RADIOAUTOGRAPHIC STUDIES OF

CHOLINE INCORPORATION

INTO PERIPHERAL NERVE MYELIN

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ABSTRACT

This radioautographic study was designed to localize the cytological sites involved in the incorporation of a lipid precursor into the myelin and the myelin-related cell of the peripheral nervous system. Both myelinating and fully myelinated cultures of rat dorsal root ganglia were exposed to a 30-min pulse of tritiated choline and either fixed immediately or allowed 6 or 48 hr of chase incubation before fixation. After Epon embedding, light and electron microscopic radioautograms were prepared with Ilford L-4 emulsion. Analysis of the pattern of choline incorporation into myelinating cultures indicated that radioactivity appeared all along the length of the internode, without there being a preferential site of initial incorporation. Light microscopic radioautograms of cultures at varying states of maturity were compared in order to determine the relative degree of myelin labeling. This analysis indicated that the myelin-Schwann cell unit in the fully myelinated cultures incorporated choline as actively as did this unit in the myelinating cultures. Because of technical difficulties, it was not possible to determine the precise localization of the incorporated radioactivity within the compact myelin. These data are related to recent biochemical studies indicating that the mature myelin of the central nervous system does incorporate a significant amount of lipid precursor under the appropriate experimental conditions. These observations support the concept that a significant amount of myelin-related metabolic activity occurs in mature tissue; this activity is considered part of an essential and continuous process of myelin maintenance and repair.

INTRODUCTION

Our knowledge of the metabolic events occurring in relation to the myelin sheath is based primarily on biochemical studies of central nervous tissue. The data accumulated over the last decade have generally been interpreted as indicating that myelin, once formed, is metabolically stable: i.e., its components turn over at a very slow rate (20). The experiments from which this concept of myelin stability derives generally involved the systemic injection of lipid precursors and the subsequent analysis of central nervous system (CNS) lipids. Such experiments demonstrated (1) that lipid precursors were actively incorporated during the period of myelin formation (21); (2) that incorporated radioactivity persisted in brain lipids for long periods with little loss (74); and (3) that an adult animal incorporated less precursor into brain lipids than did an immature animal (1). As will be discussed in detail later, the interpretation of many of these experiments rested

on certain suppositions; among these were the assumptions that certain lipids were uniquely characteristic of myelin and that blood-brain barrier effects would not significantly influence the results. Recent biochemical studies using direct intracerebral injection of precursors and the analysis of lipids in the isolated myelin subfraction suggest the need for a reexamination of the concept of myelin stability.

During this same period, there has been an increasing refinement in our knowledge of the histology of the myelin sheath (reviewed in 12). Electron microscopic studies of peripheral myelin have demonstrated that the spirally disposed, compacted lamellae are derived from the plasma membrane of the Schwann cell (25, 51). The cytoplasm of the related Schwann cell is located external and internal to the lamellae, in the cytoplasmic compartments of the paranodal region, and in the Schmidt-Lanterman clefts. Therefore, it seems reasonable at present to think of a metabolic unit composed of the myelin disposed between two nodes of Ranvier (the internode) and the cytoplasm of the related Schwann cell. The present study employs the techniques of light and electron microscopic radioautography in an attempt to answer several important questions regarding the activities of this myelin-Schwann cell unit in the processes of myelin formation and maintenance. First, there is the question of the pattern of incorporation of precursors along the myelin internode. Do all regions participate, or is there increased incorporation near the nucleus, near the node, or in the Schmidt-Lanterman clefts? Second, is there any difference between the incorporation of precursor during the stage of myelin formation and that during the stage of myelin maintenance? Third, could sufficient resolution be obtained to determine whether incorporation occurs at the inner or outer layers of the myelin sheath, particularly whether the formation of myelin membranes occurs specifically at the internal or external mesaxon?

The peripheral nervous system was selected for this investigation because of the one-to-one relationship between the Schwann cell and myelin internode, and because of the significant amount of collagen-filled extracellular space. Both these factors greatly facilitate the interpretation of light and electron microscopic radioautograms. Concern over a blood-nervous tissue barrier effect, particularly an effect that may differ in its char-

acteristics during maturation, was one of the considerations that led to the use of the tissue culture system for the study herein reported. The peripheral nervous system (PNS) cultures used in this study have been documented as containing a microcosm of all elements present in the vertebrate PNS in situ (11). Generally, the histological organization of the nerve fascicle in culture is similar to the in vivo situation except that the perineurial sheath is not completely developed in all areas (11). Thus, a potential barrier for precursor penetration is circumvented.

It was possible, with the materials and techniques utilized in the present study, to answer some of the questions posed concerning the metabolic events of the myelin-Schwann cell unit (myelin-glial unit). Choline was found to be a useful precursor for the radioautographic study of PNS myelin metabolism. It was demonstrated that precursor incorporation in a myelinating system occurred all along the length of the internode, without regard for the paranuclear and paranodal regions. A comparison of the degree of incorporation into forming and mature myelin–Schwann cell units revealed a remarkable similarity in the degree of incorporation, despite the difference in maturational states. These data will be discussed in light of some recent biochemical experiments. Taken together, the morphological and biochemical results argue against the general concept of the stability of the mature myelin-glial unit; they suggest instead that this unit is involved in a substantial amount of myelin-related metabolic activity. Some of these experiments have been reported in a preliminary note (26).

