

A TIME-SEQUENCE AUTORADIOGRAPHIC STUDY OF THE IN VIVO INCORPORATION OF [1,2-³H]CHOLESTEROL INTO PERIPHERAL NERVE MYELIN

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ABSTRACT

A time-sequence study of the incorporation and distribution of cholesterol in peripheral nerve myelin was carried out by electron microscope autoradiography. [1,2-³H]Cholesterol was injected into 10-day old mice and the sciatic nerves were dissected out at 10, 20, 40, 60, 90, 120, and 180 min after the injection. 20 min after injection the higher densities of grains due to the presence of [³H]cholesterol were confined to the outer and inner edges of the myelin sheath. Practically no cholesterol was detected in the midzone of the myelin sheath. 1½ h after injection, cholesterol showed a wider distribution within the myelin sheath, the higher densities of grains occurring over the two peripheral myelin bands, each approximately 3,100 Å wide. Cholesterol was also present in the center of the myelin sheath but to a considerably lesser extent. 3 h after injection cholesterol appeared homogeneously distributed within the myelin sheath. Schwann cell and axon compartments were also labeled at each time interval studied beginning 20 min postinjection. These observations indicate that preformed cholesterol enters myelin first and almost simultaneously through the inner and outer edges of the sheath; only after 90 min does the density of labeled cholesterol in the central zone of myelin reach the same density as that in the outer and inner zones. These findings suggest that cholesterol used by the nerve fibers in the formation and maintenance of the myelin sheath enters the lamellae from the Schwann cell cytoplasm and from the axon. The possibility of a bidirectional movement of molecules, i.e. from the Schwann cell to the axon and from the axon to the Schwann cell through the myelin sheath, is noted. The results are discussed in the light of recent observations on the exchange, reutilization, and transaxonal movement of cholesterol.

INTRODUCTION

It is generally accepted since the work of Geren (1954) that myelination is the result of a process of spiral wrapping of the infolded Schwann cell surface, initiated after the Schwann cells envelop the outgrowing axons. Little is known, however, regarding the mechanism and the site of incorporation of preformed molecules into

myelin and about the general dynamics of myelin deposition and maintenance. Several speculations related to such a mechanism tended to describe myelination as an orderly addition of rather inert concentric layers of Schwann cell cytoplasmic membrane to form a metabolically stable structure.

The view that myelin is composed by molecules more or less fixed at definite locations within the lamellae has led to suggestions such as those of Simon et al. (1969) that "early myelin" (inner) or "late myelin" (outer) could be preferentially labeled by the injection of [¹⁴C]cholesterol near the beginning or near the end of the period of myelination. They further suggested that during degeneration, myelin would be broken down in an orderly sequential manner, starting with the myelin laid down last (outer lamellae).

A similar theory of myelin formation suggested that the molecular components of myelin incorporated at the periphery of the sheath were subsequently covered up by layers added at later dates with active metabolism limited to the outer layers of myelin (Davison, 1964). In contrast to these speculations, Smith (1968) has indicated that the metabolism of myelin, in the outer layers, is identical with that of the myelin laid down early in the life of the animal.

Recently, Hedley-Whyte et al. (1969) have shown from autoradiographic studies that the [1,2-³H]cholesterol incorporated in growing myelin sheath of mouse peripheral nerve, was uniformly distributed in a radial direction 3 h, 24 h, and 7 wk after injection. They concluded that the tagged cholesterol molecules were not initially incorporated in a stable lamellar unit nearer to one myelin border than to the other and that the cholesterol initially concentrated in the myelin sheath could be redistributed with time into all tissue compartments of the nerve.

Results from other investigations have also indicated that movement or exchange of molecules may occur from myelin to other compartments of the nervous tissue. Thus it has been proposed that intraperitoneally injected [³H]histidine, [³H]lysine, and [³H]uridine can reach the axon by way of the myelin sheath (Singer and Salpeter, 1966; Singer, 1968; Singer and Green, 1968). On the other hand Hendelman and Bunge (1969) reported incorporation of [³H]choline in the myelin sheaths of myelinating and fully myelinated cultures of dorsal root ganglia. These findings, together with biochemical studies done by Rawlins and Smith (1971) on the *in vitro* incorporation of [¹⁴C]acetate in lipids and proteins of the myelin fraction of central and peripheral nervous system of rats at different ages (from 25 days up to 18 mo after birth), indicate that a significant amount of myelin related metabolic

activity occurs in mature nervous tissue. Such activity can be considered part of an essential and continuous process of myelin maintenance and repair.

Other lines of evidence support the concept of sterol molecules movement in myelin sheaths. Banik and Davison (1971) suggested that the 7-dehydrocholesterol which accumulated in myelin due to the action of a hypocholesteremic drug during brain development may be converted into cholesterol outside the myelin sheath and then reincorporated in myelin by an exchange process.

Finally, convincing evidence has been published for the reutilization of [1,2-³H]cholesterol during the regeneration of peripheral nerve, as the result of transfer of molecules from myelin debris to the newly regenerating myelin (Rawlins et al., 1970; Rawlins et al., 1972).

In the present paper a time-sequence study of the *in vivo* incorporation of [1,2-³H]cholesterol in peripheral nerve was carried out by electron microscope autoradiography in an effort to localize the site of entry of preformed molecules into the myelin. Evidence is reported of the existence not only of a trans-Schwann cell transport but also of a transaxolemma movement of cholesterol into myelin.

