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FINE STRUCTURAL LOCALIZATION OF

CHOLESTEROL-1,2-³H IN DEGENERATING

AND REGENERATING MOUSE SCIATIC NERVE

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ABSTRACT

The localization of ³H-labeled cholesterol in nerves undergoing degeneration and regeneration was studied by radioautography at the electron microscope level. Two types of experiments were carried out: (a) Cholesterol-1,2-³H was injected intraperitoneally into suckling mice. 5 wk later, Wallerian degeneration was induced in the middle branch of the sciatic nerve, carefully preserving the collateral branches. The animals were then sacrificed at various times after the operation. During degeneration, radioactivity was found over myelin debris and fat droplets. In early stages of regeneration, radioactivity was found in myelin debris and regenerating myelin sheaths. Afterwards, radioactivity was found predominantly over the regenerated myelin sheaths. Radioactivity was also associated with the myelin sheaths of the unaltered fibers. (b) Wallerian degeneration was induced in the middle branch of the sciatic nerves of an adult mouse, preserving the collateral branches. Cholesterol-1,2-³H was injected 24 and 48 hr after the operation and the animal was sacrificed 6 wk later. Radioactivity was found in the myelin sheaths of the regenerated and unaltered fibers. The results from these experiments indicate that: (a) exogenous cholesterol incorporated into peripheral nerve during myelination remains within the nerve when it undergoes degeneration. Such cholesterol is kept in the myelin debris as an exchangeable pool from which it is reutilized for the formation of the newly regenerating fibers, especially myelin. (b) exogenous cholesterol incorporated into the nerves at the time that degeneration is beginning is also used in the formation of new myelin sheaths during regeneration. (c) mature myelin maintains its ability to incorporate cholesterol.

INTRODUCTION

Cholesterol-1,2-³H is biologically stable in mice and suitable for studying incorporation and location of cholesterol in the nervous system by radioautographic methods (7, 8). Up to 90% of the cholesterol-1,2-³H incorporated in the nervous tissue can be retained by using a modification of the Idelman's method for the dehydration of the tissue (9, 11). This has made possible quantitative analysis of the distribution of cholesterol-1,2-³H in radioautographs of mouse peripheral nerve at

varying stages of development (8). From such an analysis, it is known that cholesterol-1,2-^{3}H injected into mice during the period of active myelination is incorporated into peripheral nerve within 3 hr after injection and is still detectable at maturity, predominantly as part of the myelin sheaths. It is also known from light microscope radioautography that, after resecting part of such a labeled nerve and studying the subsequently regenerated segment, the radioactivity is found in the regenerated nerve, mainly over the newly formed myelin sheaths (15). From these results the authors postulated that cholesterol molecules existed as a pool that remained in bound form within the nerve during its degeneration and was available for reutilization during its subsequent regeneration (15).

The possibility that a more detailed analysis of the labeled distribution in the different compartments of the nerve at various times after injury might shed some light on the site of cholesterol "bounding" during degeneration and its transfer to newly-forming nerve structure during regeneration has led to the present study at the electron microscope level. The distribution, in normal and regenerated fibers, of cholesterol-1,2-^{3}H incorporated into the sciatic nerves of an adult mouse at the time degeneration started is also described in this paper.

MATERIALS AND METHODS

Labeling and Sampling the Nerves

Degeneration of the sciatic nerve was induced by resecting approximately a 1 mm segment from the middle branch of the nerve under diabutal anesthesia. The collateral branches were carefully preserved as controls for unaltered fibers.

Cholesterol-1,2-^{3}H was always administered as a solution of 3% dimethyl formamide (DMF) in 5% dextrose in water (SA 2 mCi/ml, New England Nuclear Corp., Boston, Mass.) Littermates injected with 3% DMF in 5% dextrose solution were used as nonradioactive controls for the radioautography.

Two types of experiments were done: (a) nine Swiss albino mice were injected intraperitoneally, at 5 and 8 days of age, with 0.1 ml of the cholesterol solution. Each animal received a total dose of approximately 400 μCi. Degeneration was induced 5 wk after the second injection. At 5, 7, 10, 14, and 28 days after the operation the proximal and distal segments of the transected middle branch as well as the collateral branches were identified and removed under lethal diabutal anesthesia. (b) Wallerian degeneration was induced in an adult Swiss albino

mouse. At 24 and 48 hr after the operation 0.2 ml of cholesterol-1,2-^{3}H solution was injected intraperitoneally. The nerves were removed 6 wk after the operation under lethal anesthesia with diabutal.

