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Myelin: Dynamic or Stable?

BETTY G. UZMAN and E. TESSA HEDLEY-WHYTE

From the Children's Cancer Research Foundation and the Department of Pathology, Harvard Medical School at the Children's Hospital, Boston, Massachusetts 02115

The concept of a spirally wrapped cell membrane as the mode of origin of the myelin sheath has made it difficult to explain (*a*) myelin sheath adjustment to growth of the axon in diameter and length, (*b*) the herniation and implied flexibility of the myelin sheath observed in normal fibers (1), (*c*) the ability of myelinated axons in tissue culture to lose and regain myelin in relatively rapid response to the application and withdrawal of noxious stimuli (2), and (*d*) the varied responses of myelin in pathologic situations (3).

Potential and actual spaces have been demonstrated between the outer surfaces of the myelin lamellae (intraperiod line) by exposure to hypotonic solutions (4) and by penetration of lanthanum solutions.[1] Cytoplasmic trapping is often observed between the inner surfaces (5). Separation of myelin layers has been induced by irradiation damage to Schwann cells in vitro (6). While recent evidence suggests that preferred orientation and positioning of Schwann or glial cytoplasmic microtubles are influential in the over-all maintenance of a flexibly stable sheath form (7), the above observations are consistent with a picture of a stable myelin sheath adaptable to necessary form changes by temporary separation, slippage, and retightening of the spirally wrapped constituent lamellae (3).

Our view of the structural form of myelin regards the electron opaque loci of the outer and inner surfaces of the cell membrane not as "leaflets" but simply as regions of charge distribution to which osmium, dichromate ions, permanganate ions, etc. may be selectively bound.[2] This, in agreement with prevailing ideas about the myelin sheath and cell membranes, supports the thesis that a structural order prevails, as demonstrated by polarization optics and X-ray diffraction (8). In this general scheme, liquid-crystalline smectic arrays of bimolecular lipid layers containing the phospholipid and cholesterol molecules alternate with protein and/or polysaccharide components. The ability of the cell surface to respond quickly to change in shape and volume, as well as the necessity during myelin formation for the Schwann (or glial)

[1] Karnovsky, M. J., and J.-P. Revel. 1967. Personal communication.

[2] We have been able to confirm with the electron microscope that chromate ions compete with osmium for binding sites in the myelin sheath (this is the basis of the lack of blackening by normal nerve in the Marchi staining reaction). Dichromate also binds to the outer surface of the cell membrane in such a way that the myelin period is larger after dichromate fixation than after osmium fixation (in our hands, 170 vs. 110 A for methacrylate imbedding) and the intermediate dense line of myelin is represented by two electron-opaque zones. Dichromate binding within the myelin sheath leads to periodic bands of electron opacity that can be reinforced by staining Araldite sections in uranyl or permanganate solutions, but not in aqueous solutions of osmium tetroxide.

cell membrane to increase by incorporation of molecular components (9), must depend on the "fluid" aspects of this state and make it necessary to consider ways in which molecules can be added to the membrane.