MATERIALS AND METHODS

Labeling of the Nerves

24 10-day old Swiss albino mice were injected intraperitoneally with 500 μ Ci of cholesterol in a solution of 3% dimethyl formamide (DMF) in 5% dextrose in water (Sp act 55 Ci/mmol; New England Nuclear Corp., Boston, Mass.). A mouse injected with the same volume of DMF solution was used as the nonradioactive control for the autoradiography.

Fixing and Dehydrating the Tissue

At 10, 20, 40, 60, 90, 120, and 180 min after the injection, the animals were anesthetized with Diabital¹ and the sciatic nerves were rapidly exposed and fixed *in situ* with 2.5% cacodylate-buffered glutaraldehyde for 5 min. They were then dissected out and immersed in a similar glutaraldehyde solution for 1 h at 4°C. Afterwards, the nerves were washed overnight in Sabatini's washing solution (Sabatini et al., 1963) and postfixated for 1 h in each of the following:

¹ Diamond Laboratories, Inc. Des Moines, Iowa.

Dalton's chromosmium solution and 1% uranyl acetate in 10% formaldehyde solution. All solutions were at 4°C. The tissue was dehydrated and embedded in Epon 812 using a modification of Idelman's procedure (Hedley-Whyte et al., 1969) to minimize the extraction of cholesterol from the tissue (Hedley-Whyte and Uzman, 1969). Nervous tissue fixed and dehydrated in such a way frequently shows disruptions of the myelin sheaths.

Processing the Tissue for Electron Microscope Autoradiography

Pale gold transverse sections from radioactive and nonradioactive nerves were prepared on a Porter-Blum Sorvall MT2-B ultramicrotome (Ivan Sorvall, Inc., Newtown, Conn.) equipped with an IVIC diamond knife (Instituto Venezolano de Investigaciones Científicas) and collected on Formvar-coated nickel grids. The sections were then stained with Reynolds' lead citrate (Reynolds, 1963) and coated with carbon. The grids were fixed onto previously cleaned standard microscope slides by means of a strip of double stick tape (Heremans, 1971). They were then coated with Ilford L-4 emulsion (Ilford Ltd., Essex, England) by dipping the slides into a beaker containing diluted emulsion (usually 1:2) to achieve a packed monolayer of silver crystals when examined undeveloped in the electron microscope.

Emulsion-coated experimental slides exposed to light were processed at the beginning and others at the end of the exposure period to assess latent image fading during the exposure time. The slides were allowed to dry for 2 h in an upright position and stored in black plastic boxes containing Humicaps dessicant (Driaire, Inc., East Norwalk, Conn.) at 4°C. After 5 mo exposure, they were developed with Microdol-X (Eastman Kodak Co., Rochester, N.Y.) for 3 min at 24°C.

Analysis of the Autoradiographs

The autoradiographs were viewed with a Siemens Elmiskop 1A electron microscope, and all areas containing grains were photographed at constant magnification ($\times 6,000$). A minimum of 900 grains were photographed for each specimen with at least three specimens for each time interval after injection. Prints of the autoradiographs were analyzed at a final magnification of $\times 18,000$.

Although pictures were taken only from those areas containing developed grains, the area photographed was large relative to the size of the grains and usually included several potential sources. It thus was essentially random relative to the tissue compartments analyzed.

Two types of analysis were carried out in order

to study the distribution of grains in the autoradiographs. In each case certain restrictions were followed.

FIRST TYPE OF ANALYSIS: In this analysis the radial distribution of grains in the myelin sheaths was recorded. For this analysis all those myelin sheaths that had a minimum width of 3 half-distances (HD) were studied.

1 HD is the distance from a line source in an autoradiographic specimen within which half of the developed grains fall. HD for the material used in this study should be 1,600 Å according to Salpeter et al. (1969). The values for HD were obtained by Salpeter et al. (1969) using the "flat substrate" method for specimen mounting and emulsion coating introduced by Bachmann and Salpeter (1965); however, they stated (Salpeter et al., 1969, p. 9) that the same HD values should apply with reasonable accuracy to any technique "as long as it employs tritium labelling, section thickness usual for electron microscopy, and closely packed, uniform emulsion layers calibrated for thickness." These requirements were always fulfilled in the technique used in the present work and so the value of 1,600 Å for 1 HD was used.

Myelin sheaths of suitable thickness were divided into three subcompartments of equal width, named outer third, middle third, and inner third compartment. The midpoint of each silver grain was determined by placing over the pictures a plastic lamina which has series of circles of different size. The center of the smallest circle enclosing the grain (these are not the white circles drawn in the picture) was punctured with a needle, and the grain position was tabulated according to the myelin subcompartment over which the midpoint lay. The number of grains over each subcompartment was then expressed as percent of total number of grains over all myelin subcompartments.

SECOND TYPE OF ANALYSIS: In this analysis the density of grain distribution was recorded following the method of Salpeter and co-workers (Salpeter et al., 1969). In order that density difference through the myelin sheath could be detected with validity only those myelin sheaths approximately 6 HD thick were studied; in order that these density differences could be compared with theoretical density distributions in solid or hollow band sources (Figs. 11 and 12 of Salpeter et al., 1969), only fibers with axon diameter ≥ 6 HD could be used so that the radius of curvature in the electron micrographs could be ignored.