Processing the Tissue for Electron Microscope Radioautography

The nerves were immersed in 5% cacodylate-buffered glutaraldehyde for 1 hr, washed overnight in Sabatini's washing solution (17), and postfixed for 1 hr in Dalton's chrome-osmium solution. All solutions were at 4°C. The nerves were dehydrated and embedded in Epon 812, using Idelman's procedure (11) to decrease to a minimum the extraction of cholesterol from the tissue (9). Pale gold sections from radioactive and nonradioactive nerves were placed on collodion-coated slides, covered with a carbon layer and coated with a monolayer of Ilford L-14 emulsion, according to the method of Salpeter and Bachmann (18, 19). Several emulsion-coated, experimental slides exposed to light were processed to assess latent image fading during the long exposure time. The slides were stored in black plastic boxes, containing Drierite (W. A. Hammond Drierite Co., Xenia, Ohio), at 4°C and exposed for 13 months or 20 months afterwards. The slides were developed with Microdol-X for 3 min at 24°C. Electron micrographs were obtained with a Siemens Elmiskop IA electron microscope.

Analysis of the Radioautographs

The basic considerations for the analysis of the electron microscope radioautographs have been extensively described in a previous paper (8): Half distance (HD), a measure determined experimentally by Salpeter et al. for electron microscope radioautographs, is the distance from a radioactive line source containing half the developed grains due to that source (2, 19). In the present analysis, as in our previous work (8), we used 0.14 μ as the compartment size, which is less than the expected HD for the preparation used in this study, and considered the myelin sheath to be a band source. Electron micrographs of many sections from each block were analyzed and the results were expressed as unit densities for each tissue compartment of the specimen as previously described (8). The problem of extraction and possible redistribution of cholesterol has also been fully discussed (5, 8). The grain density values obtained were compared to the theoretical values previously calculated for this geometrical situation (8).

The nerve fibers have been described in three categories: (a) Undegenerated fibers. This category includes all myelinated fibers in the uninjured col-

TABLE I

Distribution of Radioactivity Expressed as Density of Developed Grains Per Lattice Points (× 10) in the Sciatic Nerve from the First Type of Experiment*

Tissue compartments	Fiber type	5 days		7 days	10 days		14 days			28 days		
		Proximal	Distal	Distal	Proximal	Collateral	Proximal	Distal	Collateral	Proximal	Distal	Collateral
Myelin	Undegenerated	5.1 ±0.5			4.9 ±0.5	4.3 ±0.2	6.7 ±0.5		6.3 ±0.3	5.8 ±0.4		5.2 ±0.3
	Degenerated Regenerated				6.6 ±0.7		7.5 ±1.2			6.6 ±0.9	5.5 ±0.6	
Myelin debris	Degenerated	4.9 ±0.5	7.3 ±0.5	7.4 ±0.5	6.9 ±0.6		6.9 ±0.9	9.4 ±0.6			4.7 ±0.4	
Lipid droplets	Degenerated	2.3 ±0.4	4.6 ±0.6	4.7 ±0.8				4.6 ±0.4				
Axon	Undegenerated	0.9 ±0.2			0.6 ±0.2	0.6 ±0.1	1.2 ±0.3		1.3 ±0.2‡	1.0 ±0.2		1.1 ±0.2‡
	Degenerated Regenerated			0.7 ±0.5	0.7 ±0.4		1.3 ±0.4			1.3 ±0.4	0.7 ±0.3	
Schwann cell	Undegenerated	0.5 ±0.3			0.5 ±0.2	0.5 ±0.2	0.3 ±0.2		1.7 ±0.3‡	1.1 ±0.3		1.4 ±0.4
	Degenerated	0.5 ±0.3			0.8 ±0.2		1.9 ±0.4	1.7 ±0.4				
	Regenerated				0.6 ±0.2		1.4 ±0.4			2.5 ±0.2	2.6 ±0.6	
Extra fiber	Macrophages		5.8 ±1.2	4.0 ±0.7								
	Fibroblasts	2.6 ±0.8	2.2 ±0.5	2.5 ±0.7							0.9 ±0.4	
	Extracellular	0.2 ±0.1	0.1 ±0.1	0.3 ±0.1	0.2 ±0.1	0.3 ±0.2	0.3 ±0.1	0.3 ±0.1	0.3 ±0.2	0.1 ±0.1	0.2 ±0.1	0.1 ±0.1

* Values are ±standard deviation.