MATERIALS AND METHODS

Precursor

Choline was selected for this radioautographic study after experience with a number of lipid and protein precursors (glucose, galactose, acetate, and serine). Choline has been used successfully for labeling of the lecithin phospholipids of mitochondrial membranes (30). As shown in Table I, choline is metabolized into three phospholipids which are known to be components of the biochemically prepared [for example, (23)] myelin subfraction-sphingomyelin, phosphatidyl choline (lecithin) and phosphatidal choline (a plasmalogen). It is assumed that this is a major metabolic pathway for choline in the tissue culture system employed. According to the only available study on PNS myelin (41), these choline phos-

pholipids constitute about 23% of the molar percentage of the total lipids of the myelin fraction, versus 13–14% for CNS myelin (39). These data also showed that PNS myelin contains approximately

(Details of the fixation and embedding procedures are given in reference 14.) The following table summarizes the experimental design to the time of fixation.

	Wash in BSS	Pulse period	Wash in BSS	Further incubation	Final step
				hr	
Group 1	3 times	30 min	3 times	0	Fixation
Group 2	"	"	"	6	Fixation
Group 3	"	"	"	48	Fixation

twice as much sphingomyelin as does CNS myelin and correspondingly lower proportions of cerebroside and cerebroside sulfate. The analysis of the myelin fraction has demonstrated that there is no single lipid which is unique for myelin (40); choline phospholipids have been found in other tissue fractions, such as microsomes (18) and nonmyelin white matter (39).

Tissue Culture

The standard Maximow techniques were used for the culture of fetal rat (18–20-day) dorsal root ganglia (11). Two spinal ganglia were placed on a collagen-coated coverslip; these were washed and refed twice weekly. Neuronal development and fascicle formation occurred during the early weeks in vitro, followed by myelin formation. Myelin was first seen around 18–24 days in vitro (DIV), and continued to form slowly for another 2–4 wk, until the mature state was reached at 40–50 DIV. Cultures used in the present study were 35 DIV (during myelination), and 70 and 94 DIV (fully mature).

Pulse Labeling

A group of sister cultures was exposed to a pulse period of tritiated choline and either fixed immediately or chased with unlabeled precursor for 6 and 48 hr. The pulse period consisted of 30-min incubation with methyl-choline-³H-chloride (New England Nuclear Corporation, Boston; Specific activity = 100 mc/mmole; purity assayed over 99%) at a concentration of 5 μc/drop of feed/coverslip (100 μc/cc). The feed thus contained a choline concentration of 0.14 mg/cc. The chase feed contained the same concentration of nonradioactive ("cold") choline. After the pulse period, the cultures were washed three times in Earle's balanced salt solution (BSS) to rid the coverslip of any unincorporated radioactive choline. The cultures were fixed in a solution of 2% osmium tetroxide buffered with veronal acetate (pH 7.4) with added calcium chloride (0.05%). Fixation was preceded by 3–4 rinses in BSS and followed by dehydration in ethanol (50, 75, 95, and 100%) and propylene oxide, and embedding in an Epon mixture.

Liquid Scintillation Counting

½-ml aliquots of each of the washing and fixation solutions were mixed with 15 ml of Bray's solution (9) and counted in a Packard Tri-Carb Model 500 C Liquid Scintillation Counting System. The counting efficiency for tritium in this system was determined to be approximately 10%. The data obtained were meaningful for general comparative purposes, but not in absolute terms because of the variable quenching effects for the different types of solutions used.

Radioautography

Many procedures and modifications have been recommended for light microscopic (LM) and electron microscopic (EM) radioautography (reviewed in 8, 52, 64). The present study employed the techniques recommended by Caro (15) for use with Ilford L-4 nuclear emulsion, with minor modifications as discussed below.

For LM radioautography, slides containing semithin Epon sections approximately 1.0–1.5 μ in thickness were coated in a 1:1 dilution of L-4 emulsion. The slides were stored at 4°C for 11 days, then developed in D-19 (20°C for 4 min). Most preparations were examined under phase microscopy after glycerin mounting; staining with 1% toluidine blue often made interpretation of the radioautograms more difficult.

For EM radioautography, diamond knife sections approximately 1000 A in thickness were picked up on collodion-coated copper grids. Most grids had been carbon-coated prior to section application, then subjected to the radioautographic procedures, and stained after development as discussed below. Other grids were first stained with full strength Reynolds' (50) lead citrate (0.16 N NaOH), then carbon-coated, and subsequently coated with emulsion. The latter preparations required examination with 100 kv in order to visualize the material through the gelatin layer of the emulsion. It has been observed that Reynolds' lead stain may remove developed silver grains (15, 49). Therefore, the standard Reynolds' stain was diluted 1 in 8 to the recommended con-

centration of 0.02 N NaOH for staining after developing the radioautograms (15, 49). This technique was successful for staining the tissue and removing the bulk of the gelatin without evident loss of developed grains.

For application of L-4 emulsion onto EM grids, the loop technique was used. The emulsion was diluted (usually 10 g in 35–40 cc of water) to achieve a packed monolayer of silver crystals when examined undeveloped in the electron microscope. The grids were stored at 4°C in a desiccator for 6–9 wk and developed in Microdol X (4½ min at 19–20°C). With selection of low background emulsion (less than 2–3 grains per 1000 μ^2), the background of the developed EM radioautograms constituted less than 10% of the average grain counts over the tissue.

Kodak NTE emulsion was used according to the

NTE emulsion and with the use of fine-grain developers, the resolution has been calculated to be 770 A (6) to 950 A (56). In actual practice, Caro (15) obtained a resolution of approximately 1000 A with Ilford L-4 emulsion Using their own techniques, Salpeter et al. (57) have found that the resolution actually obtained with NTE and L-4 emulsions is 850–1600 A, and that for equal section thickness the resolution is about 600 A poorer with the L-4 emulsion. In the present study a circle of 1500 A radius was drawn from the midpoint of the developed silver grain of the L-4 emulsion. The assignment of grains was determined by assessing which cytoplasmic constituent (axon, Schwann cell cytoplasm, or myelin) occupied more than half of this circle (Fig. 4).

