BBA 75528

MYELIN STRUCTURE AND RETENTION OF CHOLESTEROL IN FROG SCIATIC NERVE EMBEDDED IN A RESORCINOL–FORMALDEHYDE RESIN

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(Received June 1st, 1970)

SUMMARY

1. The suitability of a resorcinol–formaldehyde resin as an embedding material for electron microscopy was examined. The method used was to embed frog sciatic nerve and to examine the myelin structure using electron microscopy of thin sections and the degree of extraction of cholesterol using quantitative thin-layer chromatography and colorimetric analysis.

2. The major portion of cholesterol was retained in nerves embedded in resorcinol–formaldehyde resin. The periodicity of the myelin was characteristically around 160 Å in comparison with 130 Å obtained for nerves embedded in Epon where a major portion of the cholesterol was extracted during dehydration with acetone.

3. The resorcinol–formaldehyde resin did not penetrate efficiently into the lumen of the axons which were invariably partially or completely collapsed. The poor penetration and staining properties of the resin appear to preclude its routine use in electron microscopy.

INTRODUCTION

The extraction of lipids from tissues during preparation of thin sections for electron microscopy has been well documented[1–24]. The extent of extraction varies according to the tissue and the method of fixation and embedding. Although organic dehydrating solvents and solvents used for staining reagents are the most obvious cause of lipid extraction, all the commonly used embedding materials also appear to be powerful lipid solvents[15,16]. The use of water soluble embedding materials even at low temperatures has not yet solved this problem[2,13].

In the present study a search was made for a material in which myelinated tissue could be embedded without extraction of cholesterol. A preliminary note about this study has been published[25].

MATERIALS AND METHODS

Tissue

Freshly excised sciatic nerves from grass frogs (*Rana pipiens*) killed by cervical dislocation, were rinsed briefly in phosphate-buffered saline[26], at 4°, dried lightly with

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filter paper and weighed. Dry weights were determined by drying the nerves to constant weight in a vacuum over P_2O_5 at 23°.

Electron microscopy

Frog sciatic nerves were fixed with glutaraldehyde (1.25 % in phosphate-buffered saline) for 3 h at 4° and/or in osmium tetroxide[27] at pH 7.4 for 1.5 h at 4°. The fixed nerves were rinsed in cold veronal–acetate buffer[27] for 30 min, cut into small pieces, dehydrated using acetone and embedded in Epon[28]. The acetone dehydration included a brief rinse in 30 % acetone at 4°, 2 min in 50 % acetone at 4°, 2 min in 90 % acetone at room temperature and then three changes each of 2 min in 100 % acetone at room temperature.

TABLE I

PROPERTIES OF RESORCINOL–FORMALDEHYDE RESINS

	Cascophen RS788-160	*Cascophen RS788-77*
Number of resorcinol molecules per chain	5–7	6–8
Water content	25 %	35 %
pH	6.4	6.6
Miscible with	Water, ethanol, acetone	
Non-miscible with	Ether, light petroleum, chloroform	
Solubility of cholesterol and tristearin (37°)	< 1 mg/ml	
Loss of weight and volume during polymerization	20–25 %	30–35 %

Alternatively the nerve pieces, without prior dehydration in acetone, were embedded in the resorcinol–formaldehyde resins; Cascophen RS788-77 or Cascophen RS788-160 (Borden Inc., 14 Johnson St., Bainbridge, New York 13733). The properties of the resins are described (Table I). Impregnation of fixed nerve with Cascophen was carried out over a period of 12 h at 37° using solutions of Cascophen in water (20, 40, 69, 85 % (w/w)). The final impregnation was carried out by rotating the pieces of nerve for 1 h at room temperature in a mixture containing a maximum concentration of Cascophen and 6 % formaldehyde for Cascophen RS788-77 and 7 % for Cascophen RS788-160. Formaldehyde was prepared as a 37 % solution from paraformaldehyde[29]. The final preparation of the embedded tissue, for sectioning, has been previously described[25].