‡ Values greater than could be accounted for by spread from myelin radioactivity using calculation based on a band source and 1.75 HD (8).
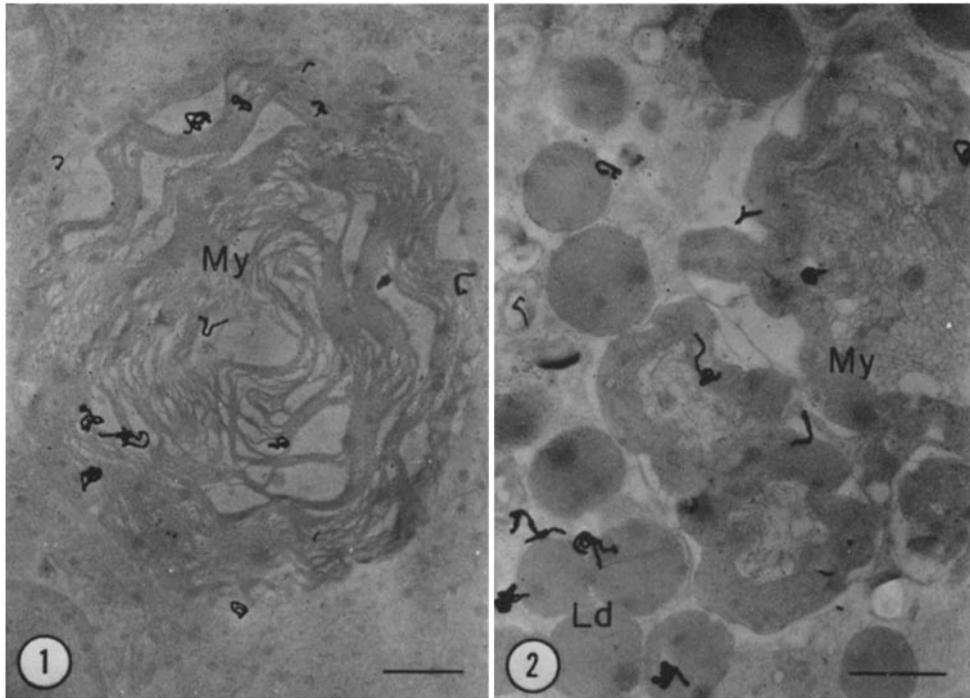
FIGURE 1 Electron microscope radioautograph of a distal segment from the sectioned middle branch of a sciatic nerve, 5 days after operation. All the radioactivity is detected over myelin debris (My). Scale bar equals 1 μ. $\times$ 10,800.

FIGURE 2 Electron microscope radioautograph of a proximal segment, 5 days after operation. Grains are associated with lipid droplets (Ld) and degenerating myelin (My). Scale bar equals 1 μ. $\times$ 13,000.

lateral branches and those myelinated fibers in the proximal segments which show no evidence of degeneration or regeneration, i.e. no morphological damage or refasciculation. (b) Degenerated fibers. This category includes those fibers from the proximal and distal segments of the interrupted branch in which morphological evidence of nerve degeneration was present. The number of recognizable axonal remnants found in this category was too small to analyze. (c) Regenerated fibers. This category includes all refasciculated fibers in the proximal and distal segments.

A lattice with uniformly spaced points was superimposed over the radioautographs (4), and the number of developed grains and lattice points within the various tissue compartments was determined.

The following tissue compartments within each category of fibers were recognized: (a) Myelin, including the grains and lattice points over all myelin sheaths; (b) Myelin debris, including the grains and lattice points over the lamellar and electron-opaque material associated with degenerat-

ing fibers; (c) Lipid droplets, including all grains and lattice points over electron-opaque bodies in Schwann cells, fibroblasts, and macrophages associated with degenerating fibers; (d) Axon, including only those grains and lattice points found in the axon more than 0.28 μ from the nearest inner edge of the myelin sheath; (e) Schwann cell, including only those grains and lattice points coinciding with Schwann cell cytoplasm or nucleus more than 0.28 μ from the outer edge of the nearest myelin sheaths, myelin debris, or lipid droplets. Schwann cells without recognizable axons were differentiated from macrophages by the presence of a basement membrane; (f) Macrophages, fibroblasts and extracellular space, and fibrils, including those grains and lattice points lying over fibroblasts and extracellular space and collagen more than 0.28 μ away from the outer edge of any myelin sheath. These are classified in Table I as "extra-fiber compartments". Blood vessels were excluded from the analysis.