Only the myelin sheath, Schwann cell, and axon compartments of the nerve were quantitatively assessed, and each fiber was divided into subcompartments of myelin, Schwann cell, and axon. The density of silver grains over each of these subcompartments was tabulated with each subcompartment equivalent

to HD increments; the relative area of each subcompartment was obtained by superimposing over the picture a lattice with regularly spaced points. The grain density distribution within each subcompartment was obtained by dividing the number of silver grain midpoints over the "HD subcompartment" by the number of lattice points found in the same HD subcompartment. Results were tabulated as number of developed grains per number of lattice points in each HD subcompartment. HD subcompartments within the myelin sheath were analyzed starting from the outer edge of myelin and proceeding inward to the axolemma. From the axolemma inward, three axonal HD subcompartments were tabulated; and from the outer edge of the myelin sheath, four Schwann cell HD subcompartments were tabulated. No grains or points over endoneurial tissue or blood vessels were recorded in this study. The standard deviation was estimated from the formula:

$$SD = G/P((1/G) + (1/P))^{1/2}$$

where G equals the number of grains and P equals the number of lattice points (see Hedley-Whyte et al.,

1969). The experimentally obtained distribution of grain densities was then compared with the appropriate universal density curves provided by Salpeter et al. (1969) for radioactive sources of different shapes.

RESULTS

Densities of silver grains over nonradioactive control sections were similar to the background in the experimental autoradiograms, indicating that there was no positive chemography. No evidence of latent image fading was detected from the emulsion-coated experimental slides exposed to light, and they were exposed and developed in the same way as the experimental slides. Furthermore, it has been well established that with the emulsion and developer used in the study, latent image fading is negligible during the exposure period used (Bachmann and Salpeter, 1965).

We know from a previous work (Hedley-Whyte et al., 1968) that [1,2-³H]cholesterol is incorporated directly in brain and periphera

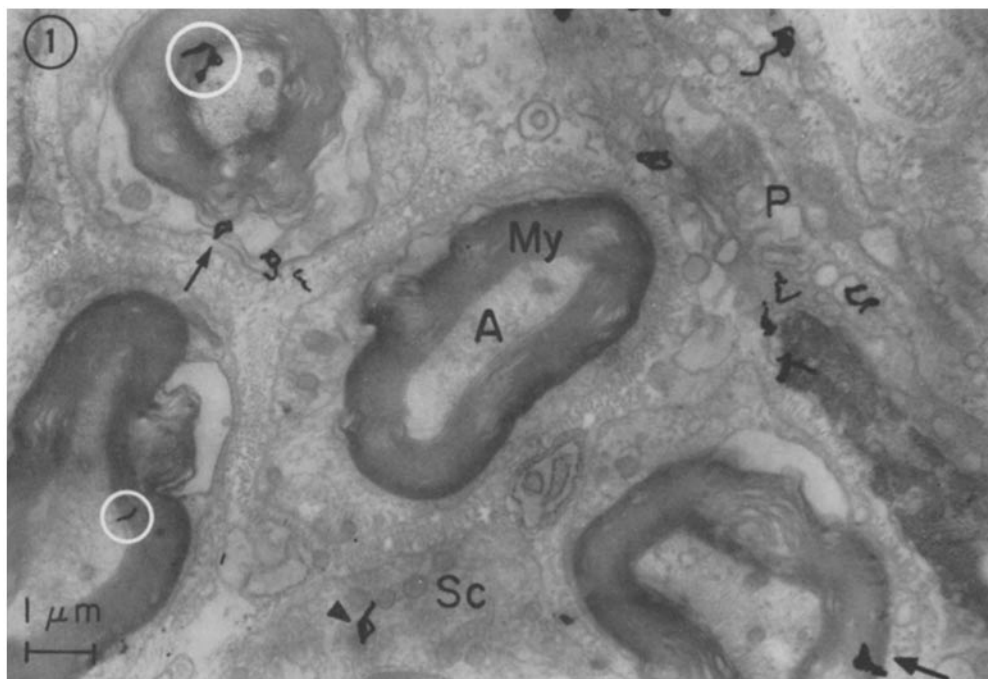
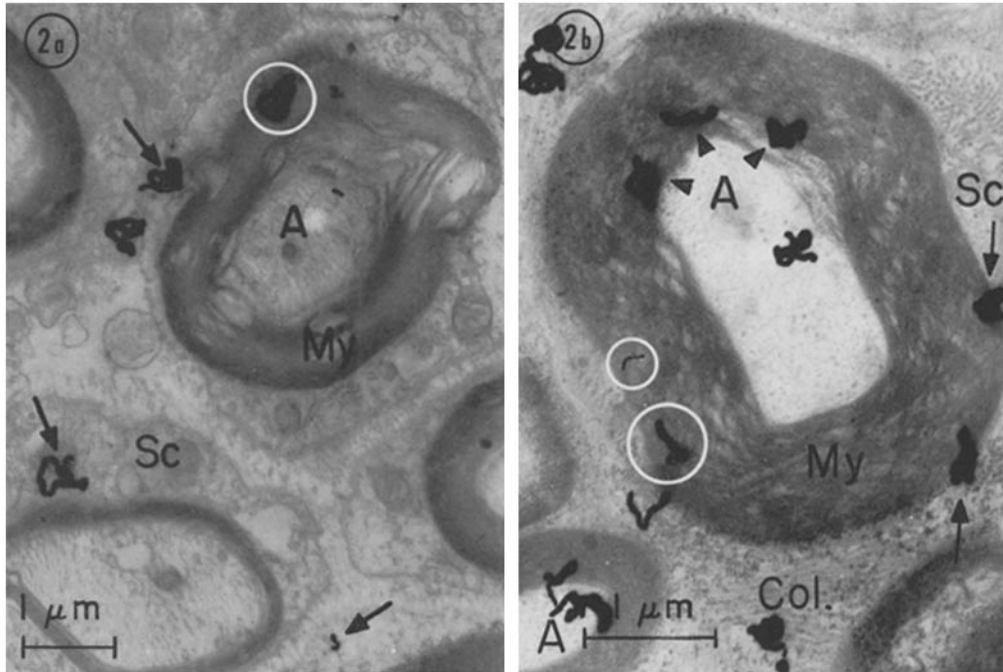