Sections were cut on an LKB ultramicrotome using a diamond knife. Both Cascophen and Epon sections were post-stained with lead citrate[30] and viewed in a Philips EM300 at 100 KV.

Lipid extraction and analysis

Total lipids were obtained from fresh nerves or nerves fixed with glutaraldehyde osmium tetroxide by exhaustive extraction using chloroform–methanol (2:1, by vol.). The extracts were taken to dryness by rotary evaporation at room temperature. The residue was extracted with chloroform–methanol (2:1, by vol.), and the solution

containing the total lipids was dried down and weighed and then made up to 2 ml with chloroform–methanol (2:1, by vol.).

The level of cholesterol was determined by the method of SEARCY AND BERGQUIST[31]. Extraction of lipids from frog sciatic nerve fixed with osmium and embedded in Cascophen was carried out using chloroform–methanol (2:1, by vol.). The level of cholesterol was determined following fractionation of the extract by quantitative thin-layer chromatography. The amount of cholesterol extracted during acetone dehydration was determined after the acetone solutions had been taken to dryness and the residue extracted with a total volume of 2 ml chloroform–methanol (2:1, by vol.).

Thin-layer chromatography was carried out on silica gel G plates which were washed, prior to application of the samples, in the same solvent system used in the subsequent chromatography. The washing solvent was absorbed by a wad of filter paper clipped to the top of each plate. Lipid samples were applied to plates using an applicator similar to that described by MONTEIRO[32] and samples were chromatographed using the solvent system; light petroleum–diethyl ether–acetic acid (60:40:1, by vol.). Components were detected by exposing the plates to iodine vapour or by spraying the plates with a 50 % aqueous solution of H_2SO_4 or a 15 % solution of phosphomolybdic acid in ethanol. Sprayed plates were developed by heating at 140°. Components were isolated from plates stained with iodine, by pushing a piece of square hollow stainless steel tubing up the plate so that the silica gel fell through the tube into a test tube. The cholesterol was eluted from scrapings with chloroform-methanol (2:1, by vol.).

RESULTS

Electron microscopy of frog sciatic nerve embedded in Cascophen RS788-160 (Fig. 1) or in Cascophen RS788-77 showed considerable collapse of the axons. That this collapse occurred during impregnation with Cascophen was confirmed by a light microscopy study in which it was observed that the axons became increasingly wrinkled with

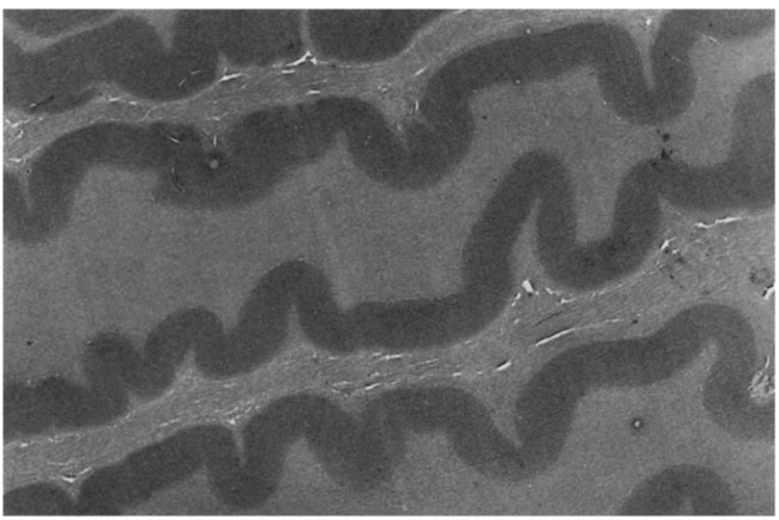

Fig. 1. Longitudinal section of a frog sciatic nerve axon fixed with osmium and embedded in Cascophen RS788-160. 4050 ×.

each successive Cascophen solution. If less than a 12-h period was used to impregnate the nerves the axons were more collapsed and if the nerves were tied off with cotton prior to dissection from the frog and were not cut up until the final polymerization step the axons were completely collapsed. Cascophen RS788-160 appeared to cause slightly