In each compartment the number of grains was divided by the number of lattice points, and the
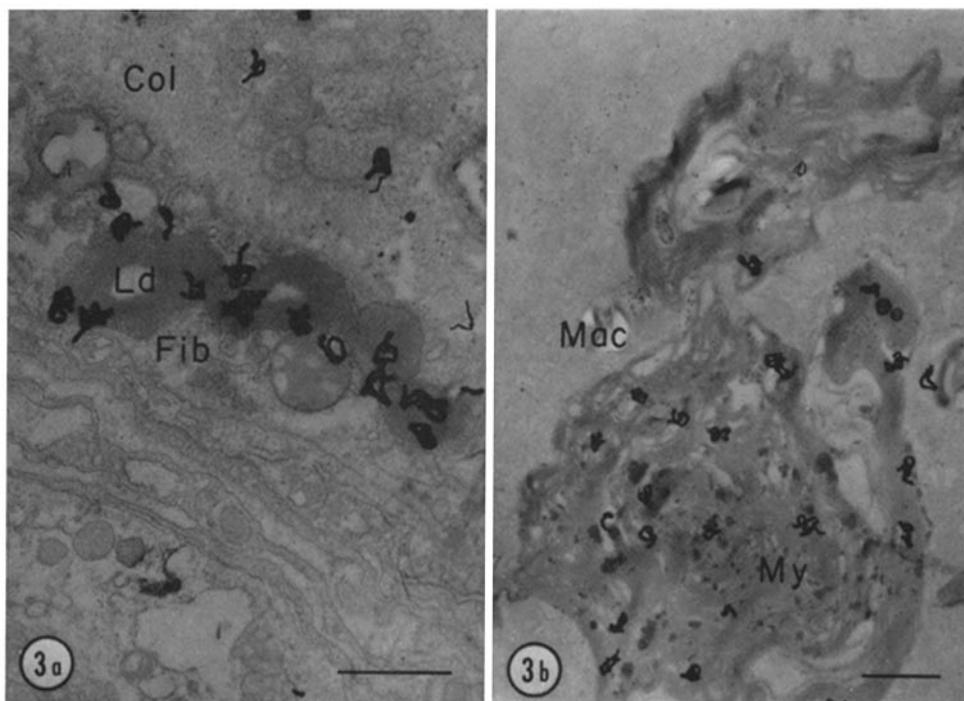
FIGURE 3   Electron microscope radioautographs of a distal segment, 2 wk after operation. In Fig. 3 a, the highest density of grains is detected over the lipid droplets (*Ld*) found within a fibroblast (*Fib*). *Col*, collagen. Scale bar equals 1 μ. $\times$ 14,950. In Fig. 3 b, all the radioactivity is localized over myelin debris (*My*) in a cell resembling a macrophage (*Mac*). Scale bar equals 1 μ. $\times$ 10,400.

results are expressed as densities (grains/lattice points). A sample area of approximately 15,000–20,000 μ^2 of tissue was counted for each sample.

RESULTS

General

The operated middle branch of each sciatic nerve was divided into proximal and distal segments relative to the operative site. Densities of silver grains over nonradioactive control sections were similar to the background in the experimental radioautograms, indicating that there was no positive chemography. No evidence of latent image fading was detected from the emulsion-coated experimental slides exposed to light.

The spotted appearance of some electron micrographs is due to the collodion used in making the supporting films for the specimen grids. The long-term exposure (13 and 20 months) used in this work to obtain a sufficient number of developed

grains for quantitative analysis of grain distribution precluded repetition of the radioautography. Micrographs with such defects have been used throughout the work.

Distribution of radioactivity is expressed as densities, i.e., number of developed grains/lattice points, in each tissue compartment. The standard deviation was calculated from the formula:

$$\frac{G}{P} \sqrt{\frac{1}{G} + \frac{1}{P}}$$

where G = number of grains and P = number of lattice points (8).