Similar considerations apply to LM radioautograms. Since the average range of beta emissions

TABLE I

Biochemical Pathways for Choline (54)

Precursor		Choline	
Intermediaries		Cytidine diphophosphate	(CDP) Choline
	D-a,b,-diglyceride	Choline plasmalogen	Ceramide
Phospholipid	Lecithin		Sphingomyelin
Molar percentage of total lipids of myelin			
1) CNS (39)	7.2– 9.2	0.2	4.3–6.2
2) PNS (41)	9.4–10.0	—	12.1–12.3

procedures recommended by Salpeter and Bachmann (55), with minor modifications. Thin sections (about 500 A, grey interference color) were picked up on collodion-coated copper grids, stained with Reynolds' lead citrate, and then carbon-coated. After centrifugation of the commercial product, the appropriate dilution was made so as to achieve a packed monolayer of emulsion when applied with a dropper onto the grids, followed by blotting to remove excess emulsion. These grids were stored in helium (4°C) for 7–8 wk.

Resolution

The problem of radioautographic resolution at the level of the electron microscope has been discussed repeatedly (6, 8, 15, 36, 52). Resolution has been defined by Bachmann and Salpeter (6) as that radius from a point source which defines a circle containing one-half the developed grains originating from that source. With a tritium label and Ilford L-4 emulsion, the resolution has been calculated to be between 1270 A (56) and 1850 A (6). For Kodak

from tritium in tissues is approximately 0.5 μ (8), only a limited portion of a tissue section will contribute significantly to an radioautogram. In practice, thicker sections are often used to facilitate light microscopic visualization of the tissue components. If, as has been suggested (43), the first layer of emulsion (silver and gelatin) absorbs the majority of the beta emissions from tritium, one may assume an effective monolayer of the L-4 emulsion. With these considerations in mind, the resolution, as defined above, was calculated from data given by Salpeter and Bachmann (56) to be approximately 0.3 μ radius from a point source. In this case, the photographic error is thought to be contributing minimally to the total error. In our system, the developed LM silver grain (using D-19) was found to measure about 0.5 μ, thereby occupying almost the whole of the (50%) probability circle. As will be documented in the Results, the nature of the experiment and the material, plus this estimate of the resolution, allowed light microscopic localization of some grains to the myelin-glial unit with a high degree of assurance.
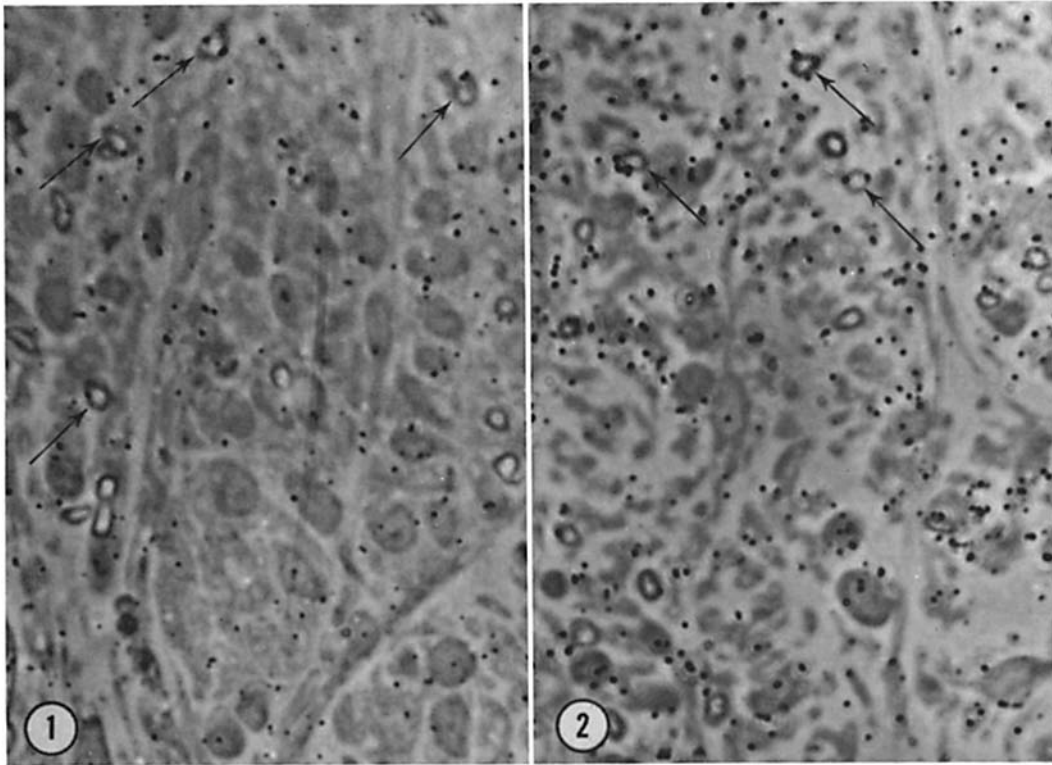
FIGURE 1   Light microscopic radioautogram of a culture 35 days in vitro (DIV) exposed to choline-^{3}H for 30 min and fixed immediately. Grains directly overlie a number of myelin sheaths, examples of which are indicated by arrows. Additional grains overlie Schwann and connective tissue cells. The lighter areas represent noncellular collagen-containing space (c). Semithin Epon section exposed to Ilford L-4 emulsion and photographed with the phase microscope. × 1,200.