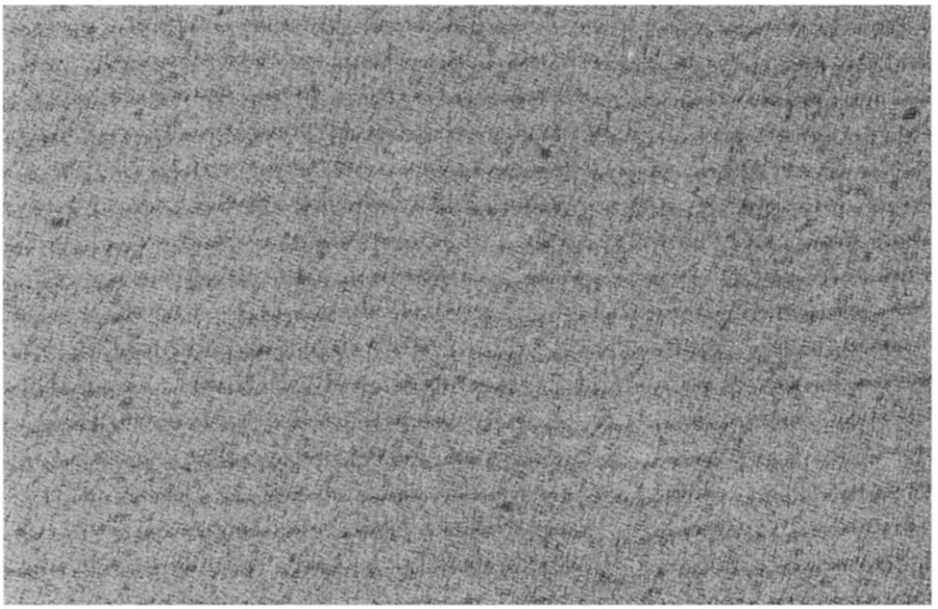

Fig. 2. Myelin structure of frog sciatic nerve fixed with osmium and embedded in Cascophen RS788-160. 278000 ×.

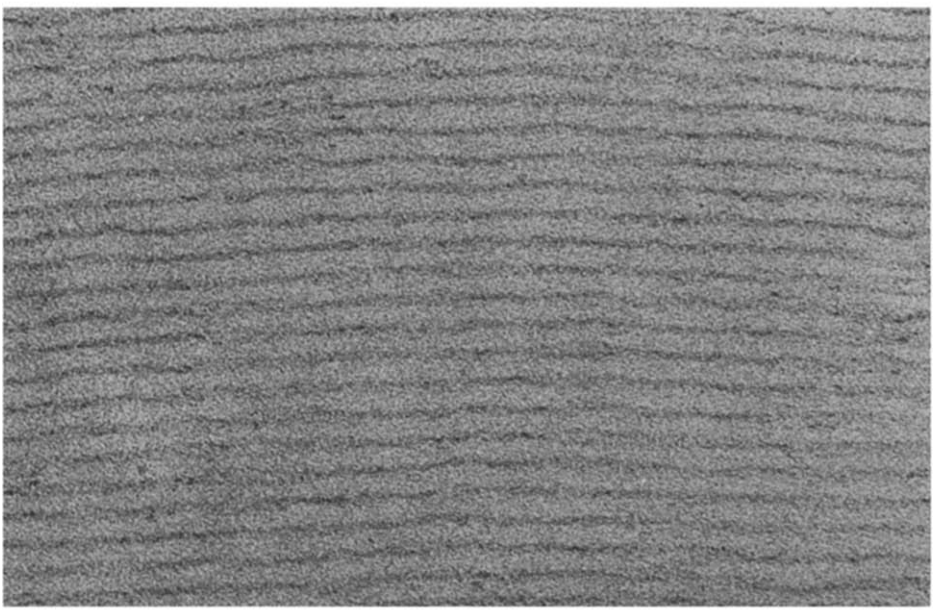

Fig. 3. Myelin structure of frog sciatic nerve fixed with osmium and embedded in Epon. 278000 ×.