First Type of Experiment

Table I shows the results of the analysis of the distribution of radioactivity in the sciatic nerves from the first series of experiments, i.e. animals injected with cholesterol-1,2-^{3}H before nerve transection.
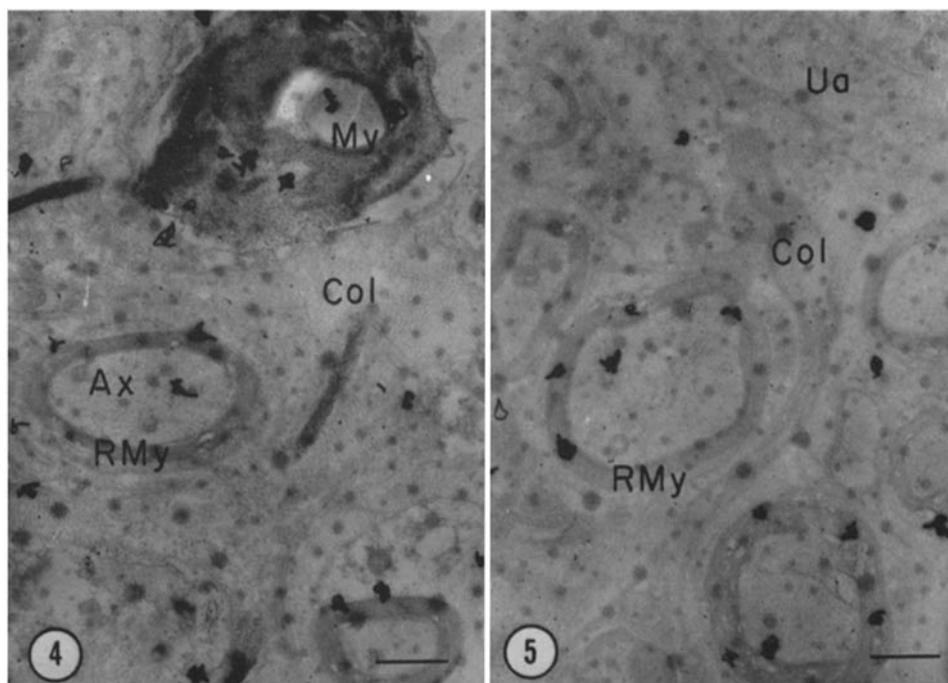
FIGURE 4 Electron microscope radioautograph of a distal segment, 4 wk after operation. Myelin debris (*My*) appears highly labeled. Grains are also detected over regenerating myelin (*RMy*) and regenerating axons (*Ax*). Occasionally, grains are seen over collagen (*Col*). Scale bar equals 1 μ. $\times$ 9100.

FIGURE 5 Electron microscope radioautograph of the proximal segment, 4 wk after operation. All the fibers shown in this micrograph are in the process of regeneration. The highest density of grains is associated with regenerating myelin (*RMy*). *Col*, collagen. *Ua*, unmyelinated axons. Scale bar equals 1 μ. $\times$ 9100.

Comparison of grain densities can be made with greatest confidence between the undegenerated and regenerated fibers in the same sections of a nerve segment. Comparison of grain densities between different segments of nerve from the same animal has less validity, although attempts were made to keep section and emulsion thickness constant. Direct comparison of grain density from animal must take into account the possible variation in dosage of administered cholesterol due to the leakage that sometimes occurred around the injection site and the variability in cholesterol extraction during dehydration.

Distal Segment of the Sectioned Branch

No remyelination was observed in the distal segment until 14 wk after operation. Up to 2 wk after operation, the highest grain density was detected in relation to myelin debris and lipid droplets within Schwann cells of the degenerated fibers (Fig. 1), fibroblasts (Fig. 3 a), and cells resembling macrophages (Fig. 3 b). 4 wk after operation (Fig. 4), labeled myelin debris was still present within the distal segment together with labeled regenerating fibers. Grain density over such newly formed myelin sheaths (5.5 $\pm$ 0.6) was not significantly different from that over myelin debris (4.7 $\pm$ 0.4).