FIGURE 1 An electron microscope autoradiograph of a cross section from the sciatic nerve of a mouse 20 min after intraperitoneal injection of [1,2-³H]cholesterol. Developed silver grains are located over the outer (arrow) and inner (encircled) edges of the myelin sheaths. A grain is also seen in the Schwann cell cytoplasm (arrowhead). Note the perineurium (P) highly labeled. A , axon; Sc , Schwann cell cytoplasm; My , myelin. $\times 9,300$.



FIGURES 2 a and b 20 min after injection. Fig. 2 a shows grains located in the Schwann cell cytoplasm (arrow) close to the outer myelin lamellae. A grain (encircled) is also seen within the myelin sheath. $\times 11,800$. In Fig. 2 b a fiber is shown with grains located over the outer (arrow) and inner (arrowhead) edges of the myelin sheath. Two other grains (encircled) are located within the sheath and one in the axon (A). My, myelin; Sc, Schwann cell; Col, Collagen. $\times 16,000$.

TABLE I
Grain Distribution in Electron Microscope Autoradiographs of Mice Sciatic Nerve at Various Times after Injection

Time after injection	Total number of grains over myelin		
	Outer 1/3	Middle 1/3	Inner 1/3
min	%	%	%
20	53	8	39
40	51	6	43
60	45	14	41
90	43	22	35
120	41	28	31
180	36	34	30

nervous tissue of mice without alteration of the label in the molecule. From the time of administration at 12 days of age until maturity, the molecule (at least the ring structure) remains stable, after dehydration and embedding over 99% of the radioactivity in the embedded nervous tissue has been shown to be due to $[1,2\text{-}^3\text{H}]$ cholesterol. This means that the developed silver grains in

these radioautographs are indeed due to $[1,2\text{-}^3\text{H}]$ cholesterol activity.

No radioactivity was found in the autoradiographs of the nerves from animals sacrificed 10 min after injection; therefore, the analysis was carried out with nerves removed 20 min or longer after the injection.

First Type of Analysis

20 min after injection (Figs. 1 and 2) almost all the grains over myelin are located within the outer and inner thirds of the sheath. The radial distribution of the grains in the myelin sheaths (Table I) shows that 53% of the total number of grains within myelin is found over the outer third, 39% over the inner third, and only 8% was over the middle third subcompartment. 40 min after injection, the percentage of grains over the outer third (51%) was similar to that over the inner third (43%) while the amount of grains over the middle third (6%) was still very low (Table I).

This pattern of distribution within the myelin

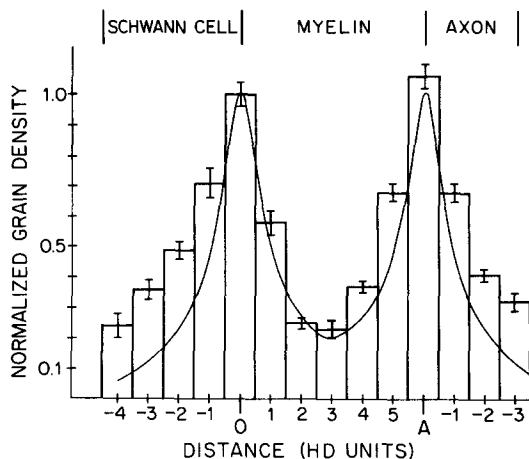


FIGURE 3 Histogram (bars) of the density distribution of silver grains over and around myelin sheaths approximately 6 HD units wide (1 HD unit is 1,600 Å) in mice sciatic nerves 20 min after injection of [1,2-³H]-cholesterol. O and A on the x axis indicate the outer edge of the myelin sheath and the axolemma, respectively. Grain density was normalized to 1 at the outer edge of the myelin sheath. Distance is measured in HD units: (a) from the outer myelin lamellae to the axolemma (myelin compartment), (b) from the outer myelin lamellae up to 4 HD units outside myelin (Schwann cell cytoplasm), (c) from the axolemma up to 3 HD units inside myelin (axon compartment). Two peaks of radioactivity are seen: one is located at the outer edge of the myelin sheath and the other is localized at the inner edge of the sheath including the periaxonal gap. The smooth curve superimposed on the histogram represents the expected distribution if the radioactivity were confined to a hollow band 6 HD units wide (Fig. 11 of Salpeter et al., 1969). There is a close correspondence between experimental and theoretical distribution of grain over myelin. The experimental values outside myelin are higher than the expected densities by more than 2 SD indicating that the Schwann cell cytoplasm and axon compartments are also labeled.

sheath changed with time. The middle third activity increased to 14% of the total at 1 h (Fig. 4); 22% at 1½ h; 28% at 2 h (Fig. 6); and finally 34% at 3 h after injection, a value similar to that for the outer third (36%) and inner third (30%) subcompartments (Table I).