less collapse than Cascophen RS788-77 under identical embedding conditions. Glutaraldehyde fixation, prior to fixation with osmium tetroxide, reduced the degree of impregnation of small pieces of nerve. Increasing the time of impregnation beyond 12 h

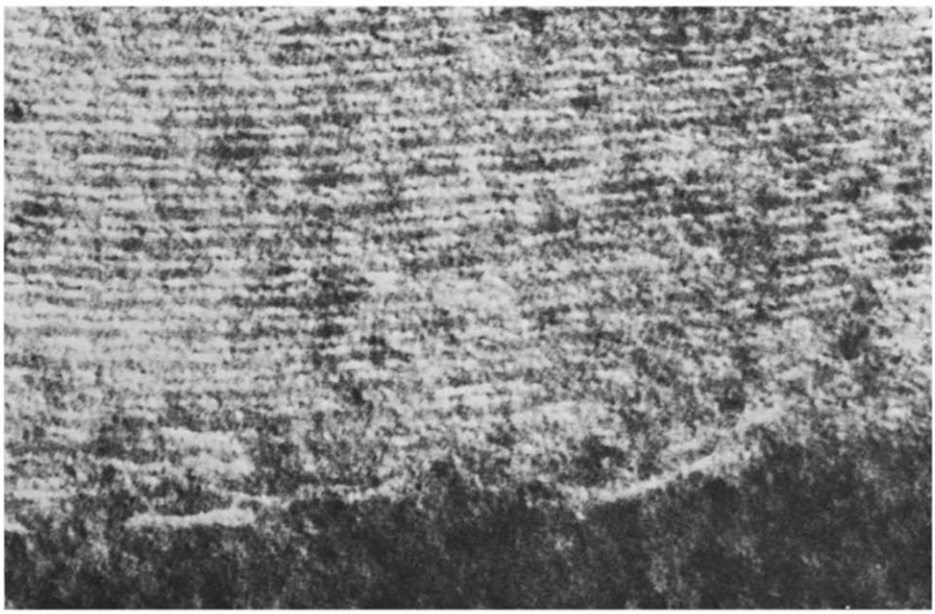

Fig. 4. Myelin structure of frog sciatic nerve fixed with osmium and embedded in Cascophen RS788-160. The section was post-stained with lead citrate. 278000 ×.

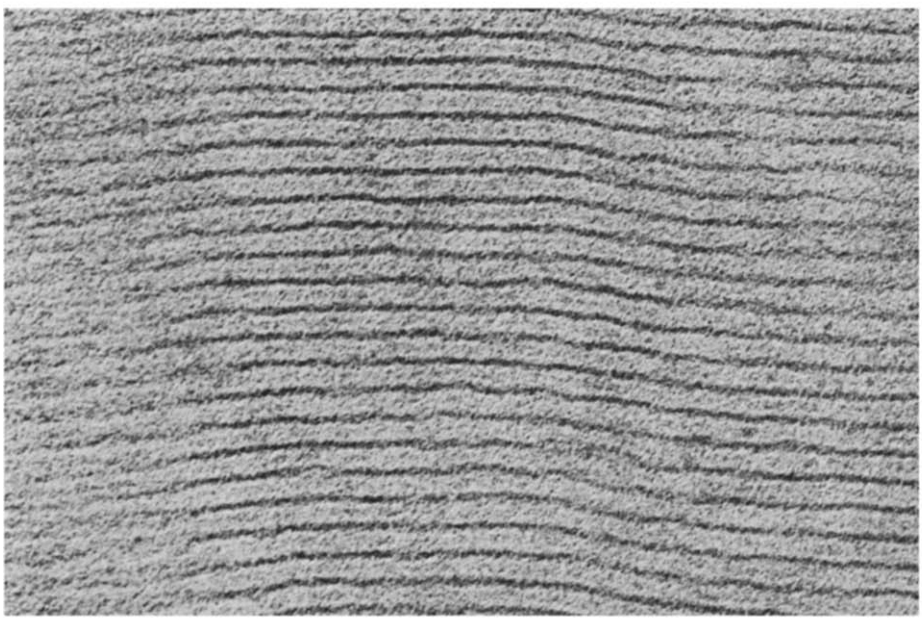

Fig. 5. Myelin structure of frog sciatic nerve fixed with osmium and embedded in Epon. The section was post stained with lead citrate. 278000 ×.

caused a deterioration of the myelin fine structure as did increasing the temperature above 37°.