Proximal Segment of the Sectioned Branch

5 days after operation (Fig. 2) the density of grains over myelin debris (4.9 $\pm$ 0.5) was similar to that found over the myelin sheaths of the undegenerated fibers (5.1 $\pm$ 0.5). Lipid droplets were also distinctly labeled (2.3 $\pm$ 0.4) while activity over Schwann cells of both undegenerated (0.5 $\pm$ 0.3) and degenerated fibers (0.5 $\pm$ 0.3) could be accounted for by the spread of radioactivity from myelin. Remyelinating fibers were detected in the

Table II

Distribution of Radioactivity Expressed as Density of Developed Grains Per Lattice Points ($\times$ 10) in the Sciatic Nerves from the Second Type of Experiment*

Tissue compartments	Fiber type	Proximal segment	Collateral branch
Myelin	Undegenerated	8.5 ± 0.6	9.0 ± 0.3
	Regenerated	9.6 ± 0.8	
Axon	Undegenerated	1.3 ± 0.2	1.5 ± 0.1
	Regenerated	1.5 ± 0.3	
Schwann cell	Undegenerated	1.8 ± 0.4	1.4 ± 0.2
	Regenerated	2.4 ± 0.4	
Extra fiber	Undegenerated	0.4 ± 0.1	0.5 ± 0.1
	Regenerated	0.3 ± 0.1	

* Values are ±standard deviation.

proximal segment 10 days after operation. The newly formed myelin sheaths showed a grain density (6.6 ± 0.7) similar to that over the myelin debris (6.9 ± 0.6) and higher than that detected in the myelin sheaths of the undegenerated fibers (4.9 ± 0.5). 2 and 4 wk after operation the highest grain densities (Table I) were found over the newly formed myelin sheaths (Fig. 5) as well as over myelin sheaths of undegenerated fibers from the proximal segments and the normal fibers of the collateral branches.

Collateral Branch

The unoperated collateral branches of the sciatic nerve were used to determine cholesterol distribution in normal myelinated fibers. The highest density of grains was always localized over the myelin sheaths (Table I). Grain densities over axons and Schwann cells more than 0.28 μ from myelin were low compared with the density detected over myelin, but three of six instances (see legend to Table I) were significantly greater than could be accounted for by spread from myelin radioactivity using calculations based on a band source and 1.75 HD as previously described (8).

Extra-Fiber Compartment

Macrophages and fibroblasts contained significant amounts of labeled cholesterol 5 and 7 days after operation. Lipid droplets were seen in both macrophages and fibroblasts at these stages. Fibroblasts in the distal (and regenerating) segment, 4 wk after operation, were not labeled more than could be accounted for by spread of radioactivity from myelinating fibers.

Second Type of Experiment

Table II shows the results of the quantitative analysis of grain distribution in the sciatic nerve from the second kind of experiment, i.e. animal injected with cholesterol-1,2-³H after nerve transection. In the undegenerated fibers (Fig. 6 a) of both the proximal segment of the sectioned middle branch and intact collateral branches, the data clearly indicate that radioactivity is localized predominantly over the mature myelin sheaths. The density of grains over axon and Schwann cell cytoplasm is low and can be accounted for by radioactivity spread from myelin (8).

In the regenerating fibers (Fig. 6 b) the highest density of radioactivity was found over the myelin sheaths just as in the undegenerated fibers (Fig. 6 a). The values in both cases were not statistically different (Table II).

DISCUSSION

A preliminary study (15) using light microscope radioautography showed that cholesterol-1,2-³H incorporated into mouse sciatic nerve during the period of rapid myelin formation was reutilized in the mature animal for regeneration after resection of approximately 1 mm segment of the nerve. The present experiments confirm and expand this work in several important respects. In the first place, a wider analysis was carried out by studying nerves at different times after operation. This experimental setting allowed us to follow the cholesterol molecules throughout the degeneration-regeneration process. Secondly, the distal as well as the proximal segments of the sectioned nerve were studied. Thirdly, a more accurate analysis was made by using electron microscope radioautography, since it allowed a finer localization of the radioactivity within the different nerve compartments.

