessary for stabilization. However, there is also some evidence that OsO_4 remains attached to the double bond site[41,42], creating additional polar groups which attach the fatty acid hydrocarbon chain to the aqueous surface (essentially "everting" the hydrocarbon chain). Studies by DREHER *et al.*[42] using negatively stained fixed and unfixed lung lipoprotein bilayers indicate alteration of both the protein and the lipid structures (presumably due to eversion of the hydrocarbon chain).

A re-arrangement of membrane lipid during OsO_4 fixation is supported by the combination of the X-ray data and electron microscope data given in this report. The periodicity changes observed by X-ray diffraction of fixed nerve (without further processing for electron microscopy) were found to vary from no change with very small amounts of OsO_4 taken up by the nerve (Fig. 1a) to a shrinkage of from 171 to 155 Å (about 11%). This relatively small change would appear compatible with thickness reduction due to partial exteriorization of the unsaturated hydrocarbon chain as a result of conversion of essentially non-polar double bonds to polar complexes with osmium.

The modified intensity distribution due to OsO_4 will be discussed in relation to the fixed and embedded nerve.

$KMnO_4$

Results of fixation with $KMnO_4$ (Fig. 4a) agree with the results of FERNANDEZ-MORAN AND FINEAN[49], but a contraction of the periodicity to 148 Å was observed by FINEAN[50]. The intensity distribution from $KMnO_4$-fixed nerve will be discussed together with the effect of dehydration and embedding.

$KMnO_4$ reacts with a variety of protein groups[40,47], but does not create cross-

links within and between proteins. Instead, $KMnO_4$ acts as a strong denaturant[45] causing a degradation of the tertiary structure[40,45]. The mechanism of $KMnO_4$–lipid reactions is not well understood, although the reaction apparently occurs at the double bonds and polar groups of unsaturated lipids[10,47], and some cross-linking of lipids is effected[10].

Dehydration and embedding

Further shrinkage of the myelin membrane periodicity and also increased membrane disorder is indicated by the reduction in the scattered intensity which occurs after dehydration (Fig. 1c–1e). The dehydration might remove a water layer from between adjacent membranes. Alternatively, shrinkage might be due to extraction of unfixed or partially fixed lipid by the dehydrating reagent.

A freeze-etching study of myelin by BISCHOFF AND MOOR[27] suggests that a significant water layer exists between the apposed cytoplasmic surfaces. This is based on the presence of irregular projections on the cytoplasmic surface of the membrane which seem to require a water space between the membranes. However, the projections could be explained as due to the tearing away of portions of the adjacent membrane, in which case, a water space does not need to be postulated.

The significance of water in myelin membrane structure has been studied by FINEAN *et al.*[51,52] and LADBROOKE *et al.*[53]. In the work of FINEAN *et al.*[52], pellets of isolated myelin were dried and the X-ray diffraction patterns examined at different residual water contents. Its was concluded that a residual 30–40% of water was closely associated with the myelin membrane. However, in the unsonicated material, and, to some extent in the sonicated material, the myelin vesicles are liable to contain soluble salts and other substances. In the final stages of dehydration, the flattened and close-packed vesicles may contain a significant amount of concentrated solution. This may cause preferential loss of water from the membrane before water is finally lost from between the membranes.

LADBROOKE *et al.*[53] examined differential thermal analysis of isolated myelin pellets at different stages of dehydration. They concluded that a minimal amount (20%) of water was required in the myelin preparation to maintain the normal lipid structure of the membrane. As already discussed, this figure could be too large if the myelin vesicles contain concentrated solutions of salts and other substances. However, accepting the 20% myelin water content figure, calculation of the equivalent average thickness of water between adjacent membranes gives a thickness of the water layer of only 17 Å. Assuming that the protein of myelin (20% of the myelin dry weight) has a normal hydration of 30%, the intervening water layer has a thickness of only 13 Å. Such thicknesses of water layers could not account for the observed shrinkages in periodicity that occur on dehydration. Following fixation and dehydration shrinkage of from 41 to 55 Å or from 25 to 35% of normal periodicity occurs. The electron density map calculated by AKERS AND PARSONS[51] does not indicate a significant water layer in the two membrane repeat structure. Inverse Fourier transform calculations by C. K. AKERS AND D. F. PARSONS (personal communication) indicate that water layers of 3–4 Å thickness between membranes could have been detected as a dip in the electron density distribution.