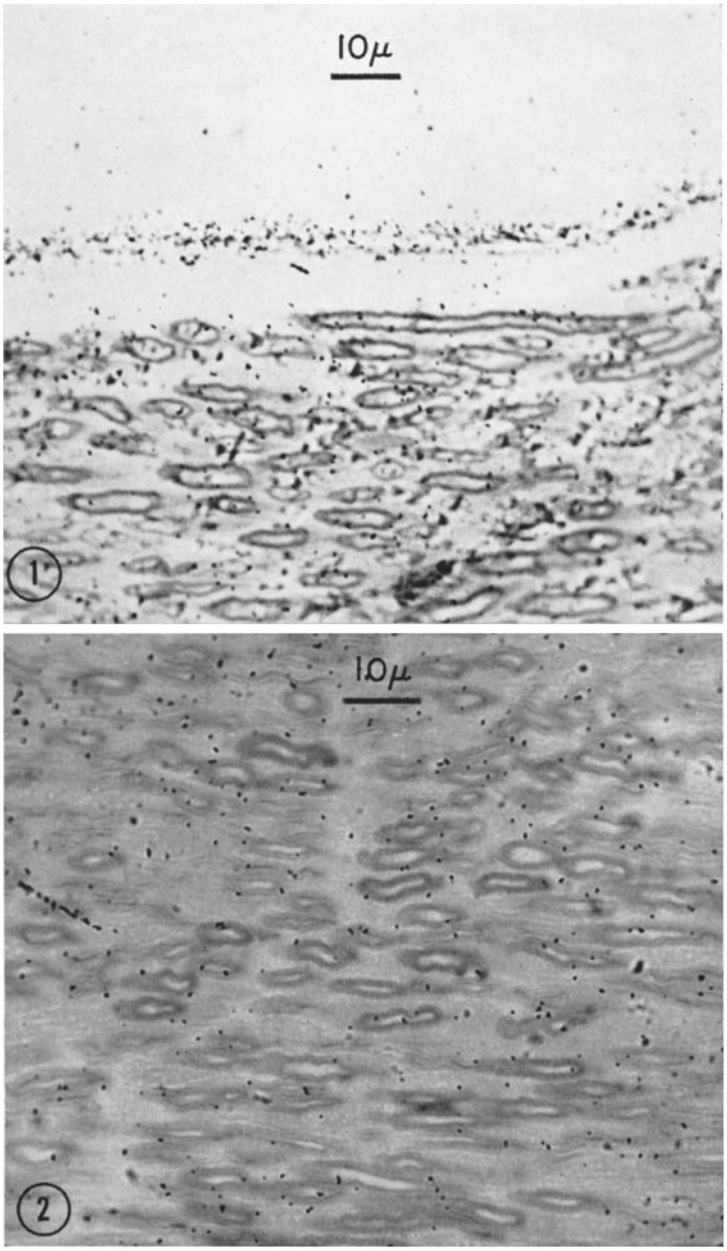
During the past decade, Davison, Dobbing, and their coworkers have provided evidence for the stability of myelin constituents in the central and peripheral nervous systems (10–22). In general, their conclusions have been based on the incorporation into brain or white matter of parenterally administered, labeled lipids or other myelin precursors during myelination and their retention until adult life in chicks, rats, and rabbits. Comparison of the incorporation data in young animals with similar data in adult rats led them to consider the existence of a "fast" (quickly turned over) metabolic pool in myelin as well as a relatively inert but slowly turned over class of myelin components. They have attributed the metabolic inertness of myelin to its membranous lamellar components. Further, they have recently emphasized the vulnerability of the myelinating nervous system to undernutrition as well as the known resistance of mature brain components to starvation (23).

Although the composition of mature myelin suggests an inert state, significant molecular movements may take place. Davison and Wajda have conclusively demonstrated that cholesterol enters white matter (24). Salpeter and Singer have published evidence that, while isotopically labeled leucine enters the axon from the neuronal end (25), radioactive histidine may "move" through myelin anywhere along the length of the nerve fiber (26). On the basis of these observations, one is led to inquire where and in what form new molecules enter myelin. Further, if myelin in vivo is relatively metabolically inert as concerns its complement of chemical constituents, is it subject to a type of internal molecular rearrangement or even reuse from sheath to sheath?

We have observed that lamellar bodies, normally present in mouse optic nerves during active myelination (12–21 days of age) are found only in very small numbers in the mature animal. Since the accumulation of myelin lipids in mouse brain proceeds more quickly than myelin sheaths can be detected by Luxol fast blue staining or by electron microscope survey (27), we have come to regard the lamellar body as a transient storage depot of myelin lipid constituents. This suggests that the lipid components of myelin are accumulated in one morphologically recognizable form (the lamellar body) before being incorporated into the myelin sheath. A probable example of failure of this system is the wobbler-lethal mouse in which the lamellar body fails to disappear and is caught within the myelin lamellae where it undergoes degeneration. In this site it becomes Marchi positive, and is associated with single fiber degeneration, presumably by axonal tamponade.

The advent of stable, isotopically labeled myelin constituents and the development of high-resolution, light and electron microscopic, radioautographic methods have led us to undertake a detailed assessment of the location and route of entry of cholesterol in myelin. Incorporation of labeled cholesterol is now being studied at various stages during myelin formation, as well as at various time intervals after injection in normal mice, in the wobbler-lethal mouse, and during degeneration and regeneration of myelin.

We report here preliminary observations based on data obtained in Swiss albino



All figures are radioautographs of sections of sciatic nerves of Swiss albino mice injected intraperitoneally with cholesterol-1,2-^{3}H.