The perineurium (Fig. 1) was always highly labeled. Grains were also seen over red blood cells and unmyelinated single fibers (Fig. 7). Some grains were seen over the outer mesaxon but no attempt was made to quantify them be-

cause of the difficulty in observing the mesaxons in most of the myelinating fibers.

Second Type of Analysis

The analysis of the density distribution of silver grains also shows that at 20 min after injection (Fig. 3) the highest density values are located at the outer and inner edges of myelin. Density values decrease toward the center of the sheath and toward the Schwann cell and axonal compartments.

1½ h after injection (Fig. 4) cholesterol has acquired a more even distribution within the sheath and the experimentally determined densities are distributed as shown in Fig. 5.

3 h after injection (Figs. 6 and 7) cholesterol appears almost homogeneously distributed across the myelin sheath as shown in Fig. 8. The tissue compartment external to myelin in each of the three time periods examined (20, 90, and 180 min) showed significantly higher (>2 SD) grain densities than those expected if all the radioactivity were confined to myelin (Figs. 11 and 12 of Salpeter et al., 1969).

DISCUSSION

The results of these experiments indicate that cholesterol injected intraperitoneally into suckling mice is incorporated in the sciatic nerve in amounts sufficient for quantitative analysis by electron microscope autoradiography as early as 20 min after injection and that cholesterol initially enters myelin through its outer and inner edges. In Fig. 3 we can see that there is a close correspondence between the density distribution obtained experimentally over myelin (bars) at 20 min after injection of [1,2-³H]cholesterol and the theoretical distribution (smooth curve) expected for the inside of a hollow band of approximately 6 HD units wide if all the radioactivity is confined to the edges of such a band (Fig. 11 of Salpeter et al., 1969).

The smooth curve in Fig. 5 represents the expected distribution for two radioactive bands each 2 HD units wide and separated by a non-radioactive band of 2 HD units (Fig. 12 of Salpeter et al., 1969). The grain density between the radioactive bands is additive. The experimental values in the center of the sheath are slightly but significantly higher (>2 SD) than the theoretical ones indicating that, although in a considerably lesser concentration, labeled chole-

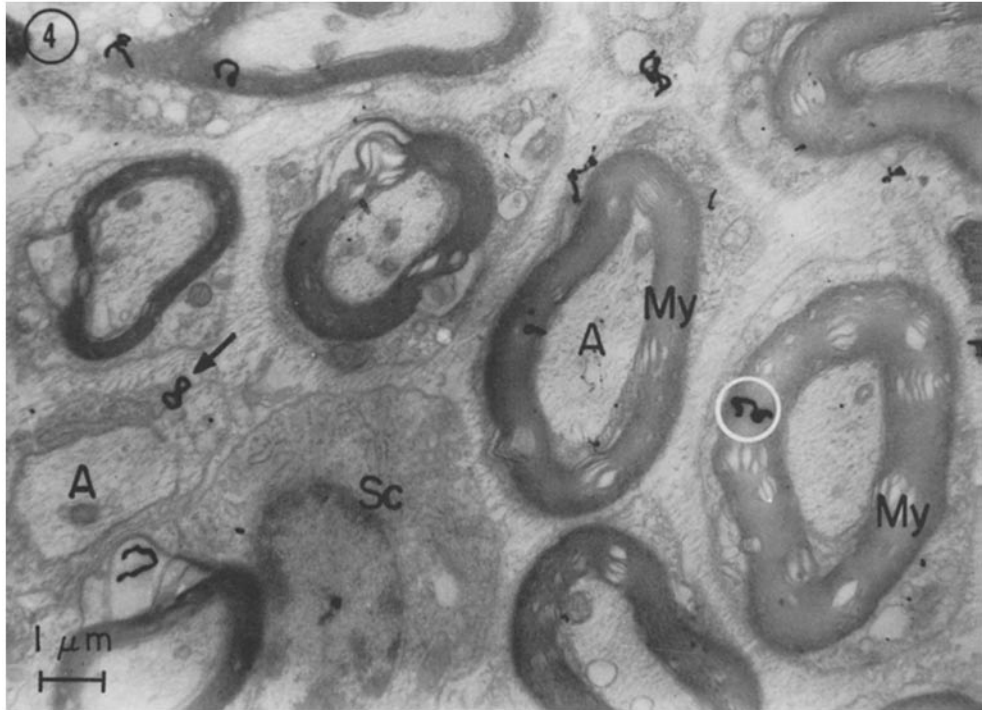


FIGURE 4 1 h after injection: grains are located over the outer and inner edges of the myelin sheaths and in the Schwann cell cytoplasm. A grain is also seen toward the midzone of the myelin sheath (encircled). Note a grain over the outer mesaxon of an unmyelinated fiber (arrow). *A*, axon; *Sc*, Schwann cell; *My*, myelin. $\times 8,400$.