If a significant water layer existed between membranes, the previous work of BAHR *et al.*[55,56] would suggest that OsO_4 fixation should be accompanied by an in-

crease in volume of the membrane bounded water space. Our X-ray data indicate a shrinkage from normal periodicity of about 11 %.

A water layer could also be associated with a loose polysaccharide coat on one or both sufaces of the myelin membrane. In this case the observed X-ray changes might be attributed to breakdown of the polysaccharide due to the fixative and dehydration solvent. Studies have shown that polysaccharides are associated with some membranes (*e.g.* see RAMBOURG AND LEBLOND[20] and ITO[57]). BRANTON[24] and STAEHELIN[26] have proposed models, based on freeze-etch studies, which incorporate a superficial layer of non-lipid materials. Biochemical[58] and histochemical[59] studies of myelin have not given evidence that mucopolysaccharides are associated with peripheral myelin.

The available evidence does not give good support for the possibility that the large X-ray changes observed following dehydration are due to removal of a water layer between membranes.

The second main possibility is that the dehydration solvent extracts out a significant amount of membrane lipid.

Quantitative studies on various tissues have demonstrated that 0–40 % of the saturated lipids and 20–100 % of the neutral lipids are extracted by ethanol and acetone after OsO_4 fixation[5,7,8,10–13]. MORGAN AND HUBER[8] and STEIN AND STEIN[9] reported that glutaraldehyde pre-fixation *plus* OsO_4 fixation lowered the extractability of both phospholipids and neutral lipids. However, KORN AND WEISMAN[7] and Dr. J. G. Robertson of our laboratory report that pre-fixation with glutaraldehyde *plus* OsO_4 does not affect the extractability of lipids by acetone and ethanol. A similar lipid solvent action is known to occur with some of the embedding reagents[9,60]. Our X-ray data on Epon embedding indicate (Fig. 1f) that little further change occurs either on immersion in unpolymerized Epon or following polymerization of the Epon.

Biochemical studies uniformly indicate that OsO_4 fixation gives the best stabilization of lipids. Additional stabilizing by a stage of aldehyde fixation seems doubtful. Nevertheless, the X-ray results show that OsO_4 fixation is not enough to prevent extraction of cholesterol and consequent membrane structural changes. We compared the observed X-ray diffraction pattern with that calculated from electron microscopy of Epon embedded OsO_4-fixed nerve (Table I). The pattern calculated for two main osmium staining sites agrees, in general, with the observed pattern. However, it differs in that the observed pattern shows no significant (400) reflection. This could be explained on the basis of insufficient regularity of the stained membrane material (the intraperiod line stains in an ill-defined way).

Since there is relatively little difference between the OsO_4-fixed and dehydrated nerve, and the embedded nerve, we can assume that the dense line–intraperiod line pattern of staining exists before embedding. However, the dramatic difference between the diffraction patterns for dehydrated and non-dehydrated nerve suggests that the osmium stain may have a completely different arrangement before the dehydration step. The possibility that the alternating laminar appearance of myelin in thin sections, and possibly the "tramline" appearance of other membranes, is an artifactual re-arrangement of the remaining stained membrane material after extraction of some of the lipids by dehydration solvents must be considered. We will discuss this in detail in another report where we will compare calculated and observed

X-ray intensities for the re-distributed metal stain using the newly available electron density map of unstained myelin[29].

It should be noted that use of the technique of small angle X-ray diffraction of myelin for assessing reagents used for electron microscope preparations of material is severely limited in the case of heavy metal fixatives such as OsO_4 because the metal stain is mainly responsible for the diffraction pattern and not the membrane components. The technique shows most usefulness in examining aldehyde fixation specimen preparation methods.

Even though $KMnO_4$ fixation has the advantage that a normal X-ray periodicity was retained, it has the disadvantage that lipids are more easily extracted by ethanol and acetone following $KMnO_4$ fixation than after OsO_4 fixation[7,10]. Proteins undergo extensive denaturation with $KMnO_4$ treatment[40,45]. Some embedding reagents also extract lipids after $KMnO_4$ fixation[60].