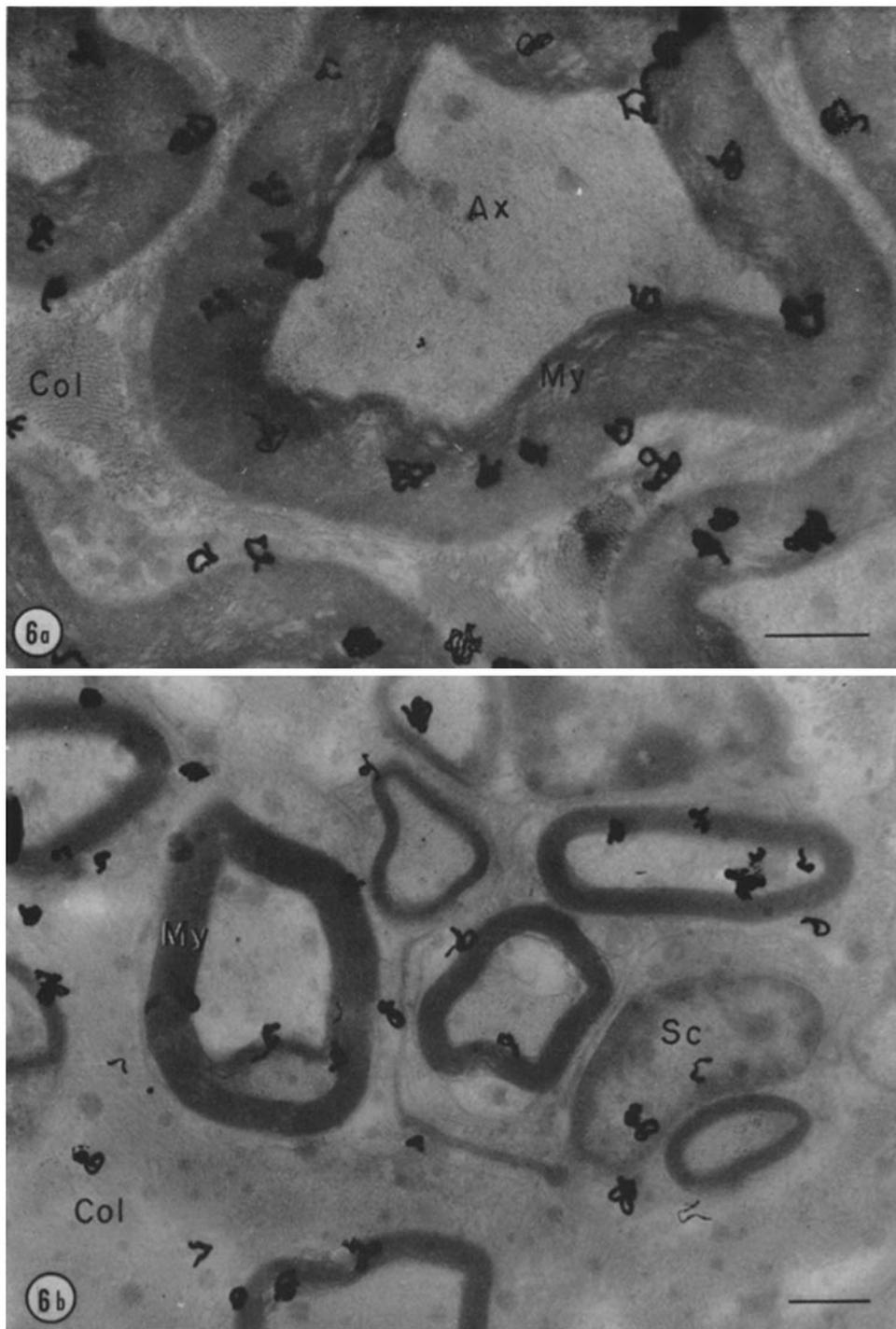In the first type of experiment, we have found

FIGURE 6 Electron microscope radioautograph of a sciatic nerve of an adult mouse injected with cholesterol-1,2-³H at 24 and 48 hr after nerve transection and sacrificed 6 wk later. Fig. 6 a shows the undegenerated fibers. All the grains can be attributed to radioactivity emanating from the myelin sheaths (*My*) *Ax*, axon. *Col*, collagen. Scale bar equals 1 μ. $\times$ 14,950. Fig. 6 b shows the regenerating fibers. Note the high density of grains associated with regenerating myelin (*My*). Occasional grains are seen over Schwann cell (*Sc*) and collagen. Scale bar equals 1 μ. $\times$ 11,700.

622

that cholesterol-1,2-³H incorporated into forming myelin sheaths is maintained within the myelin debris of such a nerve when it undergoes Wallerian degeneration. As soon as regenerated fibers are formed, cholesterol is detected in the regenerating nerves mainly over the newly formed myelin sheaths. 4 wk after operation myelin debris has largely disappeared from the proximal segment and radioactivity in the regenerated fibers has achieved a density similar to that in the undegenerated fibers, with the highest density of radioactivity being associated with the myelin sheath in both. The distal segment, however, still retained myelin debris 4 wk postoperatively. In this segment, both the myelin debris and newly regenerating fibers are labeled, the density of radioactivity over myelin debris being similar to that over regenerating myelin.