First number, age of injection; second number, age at sacrifice; third number, number of days emulsion exposed to section. Statistical validation of suggested interpretations is in progress.

FIGURE 1. 8 d/8 d + 4 hr/27 dx. Attention is drawn to heavy labeling of perineurium 4 hr after injection. Diffuse activity is apparent in the nerve with some labeling of myelin sheaths.

FIGURE 2. 5 d/9 d/10 dx. Apparent labeling of myelin sheaths.

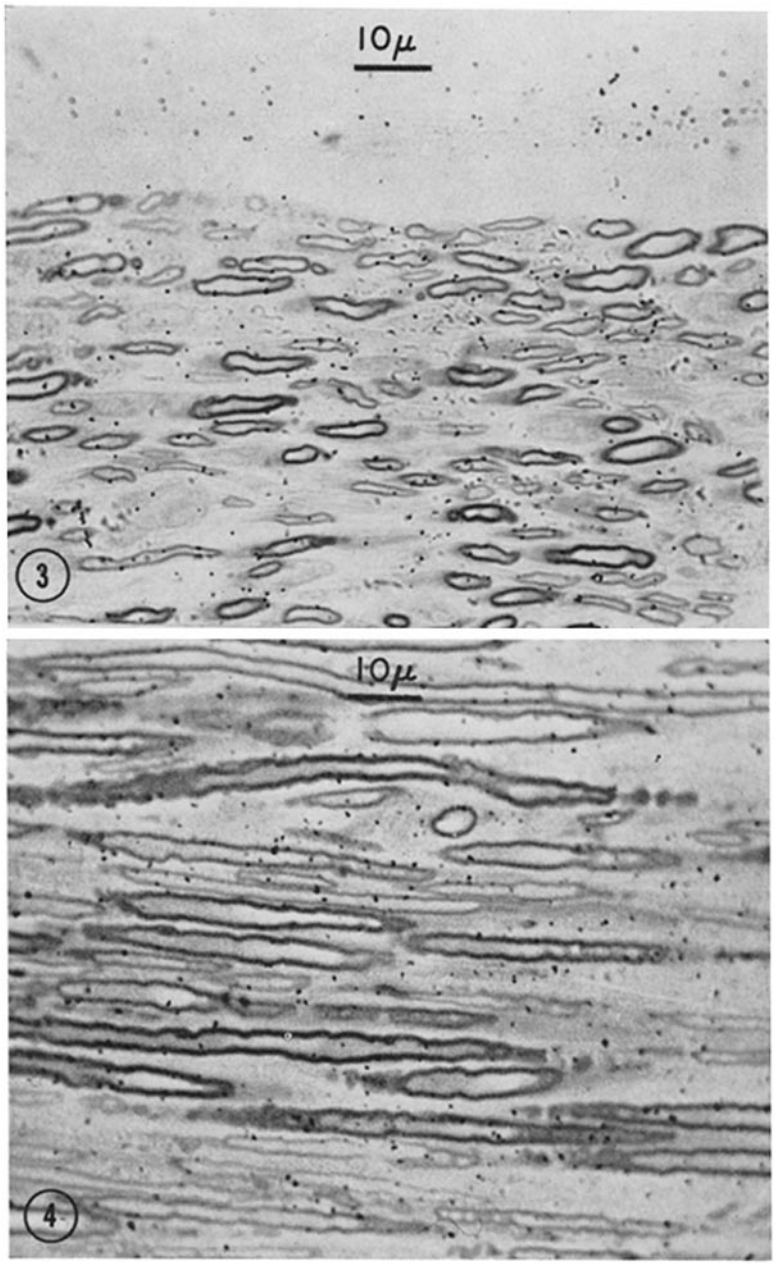
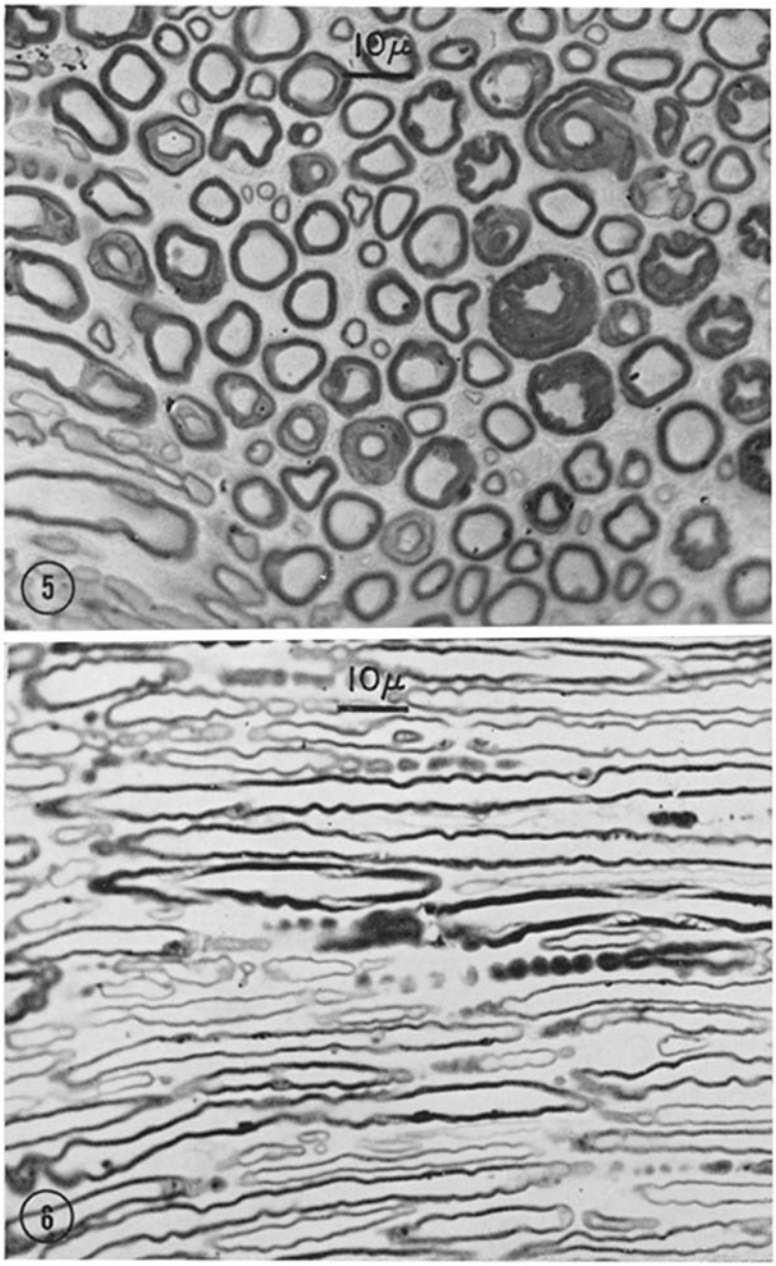