FIGURE 2   Light microscopic radioautogram of a culture 35 DIV exposed to choline-^{3}H for 30 min and fixed 48 hr later after a chase incubation. In comparison with Fig. 1, an increase proportion of myelin sheaths is overlain with grains and a number of sheaths are marked by multiple grains (see arrows). Grains over the clear intercellular areas are rare, indicating a low background. Prepared as in Fig. 1. × 1,200.

RESULTS

Morphological Observations

In the presence of nonradioactive choline (0.14 mg/cc), mature cultures showed no light microscopic evidence of damage, and developing cultures continued to form myelin in the expected manner. The dose of tritium used produced neither visible effect on the living cells nor cytological damage at the electron microscopic level.

Cultures 35 DIV

These cultures contained a substantial amount of myelin, but were still in the period of myelin formation. The choice of this phase of develop-

ment was based upon in vivo studies which have demonstrated that maximal precursor incorporation into "myelin lipids" occurs during active myelin formation. Since new myelin internodes continue to form over a period of weeks in these PNS cultures, a 30-min pulse label involves only a very small part of the total process of myelination.

LM Analysis

30-MIN PULSE: After 30 min of exposure to tritiated choline, all the cellular elements of the culture were labeled. This was anticipated, for choline-containing lipids probably occur in all cell types present. Grains were present over the

neuronal cytoplasm, the connective tissue cellular elements, and the nerve fascicles. Most significantly, there were grains directly overlying the myelin and/or its adjacent cytoplasmic component (Fig. 1). There was the impression of a paucity of grains directly overlying axoplasm, a finding which was confirmed by subsequent electron microscopic analysis (see Table II). This lack of early axonal labeling, coupled with the fact that often a considerable noncellular space surrounds and separates the individual myelinated fibers, makes it possible to positively assign the initial labeling to a specific myelin-Schwann cell unit, as opposed to the axon or to adjacent nerve fibers. This, in turn, allows a meaningful LM comparison of the degree of myelin labeling in cultures of different ages exposed to the same 30-min pulse of tritiated choline. In these myelinating cultures, approximately 40% of the cross-sectioned myelin-Schwann cell profiles were labeled, usually with one grain each.

CHASE EXPERIMENTS: After 6 and 48 hr of chase incubation, the LM radioautograms were much heavier, all the cellular elements manifesting an increased amount of labeling (Fig. 2). Grains were again localized directly over myelin sheaths, but with an increased frequency amounting

to approximately twice that of the 30-min pulse label (Table II). In addition to the fact that 70-85% of the cross-sectioned myelin sheaths were overlain with grains, there were often more grains per myelin profile. This aspect of the increased labeling was not quantitated.

EM Analysis

No single cell type or cell component contributed inordinately to the widespread labeling. Some mitochondria had grains directly over them, and grains were frequently noted in areas containing either flattened extensions of connective tissue cells or ensheathing Schwann cell processes (see Fig. 5). Most significantly, it was observed that myelin lamellae were labeled (Figs. 3-6). This was established by observations on thicker or obliquely cut myelin sheaths (Figs. 4, 6). This positive labeling of the myelin was seen in all cultures, i.e., those which had been only pulse labeled as well as those chased for 6 and 48 hr. The heavier labeling found in the LM radioautograms after the chase experiments was also observed in the EM radioautograms. Not only was the myelin labeled more frequently, but there was the same tendency for more grains to be seen per cross-sectioned profile; approximately

TABLE II

Grain Counts of Radioautograms

Technique	Cross-sectioned myelinated fibers	No chase	6-hr Chase	48-hr Chase
LM	% of myelin sheaths labeled	40%	70%	85%
EM	Total No. cross-sectioned sheaths counted	143	247	168
	Myelin			
	a. % of sheaths labeled	9%	22%	24%
	b. % of total grains located over myelin	73%	58%	56%
EM	*Schwann cell cytoplasm*			
	a. Frequency of labeling	3%	9%	9%
	b. % of total grains located over Schwann cytoplasm	17%	18%	18%
EM	*Axons*			
	a. Frequency of labeling	2%	12%	14%
	b. % of total grains located over axoplasm	10%	24%	26%