Significance of X-ray observations for improving fixation and embedding of membranes

The reported results emphasize that the fixation step, which may cause a disordering of membrane protein and lipids, should be regarded separately from the dehydration step, which may cause a different kind of disordering of membrane components as a result of extraction of cholesterol (and possibly also polar lipid). Because of the availability of a number of types of water-soluble embedding reagents[1], it would appear not difficult to prevent the cholesterol extraction. However, it is more difficult to prove that the fixative reagent does not cause disordering and, in addition, that is has stabilized the tissue sufficiently to withstand the disruptive effects of immersion in an organic medium, with possible re-orientation of polar and non-polar portions of the membranes, and the disruptive effects of the polymerization step.

It has already emphasized that the small angle X-ray method is at a disadvantage in analyzing changes during fixation by heavy metal fixatives. The membrane components contribute little to the diffraction pattern with the usual concentrations of OsO_4 in the membrane. However, the present study has also shown that there is a limited, low concentration range of OsO_4 vapor fixation in which the myelin structure is unchanged. Hence, it appears that the first step of an improved fixation procedure should consist of less than 15 min exposure to OsO_4 vapor at 4°. This might be followed by multiple treatments with small concentrations of other cross-linking reagents (particularly aldehydes) so that many different kinds of sites are cross-linked without reaching the high concentrations with any one reagent that cause structural changes.

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.

REFERENCES

1 A. M. GLAUERT, in D. KAY, *Techniques for Electron Microscopy*, Blackwell, Oxford, 2nd Ed., 1965, p. 166.
2 S. FLEISCHER, B. FLEISCHER AND W. STOECKENIUS, *J. Cell Biol.*, 32 (1967) 193.
3 L. NAPOLITANO, F. LEBARON AND J. SCALETTI, *J. Cell Biol.*, 34 (1967) 817.