FIGURE 3. 5 d/9 d/17 dx. Minimal activity over perineurium after 4 days.
FIGURE 4. 8 d/12 d/17 dx. Myelin labeling.

mice. 140–160 μc cholesterol-1,2-^{3}H (New England Nuclear Corp., Boston, Mass.) were injected intraperitoneally using 6% dimethylformamide in 5% dextrose (specific activity, 1.89 mc/ml) as the vehicle, at ages in which sciatic nerve myelination is known to be active (5–8 days after birth) and to be relatively less active (12 days after



FIGURES 5 and 6. 8 d/>35 d/7 dx and 5 d/>35 d/17 dx, respectively. Apparent persistence of a small fraction of initial activity in myelin sheaths of mature animal.

birth). The animals have been sacrificed at 1 or 4 hr and 1, 3, 4, 7, or >35 days after injection of labeled cholesterol. Tissues were fixed by immersion in 5% cacodylate-buffered glutaraldehyde, postfixed in chrome-osmium solution (Dalton's), rapidly dehydrated in graded acetones, and infiltrated and imbedded in an Epon-Araldite

mixture (28). Sections (± 1 μ) were mounted on glass slides and coated with Kodak NTB-2 emulsion. After suitable exposure times the slides were developed with D-170 (29) or Dektol (1:1), fixed in Edwal or Kodak Acid Fixer, washed, and dried. Some sections were stained with 0.2% toluidine blue and mounted in Epon-Araldite (30). Other sections were mounted in Zeiss phase-mounting medium, $n = 1.535$.