Cultures 35 DIV were exposed to choline-^{3}H for 30 min, and chased as indicated. For the LM analysis, grains were assigned to the myelin sheath only if they were located directly over the myelin itself. An example of this method of grain assignment is given in Fig. 16. For the EM analysis, grains were assigned according to the resolution considerations discussed under Materials and Methods.
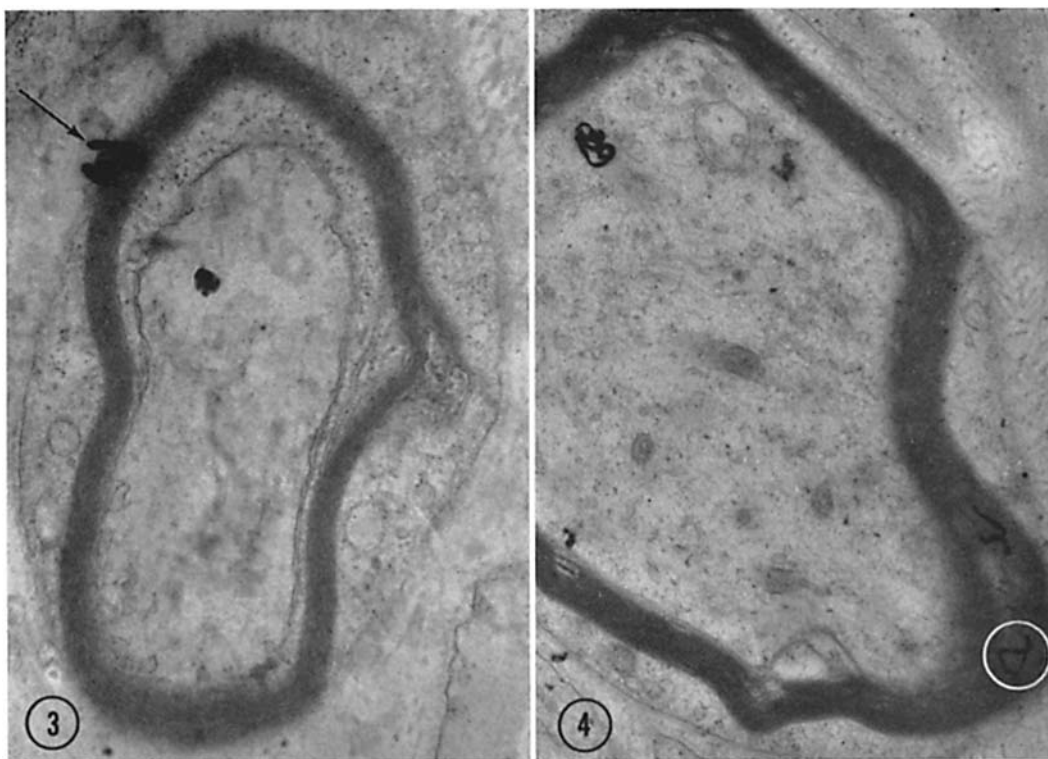
FIGURE 3 Electron microscopic radioautogram of a culture 35 DIV exposed to choline-³H for 30 min and fixed immediately. A developed silver grain directly overlies the myelin (at the arrow). The amount and nature of the cytoplasm internal and external to the myelin suggest that this Schwann cell is in an active phase of myelin formation. Radioautogram exposed to Ilford L-4 emulsion and stained, after development, with a dilute Pb stain. × 32,000.

FIGURE 4 Electron microscopic radioautogram of a culture 35 DIV exposed to choline-³H for 30 min and chased for 6 hr before fixation. Three grains overlie the myelin, as it is obliquely sectioned. There is, in addition, a grain which may be localized to the axon, which is representative of the axonal labeling seen at this stage (see Table II). The circle surrounding the grain indicates the resolution obtainable by the techniques used, as discussed in the text. It is seen that the area enclosed by this circle encompasses predominantly the myelin lamellae. Prepared as in Fig. 3. × 20,500.

twice the number of grains were present. Tabulated counts (Table II) showed that the myelin was labeled with a frequency of 9% after the pulse label, and that the frequency was 22 and 24% after the chase intervals. The lower frequency of myelin labeling in the EM radioautograms compared to the LM radioautograms may be explained partly on the basis of the thinner sections used for EM, and by the fact that with EM analysis some of the grains can be more accurately assigned to the Schwann cell cytoplasm and to the axon. In addition, L-4 emulsion as used for EM radioautography records only about one of every 10 tritium decays occurring in

the specimen (7). This figure, as well as other considerations, would mean that only a very small fraction of the total radioactivity present in the tissue is recorded in the EM radioautograms (7). Despite the decreased frequency of labeling in the EM radioautograms, the percentage of sheaths labeled approximately doubled during the chase period, just as it did in the LM radioautograms.

In some cases the increased resolution of the EM procedures allowed localization of the label to the Schwann cell cytoplasm surrounding the myelin lamellae. A comparison was made between the number of grains seen in this location and the
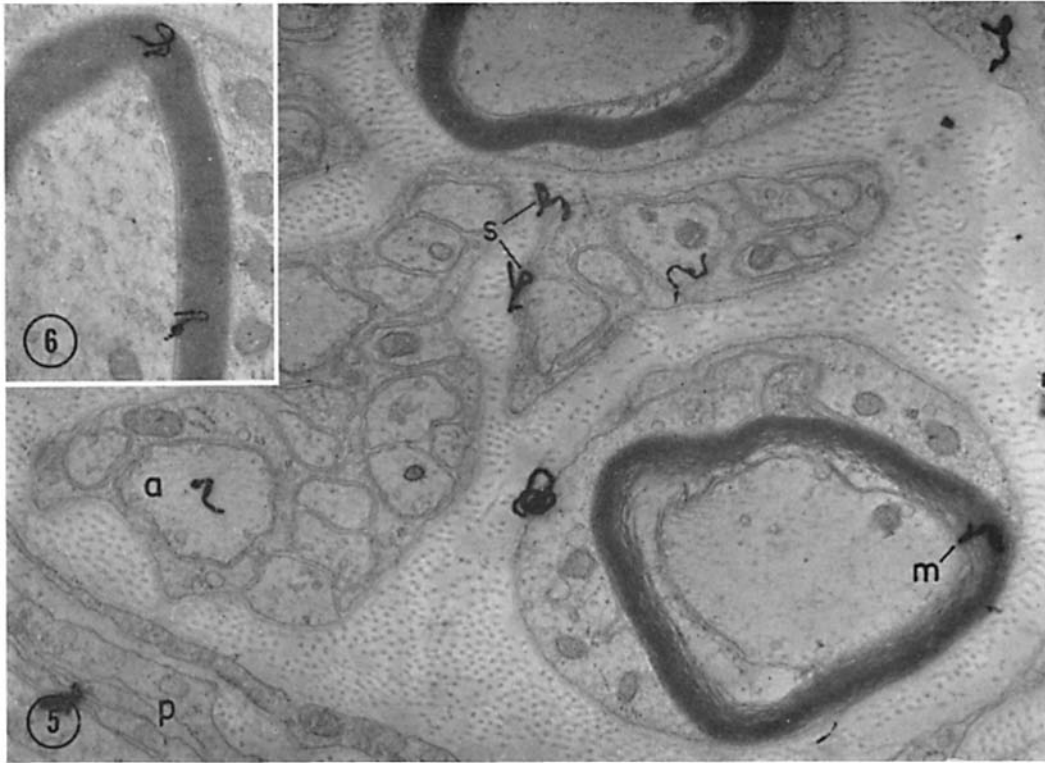
FIGURE 5 Electron microscopic radioautogram of tissue after 6 hr of chase. This nerve fascicle consists of myelinated and unmyelinated axons and their associated Schwann cell cytoplasm, as well as perineurial elements (*p*). The grains may be assigned to: (*1*) the myelin (*m*); (*2*) the unmyelinated axon (*a*); (*3*) areas of ensheathing Schwann cell processes (*s*); and (*4*) the perineurial elements (*p*). Precise localization to specific tissue elements is not possible in the latter two cases because of the resolution limitations discussed in the text. This fascicle demonstrates the noncellular collagen-filled space separating the nerve fibers from each other. Prepared as in Fig. 3. × 20,000.