4 J. B. FINEAN, in J. N. CUMING, *Modern Scientific Aspects of Neurology*, Arnold, London, 1960, p. 232.
5 R. DUNCAM DALLAM, *J. Histochem. Cytochem.*, 5 (1957) 178.
6 B. EDGREN AND B. FREDRICSSON, *Acta Morphol.*, 1 (1958) 384.
7 E. D. KORN AND R. A. WEISMAN, *Biochim. Biophys. Acta*, 116 (1966) 309.
8 T. E. MORGAN AND G. L. HUBER, *J. Cell Biol.*, 32 (1967) 757.
9 O. STEIN AND Y. STEIN, *J. Cell Biol.*, 33 (1967) 319.
10 A. ONGUN, W. W. THOMPSON AND J. B. MUDD, *J. Lipid Res.*, 9 (1968) 416.
11 G. B. DERMER, *J. Ultrastruct. Res.*, 22 (1968) 312.
12 D. R. SAUNDERS, J. WILSON AND C. E. RUBIN, *J. Cell Biol.*, 37 (1968) 183.
13 R. J. BUSCHMANN AND A. B. TAYLOR, *J. Cell Biol.*, 38 (1968) 252.
14 H. FERNANDEZ-MORAN, *Circulation*, 26 (1962) 1039.
15 D. F. PARSONS, *Science*, 140 (1963) 985.
16 D. R. MCCONNACHIE AND P. FORD, *J. Embryol. Exptl. Morphol.*, 16 (1966) 17.
17 S. S. SPICER, M. W. STALEY, H. G. WETZEL AND B. K. WETZEL, *J. Histochem. Cytochem.*, 15 (1967) 225.
18 J. H. LUFT, *Federation Proc.*, 25 (1966) 1773.
19 D. C. PEASE, *J. Ultrastruct. Res.*, 15 (1966) 555.
20 A. RAMBOURG AND C. P. LEBLOND, *J. Cell Biol.*, 32 (1967) 27.
21 J. D. BERLIN, *Radiation Res.*, 24 (1968) 347.
22 H. MOOR AND K. MÜHLETHALER, *J. Cell Biol.*, 17 (1963) 609.
23 J. K. KOEHLER, *J. Ultrastruct. Res.*, 16 (1966) 359.
24 D. BRANTON, *Proc. Natl. Acad. Sci. U.S.*, 55 (1966) 1048.
25 H. MOOR, *Protoplasma*, 64 (1967) 7.
26 L. A. STAEHELIN, *J. Ultrastruct. Res.*, 22 (1968) 326.
27 A. BISCHOFF AND H. MOOR, *Z. Zellforsch.*, 81 (1967) 571.
28 W. BERNHARD AND E. H. LEDUC, *J. Cell, Biol.*, 34 (1967) 757.
29 C. K. AKERS AND D. F. PARSONS, *Biophys. J.*, 1969, in the press.
30 R. C. MORETZ, *J. Cell Biol.*, 39 (1968) 95a.
31 R. DULBECCO AND M. VOGT, *J. Exptl. Med.*, 99 (1954) 167.
32 O. KRATKY, *Progr. Biophys.*, 13 (1963) 105.
33 J. H. VENABLE AND R. COGLESHALL, *J. Cell Biol.*, 25 (1965) 407.
34 J. S. SUTTON, *J. Ultrastruct. Res.*, 21 (1968) 424.
35 H. M. JOHNSON AND D. F. PARSONS, *Proc. 25th Annual Meeting Electron Microscope Society America, Chicago, 1967*, Claitors, Baton Rouge, La., 1967, p. 236.
36 J. H. LUFT, *J. Biophys. Biochem. Cytol.*, 9 (1961) 409.
37 G. E. PALADE, *J. Exptl. Med.*, 96 (1952) 285.
38 G. F. BAHR, *Exptl. Cell Res.*, 7 (1954) 457.
39 G. F. BAHR, *Exptl. Cell Res.*, 9 (1955) 277.
40 T. HAKE, *Lab. Invest.*, 14 (1965) 1208.
41 E. D. KORN, *Biochim. Biophys. Acta*, 116 (1966) 325.
42 K. D. DREHER, J. H. SCHULMAN, O. R. ANDERSON AND O. A. ROELS, *J. Ultrastruct. Res.*, 19 (1967) 586.
43 O. R. ANDERSON, O. A. ROELS, K. D. DREHER AND J. H. SCHULMAN, *J. Ultrastruct. Res.*, 19 (1967) 600.
44 J. C. RIEMERSMA, *Biochim. Biophys. Acta*, 152 (1968) 718.
45 J. LENARD AND S. J. SINGER, *J. Cell Biol.*, 37 (1968) 117.
46 W. STOECKENIUS AND S. C. MAHR, *Lab. Invest.*, 14 (1965) 1196.
47 G. MILLONIG AND V. MARRINOZZI, in R. BARER AND V. E. COSSLETT, *Advances in Optical and Electron Microscopy*, Vol. 2, Academic Press, New York, 1968, p. 251.
48 H. SCHIECHL, *Z. Naturforsch.*, 236 (1968) 989.
49 H. FERNANDEZ-MORAN AND J. B. FINEAN, *J. Biophys. Biochem. Cytol.*, 3 (1957) 725.
50 J. B. FINEAN, *J. Biophys. Biochem. Cytol.*, 8 (1960) 13.
51 J. B. FINEAN, *J. Biophys. Biochem. Cytol.*, 3 (1957) 95.
52 J. B. FINEAN, R. COLEMAN, W. G. GREEN AND A. R. LIMBRICK, *J. Cell Sci.*, (1966) 287.
53 B. D. LADBROOKE, T. J. JENKINSON, V. B. KAMAT AND D. CHAPMAN, *Biochim. Biophys. Acta*, 164 (1968) 101.
54 C. K. AKERS AND D. F. PARSONS, to be published.
55 G. F. BAHR, G. BLOOM AND U. FRIBERG, in F. S. SJOSTRAND AND J. RHODIN, *Proc. Stockholm Conf. on Electron Microscopy, 1956*, Academic Press, New York, 1957, p. 106.
56 G. F. BAHR, G. BLOOM AND U. FRIBERG, *Exptl. Cell Res.*, 12 (1957) 342.
57 S. ITO, *J. Cell Biol.*, 27 (1965) 475.
58 R. U. MARGOLIS, *Biochim. Biophys. Acta*, 141 (1967) 91.
59 C. W. M. ADAMS AND O. B. BAYLISS, *J. Histochem. Cytochem.*, 16 (1968) 119.
60 J. R. CASLEY-SMITH, *J. Roy. Microscop. Soc.*, 87 (1967) 463.