The discussion of our results is predicated on the assumption that radioactivity, as indicated in technically suitable radioautographs, reflects the location of labeled cholesterol. Evidence that the ^3H in the cholesterol-1,2-^3H molecule is stable is summarized in the appendix. For clarity and convenience we have employed for each specimen a notation indicating age at injection/age at sacrifice/number of days of exposure of NTB-2 emulsion to the tissue section, e.g., 8 d/9 d/20 dx.

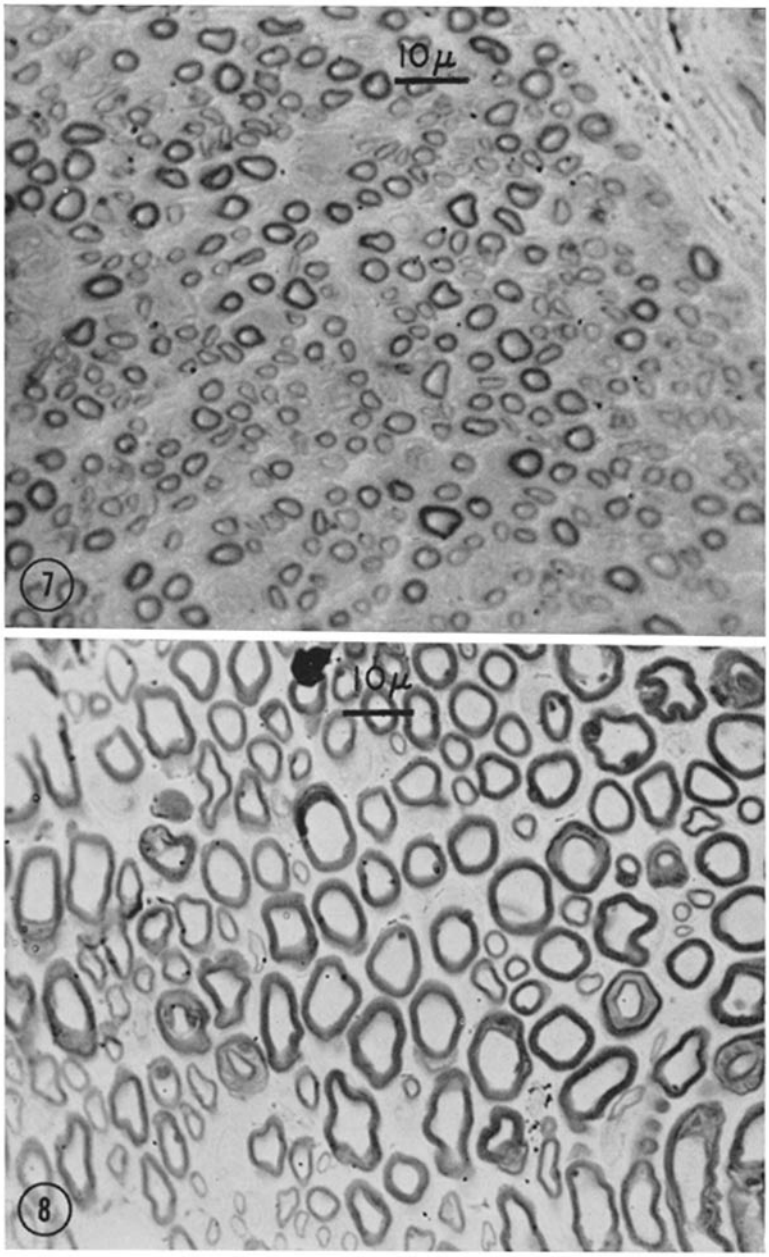
Fig. 1 (8 d/8 d + 4 hr/27 dx) indicates that shortly after intraperitoneal injection, labeled cholesterol accumulates markedly in the perineurium and to some extent is detectable in myelin sheaths. Labeling of myelin can also be seen in Figs. 2 (5 d/9 d/10 dx), 3 (5 d/9 d/17 dx), and 4 (8 d/12 d/17 dx). Fig. 3 is from a nerve removed 4 days after injection of label; there is very little persistent perineural label (compare with Fig. 1 in which the nerve was removed 4 hr after injection of label). Such perineural labeling must, therefore, be a transient phenomenon.