FIGURE 6 Inset A radioautogram of the same material as in Fig. 5. It presents a particularly clear example of two silver grains which may be assigned to myelin, here cut in cross-section. To see multiple grains per myelin profile was common in the chase experiments. Prepared as in Fig. 3. × 23,000.

number of cross-sectioned profiles of myelin examined (Table II). The frequency of cytoplasmic labeling was found to be higher in the chase intervals than after the pulse exposure. One interpretation of this finding is that precursor material present after a 30-min pulse was extracted during the fixation procedure, but this precursor was further metabolized and more securely incorporated if the cultures were refed and allowed additional incubation time. Additional evidence for this interpretation is available from the liquid scintillation-counting data, to be discussed below.

Analysis of EM radioautograms was performed to exclude the possibility that grains observed directly overlying compact myelin represented scatter from heavily labeled adjacent structures (Schwann cell cytoplasm and axoplasm). In an analysis of cross-sections, the number of grains assigned to each cytoplasmic constituent was related to the total number of grains counted over the axon-myelin–Schwann cell complex for each time interval (Table II). At all intervals the majority of grains was located over compact myelin. The second method of analysis involved cutting and weighing traced profiles of these components from longitudinally sectioned material. The grain density was found to be 2.5–8 times as heavy over compact myelin as over related

Schwann cell cytoplasm or axoplasm, for both no-chase and chase intervals.

The labeling of the myelinated axon itself could definitely be established by EM radioautography, and these observations were tabulated in terms of the number of grains assigned to the axon per number of cross-sectioned profiles of myelin examined (Table II). The frequency of axonal labeling was very low (2%) after a pulse of 30 min; after 6 hr of chase, however, the frequency was about 12%. After 48 hr of chase incubation, this figure was 14%. These data would support the current concept of proximo-distal axonal transport (discussed in 46), in that choline compounds synthesized in the perikaryon would be expected to appear, after some delay, in the axon. Some of the axonal grains were found in association with mitochondria or other membranous material; other grains were seen overlying the axoplasm (Figs 4, 5, 13). The results seem to indicate that choline did not penetrate through the myelin sheath into the axon within the 30 min of the pulse labeling, although it is possible that this penetration did occur, but that the choline was not incorporated by structures in the axon and was, therefore, extracted during fixation.

Longitudinal Sections

Longitudinal sections of all intervals were analyzed by both LM and EM radioautography for determining whether any point along the myelin internode was the initial site for the incorporation of the choline label. In the LM radioautograms, after 30 min of pulse label, it was found that grains were distributed randomly all along the myelin internode (Fig. 7). This observation was substantiated by analysis of longitudinal sections by EM radioautography. Grains were observed in the paranuclear region (Fig. 8), in the internodal region (Fig. 9), and at the myelin terminating loops (Fig. 10), but no point of concentration was noted. After the chase intervals, the myelin internode was labeled more heavily, but in the same seemingly random fashion (Figs. 11, 14). Grains were now commonly seen over the axon (Fig. 13).

Consistent with the finding of diffuse labeling all along the internode, the electron micrographs showed that at this stage of development the collar of Schwann cell cytoplasm external to the myelin lamellae contained polysomes and ele-

ments of endoplasmic reticulum (Figs. 5, 9, 13). Some of the enzymes required in the synthetic pathways utilizing choline have been localized biochemically in the soluble fraction (supernatant) and in the microsomal fraction of certain cells (58, 73). This suggests that there is present locally all along the internode the cellular machinery necessary for the incorporation of choline into complex lipids and also for the manufacture of proteins.

Mature Cultures

The PNS culture system provides the advantage that the myelin internodes in vitro do not elongate significantly after their formation (13). This contrasts with the in vivo situation in which internodal elongation during growth and maturation leads to a second prolonged phase of myelin formation (discussed in 53). There do develop in vitro, however, irregularities of the surface contour of the myelin sheath (45, 46) comparable to those described in vivo (70) (See Figs. 10, 14).

This part of the investigation employed LM analysis only. For reasons discussed above, the frequency of labeling of the myelin–Schwann cell unit could be determined accurately with LM examination, particularly in tissue pulse-labeled for 30 min. In addition, the ease of sampling allowed a large amount of data to be collected. Cultures 70 and 94 DIV were exposed to choline-^{3}H according to the protocol and procedures as presented under Materials and Methods.