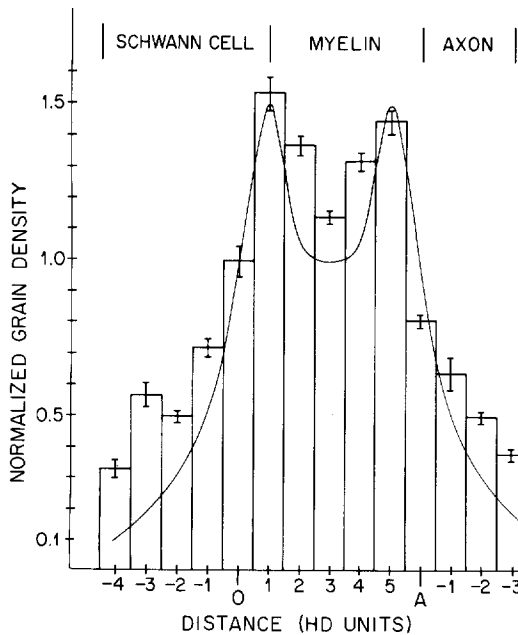
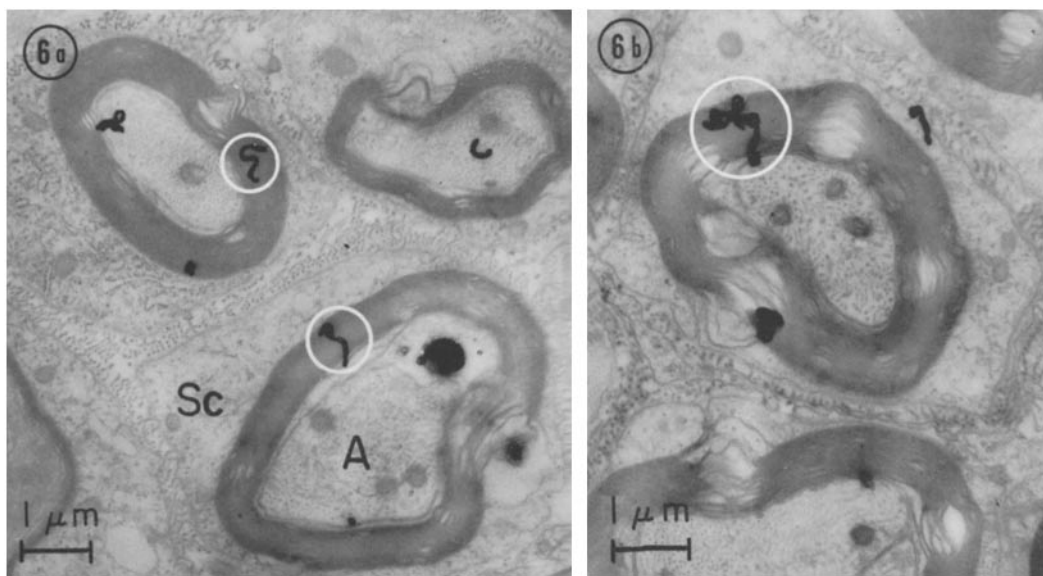


FIGURE 5 Histogram (bars) of the density distribution of silver grains over and around myelin sheaths approximately 6 HD units wide in mice sciatic nerves $1\frac{1}{2}$ h

terol must also be present in the center of the sheath. An alternative explanation is that the radioactive bands are wider than 2 HD. The experimental grain distribution over myelin at 3 h after injection (Fig. 8) is in close correspondence with the expected distribution for the inside

after injection of $[1,2-^3\text{H}]$ cholesterol. Grain density was normalized to 1 at the outer edge of the myelin sheath. The smooth curve superimposed on the histogram represents the expected distribution for two radioactive bands of 2 HD units wide each and separated by a non-radioactive band of 2 HD units (Fig. 12 of Salpeter et al., 1969) assuming that the activity between the radioactive bands would be additive. The experimental values in the center of the myelin sheath differ significantly from the theoretical ones. This is interpreted as indicating that although the greater density of radioactivity lies in two bands, one at the outer edge and the other at the inner edge of the myelin compartment, the center of the sheath is also labeled to certain extent. Experimental values for Schwann cell cytoplasm and axon compartments are also significantly higher than the theoretical ones, indicating that $[1,2-^3\text{H}]$ cholesterol is also present in those compartments.



FIGURES 6 *a* and *b* 2 h after injection the percentage of grains (encircled) located within the middle third subcompartment increased (see Table I). In Fig. 6 *a* grains are seen over the axon and middle third myelin subcompartment $\times 9,100$. A, axon; Sc, Schwann cell. In Fig. 6 *b* grains (encircled) are seen in Schwann cell cytoplasm close to the outer lamellae as well as the outer, middle, and inner third myelin subcompartments. $\times 9,600$.

of a solid band source approximately 6 HD units wide (Fig. 12 of Salpeter et al., 1969). Since the tissue compartments, other than myelin, showed significantly higher grain density than that expected if all the radioactivity were confined to myelin, it is concluded, that at all postinjection periods analyzed, there was a measurable amount of $[1,2-^3\text{H}]$ cholesterol in the Schwann cell cytoplasm and axon.

The deposition of myelin lamellae has been thought to be a relatively slow process (Geren and Schmitt, 1955), and in rats approximately 5 h are required for the formation of the myelin lamellae during the first 10 days after birth (Friede and Samorajski, 1968). Therefore, the rapid uptake of cholesterol by myelin observed in the present study may be related not only to the formation of new myelin lamellae but also to a preferential binding process possibly involved in the maintenance of myelin integrity.