On the other hand, label persists in mouse sciatic myelin until maturity, as demonstrated in Fig. 5 (8 d/>35 d/7 dx). The fact that the grains are few and far between in the mature animal could be accounted for by further growth of the myelin sheath after injection of the labeled cholesterol, by the relatively short exposure of emulsion to section, or by loss of labeled cholesterol with time. We shall have to make appropriate statistical analyses of more amply recorded data to check on the first possibility and use longer exposure times to rule out the second, before attributing lack of label to loss of cholesterol. In this regard, a nerve from an animal injected with cholesterol-1,2-^3H at 5 days of age was removed after 35 days of age. Here (Fig. 6, 5 d/>35 d/15 dx) a 15 day exposure of emulsion to section reveals little evidence of cholesterol retention until maturity.

A further point of interest in the problem of cell membrane growth as related to myelin formation concerns sites of entry of new components into myelin. We hope to be able to distinguish entry through the peripheral Schwann cytoplasmic layer from entry through the axon by examination of transverse sections. Our preliminary results as shown in Fig. 7 (8 d/9 d/7 dx) and Fig. 8 (8 d/>35 d/7 dx) show practically no label over axons. An intermediate sample, Fig. 9 (8 d/12 d/17 dx) suggests a stage in which label reaches the axon, but, as shown in Fig. 7, myelin labeling does not appear to be solely (if at all) dependent on entry via an axonal pathway.

Finally, we have examined a nerve from an animal into which labeled cholesterol had been injected at the age of 12 days. Peripheral myelination in the mouse, while incomplete at this age, is known to be well advanced. Fig. 10 (12 d/12 d + 4 hr/20 dx) shows very little incorporation of cholesterol in perineurium or myelin compared to the example of Fig. 1 (8 d/8 d + 4 hr/27 dx).

While our data is based on a limited number of observations, radioautographic evidence of the incorporation of lipids and lipid precursors into white matter of brain has been reported (31). In this study the localization of the tagged molecules in myelin sheaths was not specifically established. Work on the in vitro incorporation of choline–



FIGURES 7 and 8. 8 d/9 d/7 dx and 8 d/>35 d/7 dx, respectively. Compare lack of axonal label in these two instances (1 and 27 days after injection of label) with Fig. 9.

methyl-^{3}H chloride into myelin has not been reported in sufficient detail for precise interpretation (32). Further accumulation of evidence from experiments such as those which have been described and reviewed here should lead to an understanding of the dynamic aspects of myelin metabolism while allowing for the known stable structure.

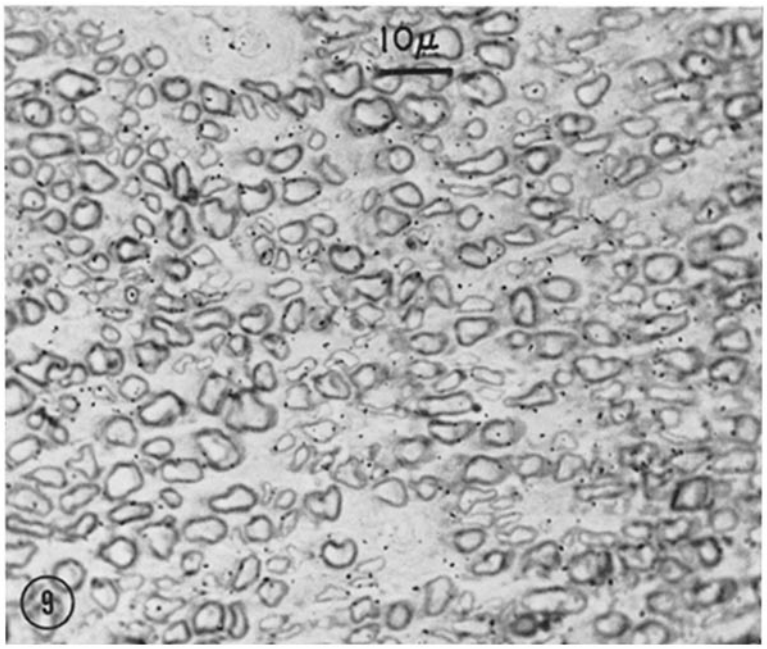


FIGURE 9. 8 d/12 d/17 dx. This cross-section from a nerve 4 days after injection indicates label can occur over axons although labeling of mid-axonal regions is not a prominent feature (Fig. 7) at earlier times after injection.