The over-all pattern of labeling was very similar to that seen in the younger cultures. All the elements of the culture showed uptake, with heavier labeling in the chase experiments. The myelin sheaths were labeled after the 30-min pulse (Fig. 15); after the chase incubation, there was more frequent labeling and often more grains per profile (Fig. 16). When frequency counts were performed, the proportion of sheaths labeled was almost exactly the same as that seen in the younger cultures (Table III). The counts indicate that about one-third of the myelin sheaths were marked with silver grains after the pulse, about one-half after 6 hr of chase, and about two-thirds after 48 hr.

These data suggest that the rate of incorporation of choline into myelin in a mature system does not differ significantly from that observed in a maturing system. This may be explained, in part, by the fact that many of the sheaths ana-
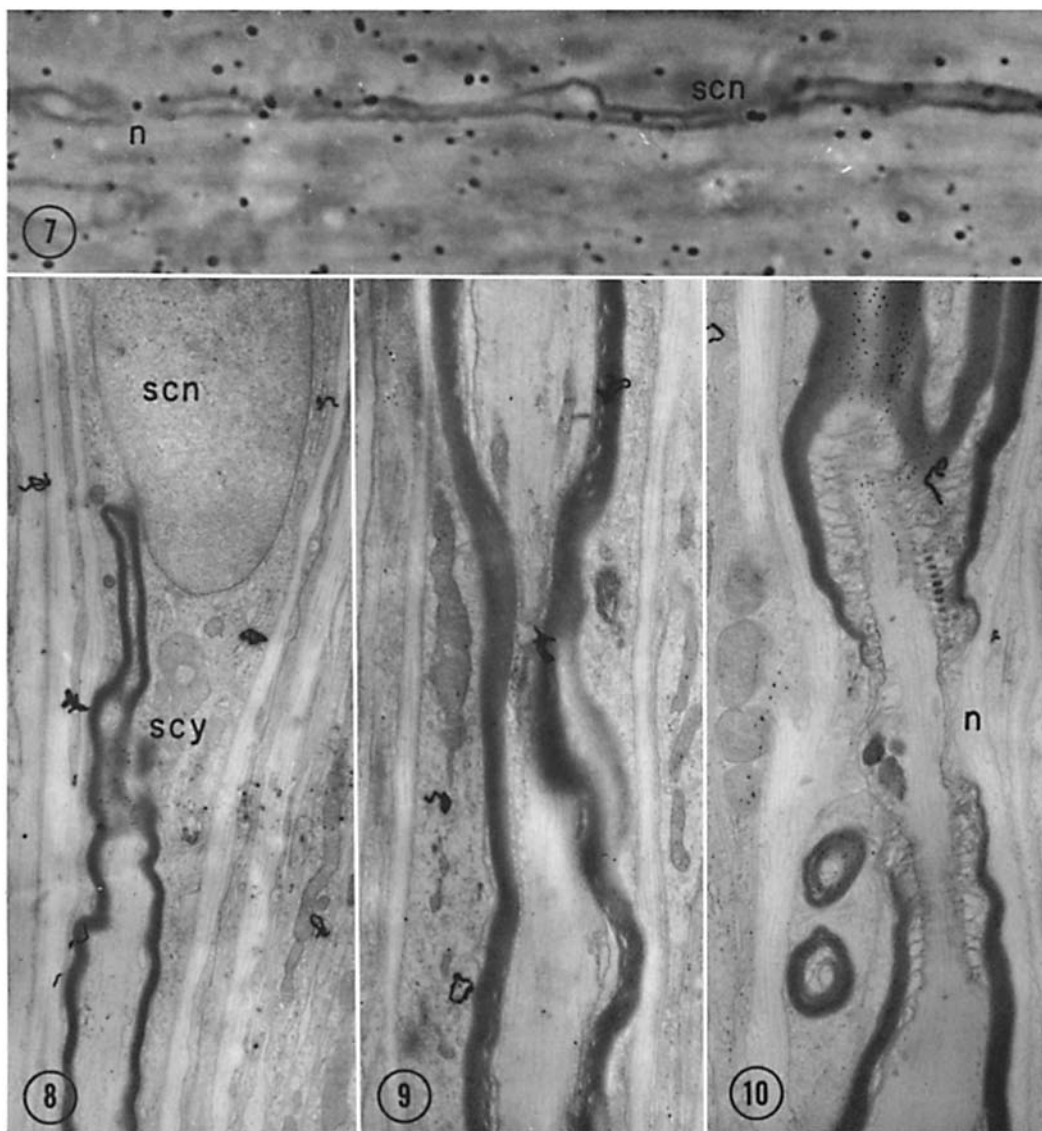
FIGURE 7   A light microscopic radioautogram of a longitudinally sectioned myelinated axon, including the Schwann cell nucleus (scn) and a node of Ranvier (n). This culture was exposed to choline-^{3}H for 30 min and fixed immediately. Grains are seen overlying the myelin profile all along the internode, with no concentration at any point. Grains also overlie the other cellular elements of the fascicle. Semithin Epon section exposed to Ilford L-4 emulsion and photographed with the phase microscope. × 1,700.