Incorporation of $[1,2-^3\text{H}]$ cholesterol through the inner surface of myelin seems to be due to a transaxolemmal movement of cholesterol and its subsequent uptake by the innermost myelin lamellae. Cholesterol may have reached the axon from the neuronal cell body. This axon \rightarrow myelin movement of molecules is supported by the fact

that the axonal compartment always appears labeled. Recent biochemical studies (Dr. L. M. H. Larramendi, personal communication) have shown that the myelin fraction from the goldfish optic nerve appears highly labeled after injection of radioactive mevalonic acid, a cholesterol precursor, into the vitreous body of the ipsilateral eye. These observations have been interpreted as indicating that the axons of the optic nerve fibers continuously leak label (presumably cholesterol) which is subsequently incorporated into myelin; an interpretation in accord with the present results.

The possibility that myelin could incorporate certain molecules of neuronal origin has been suggested by other authors, for example, by Klenk (1955) who postulated that the axon may supply material necessary for the maintenance of myelin integrity. Elam and Agranoff (1971 *a* and *b*) have reported that after intraocular injection of several ^3H -labeled amino acids and $\text{Na}_2^{35}\text{SO}_4$ the label was found in the optic nerve myelin as well as in the synaptosomal and mitochondria subfractions. Such radioactivity was undiminished in the myelin fraction purified by hypotonic washing and reisolated on a discontinuous gradient. Therefore, they raised the

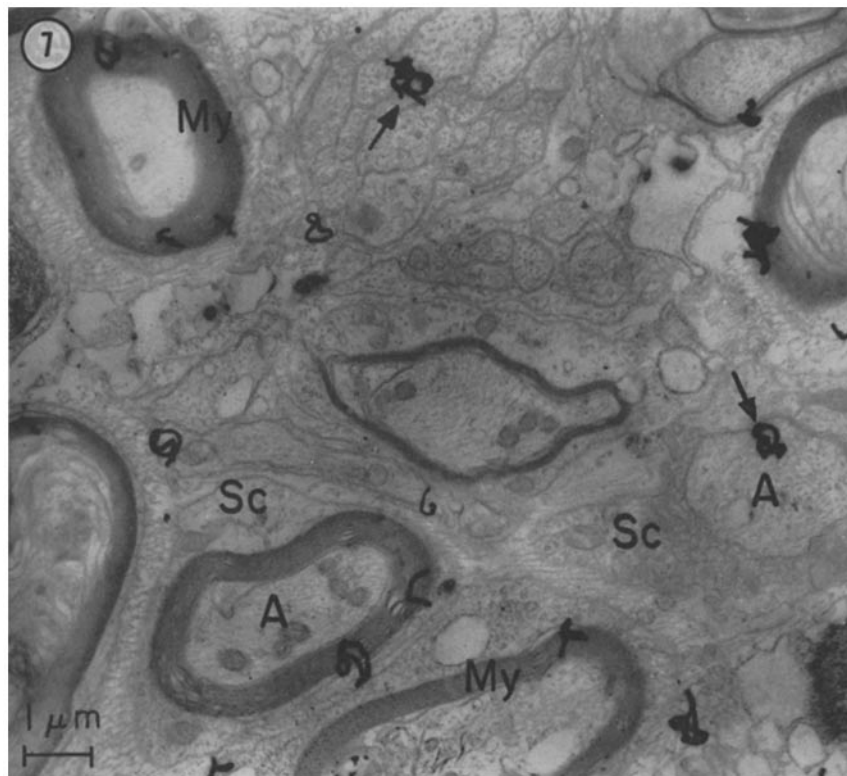


FIGURE 7 Electron micrograph showing a cross section of the sciatic nerve 3 h after injection. Grains are almost homogeneously distributed over the myelin sheaths (*My*). Grains are also seen over unmyelinated fibers and Schwann cell cytoplasm (*Sc*). *A*, axon. $\times 8,400$.

question of whether some myelin protein could have a neuronal origin.

That labeled cholesterol may enter the axon from the neuronal perikaryon is compatible with Miani's (1963) demonstration of a somatoaxonal movement of labeled phospholipids in the cervical vagus and hypoglossal nerve after labeling of the bulb with phosphate- ^{32}P . Miani reported that the rate of flow of the phospholipids was of the order of 72 mm/day and of 39–41 mm/day in the cervical vagus and hypoglossal nerve, respectively.

Axoplasmic transport in the vagus and hypoglossal nerves has also been studied by Sjostrand (1969) in the adolescent rabbit after an intramedullary injection of [^3H]leucine. He reported a rapid transport of labeled proteins in proximo-distal direction at rates of approximately 400 mm/day in the vagus nerve and approximately 350 mm/day in the hypoglossal nerve. Recently, Ochs et al. (1970, 1972) reported the existence of a fast transport system moving materials in cat

sciatic nerves at a rate of 410–424 mm/day after injection of the lumbar seventh (L7) ganglia with the precursors [^3H]leucine or [^3H]lysine.