Undegenerated fibers in the proximal segment and normal fibers in the intact collateral branches showed similar grain densities. This probably means that no significant losses of cholesterol-1,2-³H from undegenerated fibers of the proximal segment have occurred in spite of interruption of the perineurium and operative interference with the normal vascular supply.

These findings indicate that cholesterol-1,2-³H detected in regenerated fibers, at least in part, has come from myelin debris, supporting the previously proposed hypothesis (15) that cholesterol reutilized for regeneration exists in "bound" form or reservoirs from the time of degeneration until remyelination.

During regeneration, penetration of growth cones followed by remyelination has been observed in Schwann cells which still contained myelin debris (14). Although it has been proposed that such Schwann cells can digest the myelin debris (14), further breakdown of the cholesterol molecules might not occur, in keeping with the well recognized biological stability of cholesterol within the nervous system (6, 7, 8). Therefore, it is feasible that Schwann cells may directly reutilize for new myelin sheaths at least part of the cholesterol-1,2-³H that previously resided in the debris.

The possibility that labeled cholesterol detected in the regenerated nerve has come from the animal blood supply is unlikely in view of the absence of long-term visceral and muscular stores of cholesterol-1,2-³H$_x$(7). The only significant long-term store of labeled cholesterol in developing mice was found in the nervous system.

The suitability of cholesterol-1,2-³H for radioautography has been extensively discussed in previous papers, and evidence has been presented that the label detected in nerve fibers is due to the injected cholesterol-1,2-³H (5, 6, 8, 15). The same criteria hold in this work for the undegenerated as well as the unaltered fibers. However, in normal nerve all cholesterol is in a nonesterified form, while during degeneration, as free cholesterol decreases in amount, the cholesterol esters increase significantly (1, 3). Such cholesterol esters are known to persist for a long period of time in myelin debris, and this esterified form may account for the label detected in the present radioautographs of myelin debris.

The results from the second type of experiment, i.e. animals injected with cholesterol-1,2-³H 24 and 48 hr after nerve transection, indicate that exogenous cholesterol incorporated into the nerve at the time that degeneration is beginning is also used by that nerve in the formation of the newly regenerating myelin. Biochemical studies by Nicholls and Rossiter (12) have indicated that cholesterol-¹⁴C injected into rats at intervals during the period 2–8 wk after crushing the nerve, i.e. the period of maximum myelin regeneration, is also incorporated into the regenerating as well as into the intact fibers. However, no information is available from that work as to the distribution of radioactive cholesterol within the different nerve compartments.

The fact that cholesterol-1,2-³H was detected in the undegenerated mature myelin sheaths may be a reflection of a mechanism for long-term myelin deposition and maintenance as proposed by several investigators (10, 13, 16, 20, 21).

Thus, in vitro studies with rats have shown that there is incorporation of myelin precursors such as acetate-¹⁴C and leucine-¹⁴C into brain, spinal cord, and sciatic nerve myelin fractions of adult as well as young animals (16). Norton et al. (13) have found that the accumulation of myelin starts in rat brain at 12 days and continues for more than a year after birth. They suggested that there may be a slow, continual deposition of myelin throughout the life of the rat. Hendelman and Bunge (10), using tissue culture and radioautography, claim that the myelin–Schwann cell unit in a fully myelinated culture incorporates choline-³H as actively as in a myelinating culture. They suggest that such incorporation is part of a continuous process of myelin maintenance. Singer and Sal-

peter (20, 21) have also indicated that labeled amino acids enter the axons of peripheral nerve by traversing the Schwann cell and myelin sheaths. They suggested that the myelin sheath must contain some of those metabolic mechanisms needed to renew itself continuously (20), including a protin synthetic mechanism (21).

In conclusion, it is indicated from the results of the present study that: (a) part of the cholesterol-1,2-^{3}H incorporated into the sciatic nerve during myelination does not leave the nerve during its degeneration, but is kept within the myelin debris as an exchangeable pool and later reutilized for the formation of the newly regenerating fibers. (b) Cholesterol incorporated into the nerve at the time that degeneration is beginning is also used in the formation of new myelin sheaths during regeneration. (c) Mature meylin maintains the ability to incorporate cholesterol.

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