High resolution studies of axons showed a series of evenly spaced lines of periodicity varying in different micrographs of the same preparation from 140 to 180 Å (Fig. 2). This variation appeared related to the bending of the myelin during collapse of the axon. Myelin structure was always well preserved in axons showing the least degree of collapse and in such preparations a periodicity of the order of 160 Å was obtained. In comparison the periodicity of myelin embedded in Epon was around 130 Å (Fig. 3). Positive identification of other membranous structures such as mitochondria in preparations embedded in Cascophen proved impossible. This was possibly due to the very low contrast of unstained preparations. However, attempts to improve contrast by staining thin sections were not very successful. Of the stains tested (potassium permanganate, lead citrate, uranyl acetate, vanadyl sulfate, phosphotungstic acid, and vanadatomolybdate) only potassium permanganate and lead citrate reacted. Potassium permanganate caused drastic damage to the myelin fine structure and lead citrate tended to stain the resin heavily but it was still possible to detect the myelin membrane structure (Fig. 4). The intraperiod line was more heavily stained than in preparations of frog sciatic nerve embedded in Epon and post-stained with lead citrate (Fig. 5).

Fixation of nerves with glutaraldehyde prior to osmium tetroxide fixation and

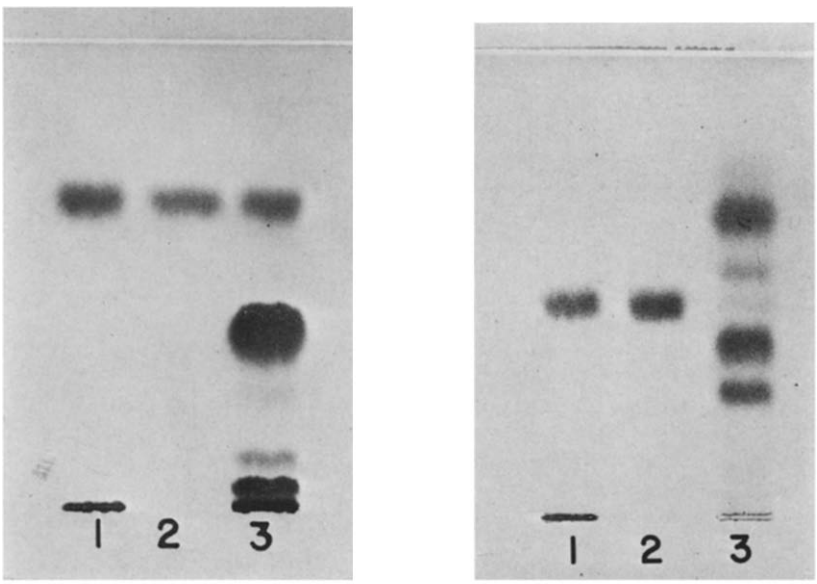

Fig. 6. Thin-layer chromatograph of lipids stained with phosphomolybdic acid reagent. (1) Total lipids from fresh frog sciatic nerve (46 μg); (2) cholesterol (8 μg); (3) chloroform–methanol extract of frog sciatic nerve fixed in osmium and embedded in Cascophen RS700-160 (equivalent total lipid applied to plate, 46 μg).

Fig. 7. Thin-layer chromatograph of lipids stained with phosphomolybdic acid reagent. (1) Chloroform–methanol extract of osmium-fixed frog sciatic nerve (equivalent total lipid applied to plate, 46 μg); (2) cholesterol (8 μg); (3) chloroform extract of combined Cascophen RS788-160 solutions used to impregnate osmium-fixed frog sciatic nerve pieces (equivalent total lipid applied to plate 46 μg).

embedding in Cascophen showed a myelin structure which did not differ noticeably from that obtained with osmium tetroxide fixation alone.