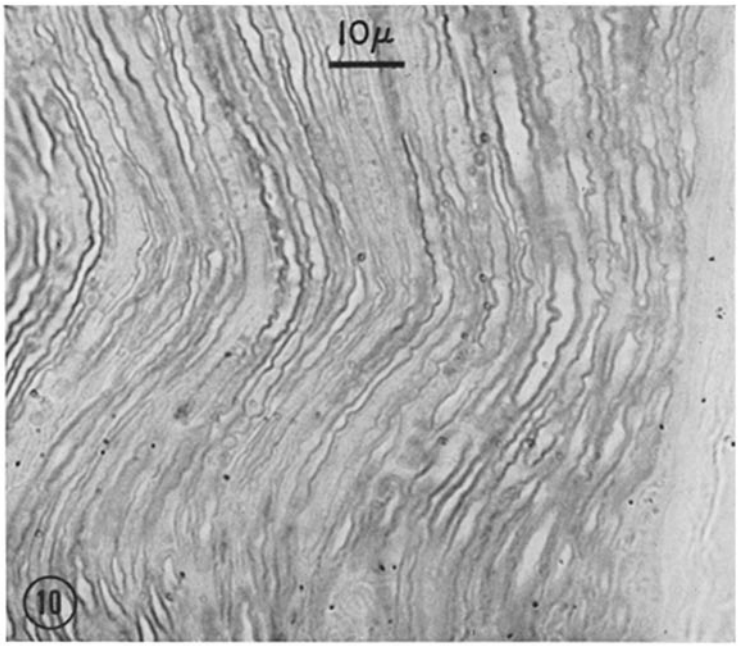


FIGURE 10. 12 d/12 d + 4 hr/20 dx. Compare with Fig. 1. More mature nerve (heavil myelinated) of 12 days shows much less perineural labeling (lower right-hand corner and much less diffusely distributed label over nerve fibers than the 8 day sciatic nerve

Appendix

Several workers have attempted to establish the biological stability of tritium-labeled steroids including cholesterol. The methods of doing this have involved the use of mixtures containing tritium and carbon 14–labeled molecules with the determination of the ratio of $^{3}H/^{14}C$ in the original mixture and in the metabolites or the same substance reisolated from men or animals.

Wood et al. (33) fed a mixture of cholesterol-1,2-^{3}H and cholesterol-4-^{14}C to a human adult and found a constant $^{3}H/^{14}C$ ratio in the plasma and duodenal aspirate over a period of 31 days. Chevallier (34) found constant $^{3}H/^{14}C$ ratios in the excreta of rats 13–18 days after subcutaneous injection of a mixture of labeled cholesterol-^{3}H and cholesterol-4-^{14}C. Werbin et al. (35) found constant $^{3}H/^{14}C$ ratios in plasma cholesterol and urinary steroids for 10 days after intravenous administration of a mixture of generally labeled cholesterol-^{3}H and cholesterol-4-^{14}C to an adult male. They concluded that tritium-labeled cholesterol was sufficiently stable for tracer studies.

Other studies can be cited as indirect evidence of the stability of tritium attached to the first ring of the sterols. Gold and Crigler (36) showed that after administration of a mixture of cortisol-1,2-^{3}H and cortisol-4-^{14}C to an adult man, the ratios of $^{3}H/^{14}C$ in the metabolites remained constant for 24 hr. Osinski (37) incubated a doubly labeled molecule cortisol-1,2-^{3}H-4-^{14}C with rat liver slices and obtained the same $^{3}H/^{14}C$ ratio in the metabolites as were present in the original substance.

The above cited work has depended on the biological stability of the fourth carbon atom. Davison and Wajda (24) demonstrated that cholesterol recovered from the brain of 1 yr old rabbits who had been injected with cholesterol-4-^{14}C at the age of 17 days was still labeled at the fourth carbon position.

Note Added in Proof—Since submission of this manuscript definitive evidence of the long term stability of cholesterol-1, 2-^{3}H in mice has been obtained; the intepretations here have been confirmed and extended by further observations and detailed statistical analysis; all work is in preparation for publication by E. T. Hedley-Whyte, B. G. Uzman, and F. A. Rawlins.

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