The assumption that cholesterol reaches the innermost part of the myelin sheath by axoplasmic flow of these molecules from the cell body within 20 min of intraperitoneal injection would imply a very rapid axoplasmic transport of approximately 720 mm/day if the distance is assumed to be only 10 mm. This speed exceeds that reported by Miani (1963), Sjostrand (1969), and Ochs (1970, 1972). Several experiments are being carried out in our laboratory to measure the axoplasmic flow rate of cholesterol in mice and rats sciatic and optic nerves. A second explanation for the presence of cholesterol in the innermost myelin lamellae, almost simultaneous with its presence in the outermost myelin lamellae, is that cholesterol incorporated into the Schwann cell rapidly reaches the adaxonal Schwann layer via the Schmidt-Lanterman clefts and/or the node of Ranvier. From there

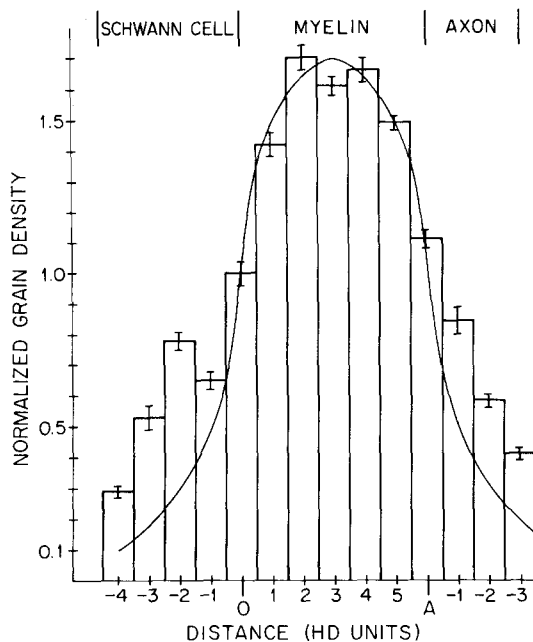


FIGURE 8 Histogram (bars) of the density distribution of silver grains over and around myelin sheaths approximately 6 HD units wide in mice sciatic nerves 3 h after injection of $[1,2\text{-}^3\text{H}]\text{cholesterol}$. Grain density was normalized to 1 at the outer edge of the sheath. The histogram over myelin is in close correspondence with the expected distribution (smooth curve) for the inside of a solid band source approximately 6 HD units wide (Fig. 12 of Salpeter et al., 1969), indicating that $[1,2\text{-}^3\text{H}]\text{cholesterol}$ is almost homogeneously distributed all across the myelin sheath. The experimental values outside and inside myelin are higher by more than 2 SD from the expected densities if myelin were the only source of radioactivity. Therefore, Schwann cell cytoplasm and axon compartment have to be considered labeled also.

it moves outward into the innermost myelin lamellae, thus accounting for the labeling of the latter before the labeling of the sheath midzone. Cholesterol from the adaxonal Schwann layer could also have entered the periaxonal space to be finally incorporated in the axon. If such is the case, one would need to invoke an adaxonal Schwann cytoplasm \rightarrow innermost myelin lamellae movement of cholesterol rather than an axon \rightarrow myelin movement of these molecules. However, a combination of both types of movements of molecules could also be occurring. Evidence for the existence of a transmyelin pathway of substances between the Schwann cell and axon has already been reported by Singer and co-

workers. They have recently found that ruthenium red (Singer et al., 1972) and peroxidase (Krishnan and Singer, 1973) penetrate from the epineurium through the myelin-Schwann sheath into the axon. These findings supplement previous observations on the transport of labeled amino acids through the myelin sheath into the axon (Singer, 1968).

The fact that at all time periods examined the Schwann cell cytoplasm appears labeled suggests that the incorporation of $[1,2\text{-}^3\text{H}]\text{cholesterol}$ through the outer edge of the sheath, as seen in these experiments, takes place by a direct exchange of cholesterol from the Schwann cell to the outermost myelin lamellae. Such an exchange of cholesterol (and other sterols) between myelin and other nervous tissue compartments has been demonstrated by several recent biochemical studies. Thus, Ramsey et al. (1972) have found that after intracerebral injection of labeled mevalonic acid into 30-day old rats, there was a shift of labeled cholesterol from the nonmyelin fraction to the myelin fraction. Banik and Davison (1971) have reported that when myelin which contained labeled sterols was incubated with an unlabeled microsoma fraction, there was a transfer of labeled sterols from myelin to the microsomal fraction.

In the present study it has been found that the density of grains in the center of myelin increased until, at 3 h postinjection, it was greater than or the same as the values found in the outer and inner bands. This indicates that after incorporation in the outer and inner lamellae a rapid exchange, diffusion, or migration of cholesterol occurs within the myelin sheath. We know from previous observations (Hedley-Whyte et al., 1969) that cholesterol, once incorporated in the developing myelin of the peripheral nerve, is still found in the mature myelin and to a lesser extent in the Schwann cell and axon of the mature nerve. It is also known that when a portion of sciatic nerve, previously labeled with $[^3\text{H}]\text{cholesterol}$, is removed and the nerve allowed to regenerate, the labeled cholesterol is found not only in the proximal stump but also in the regenerated distal part (Rawlins et al., 1970). During degeneration cholesterol is kept within myelin debris as an exchangeable pool which is later reutilized for the formation of the newly regenerating fibers (Rawlins et al., 1972). All these observations, together with the present

results, seem to indicate that exogenous cholesterol in the peripheral nerve myelin is subject to a continuous exchange and reutilization. Thus after reaching a homogeneous distribution within myelin (3 h after injection), cholesterol molecules coming from the Schwann cell compartment may traverse the myelin sheath and enter the axon and vice versa.

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