Extraction of lipids during embedding in Cascophen

Quantitative thin-layer chromatography (Fig. 6) and analysis for cholesterol (Table II) demonstrated that the major portion of the cholesterol was retained in nerves embedded in Cascophen RS788-160. In comparison approx. 50 % of the cholesterol (Table II) and a small amount of phospholipid, as detected by thin-layer chromatography, was extracted from osmium-fixed nerves during dehydration in acetone prior to embedding in Epon. Further evidence that cholesterol was not extracted during impregnation of nerve pieces with Cascophen RS788-160 was obtained by extracting the Cascophen solutions with chloroform. Quantitative thin-layer chromatography of the extract (Fig. 7) showed a minor component corresponding to cholesterol which stained only lightly with phosphomolybdic acid. This component stained with H_2SO_4 but did not show the initial pink coloration characteristic of cholesterol.

The component remaining at the origin after fractionation of the chloroform extract of the Cascophen RS788-160 solutions (Fig. 7) gave a negative test when sprayed with phospholipid reagent[33].

TABLE II

ESTIMATION OF DRY WEIGHT, TOTAL LIPID AND CHOLESTEROL IN FROG SCIATIC NERVE AT VARIOUS STAGES OF PREPARATION FOR THIN SECTIONING

Sample	*Analysis*	*Percent of nerve wet weight (mean ± S.E.* (number of analyses))*
Fresh nerve	Dry weight	24.7 ± 0.8 (2)
Fresh nerve	Total lipid	19.8 ± 0.4 (2)
Total lipid from fresh nerve	Cholesterol	3.9 ± 0.2 (3)
Total lipid from fresh nerve	Cholesterol following quantitative thin-layer chromatography	3.5 ± 0.2 (3)
Chloroform–methanol extract of nerve fixed in osmium and embedded in Cascophen RS788-160	Cholesterol following quantitative thin-layer chromatography	3.2 ± 0.3 (3)
Chloroform–methanol extract of residue from acetone extract of osmium-fixed nerve	Cholesterol	2.0 ± 0.2 (2)
Choroform–methanol extract of residue from acetone extract of glutaraldehyde and osmium-fixed nerve	Cholesterol	2.0 (1)

*S.E = σ^2/N.

DISCUSSION

The results demonstrate that little of the cholesterol or phospholipid has been lost during embedding in Cascophen and that the periodicity of the myelin embedded in Cascophen is of the order of 160 Å in comparison with 130 Å in Epon preparations

where a high proportion of the cholesterol has been extracted from the tissue. Studies using X-ray diffraction[34] indicate a repeat unit of 171 Å for fresh frog sciatic nerve, 155 Å for nerve fixed with osmium tetroxide and 130 Å for nerve fixed with osmium, dehydrated with acetone and embedded in Epon. Apparently osmium causes an initial shrinkage which is subsequently increased as a result of lipid extraction during dehydration and embedding.

Thin section preparations of nerves which have been fixed in glutaraldehyde and osmium tetroxide and then embedded in Epon, commonly show in electron micrographs, a periodicity of the order of 170 Å and a splitting of the intraperiod line[35]. This observation might be explained by crosslinking of proteins by glutaraldehyde and membrane shrinkage due to lipid extraction.

It was not determined whether the poor penetration of Cascophen through the myelin was related to some particular property of this resin or whether it substantiates the suggestion made by COPE AND WILLIAMS[2] that at least some lipid must be removed in order to permit penetration of embedding materials. It appears, however, that it is possible to use relatively low-molecular-weight materials to embed tissue without extracting neutral lipids.

ACKNOWLEDGMENTS

This investigation was conducted during the tenure by J. G. R. of a Damon Runyon Cancer Research Fellowship while on leave from the Applied Biochemistry Division, D.S.I.R., Palmerston North, New Zealand. We thank A. Hamad for technical assistance.

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