FIGURES 8–10   EM radioautographs of representative portions of a myelin internode from the same material as in Fig. 7. The random distribution of grains along the length of the internode is confirmed. Grains are seen overlying the myelin and/or the associated Schwann cell cytoplasm (scy) in the nuclear region (scn) (Fig. 8), along the internode (Fig. 9), and at the myelin-terminating region of a node of Ranvier (n) (Fig. 10). There is no concentration of grains in any of these areas. Radioautograms exposed to Ilford L-4 emulsion and stained, after development, with a dilute Pb stain, Fig. 8, × 8,500; Figs. 9 and 10, × 12,000.
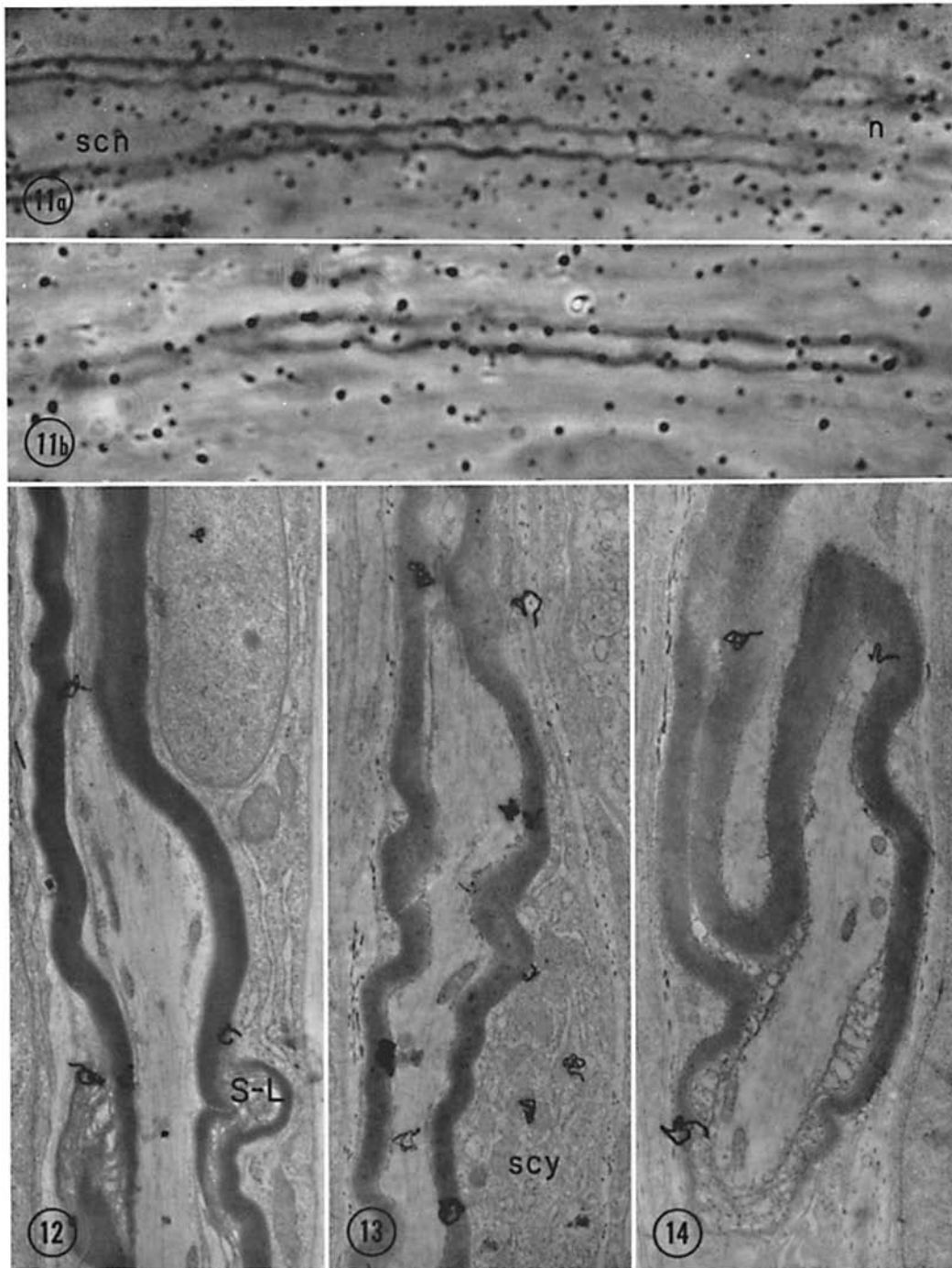
FIGURE 11 Light microscopic radioautograms of cultures 35 DIV exposed for 30 min to choline-^{3}H, followed by a chase incubation of 6 hr (Fig. 11 a) and by a chase incubation of 48 hr (Fig. 11 b). In Fig. 11 a the area from the Schwann cell nucleus (scn) to the node of Ranvier (n) is included. The pattern of grains overlying the myelin indicates diffuse labeling all along the internode. In comparison to Fig. 7, an increased labeling is seen over the myelin as well as over the other cellular elements. Semithin Epon sections of longitudinally cut myelin sheaths exposed to Ilford L-4 emulsion and photographed with phase microscopy. Fig. 11 a, × 1,700; Fig. 11 b, × 2,000.

FIGURES 12–14 EM radioautograms of representative portions of the myelin internode from the same material as in Fig. 11. The diffuse labeling of the internode is again noted. In comparison with Figs. 8–10, more grains overlie the myelin and the associated Schwann cell cytoplasm (scy). This is seen in the paranuclear region (Fig. 12) which includes a Schmidt-Lanterman cleft (S-L), along the internode (Fig. 13), and in the nodal region (Fig. 14). There is one grain which can be assigned to the axon (Fig. 13). Radioautograms were exposed to Ilford L-4 emulsion. In Fig. 12 the radioautogram was stained, after development, with a dilute Pb stain. In Figs. 13 and 14 the sections were stained first, then coated with emulsion and photographed with 100 kv. Fig. 12, × 12,500; Fig. 13, × 9,000; Fig. 14, × 11,000.

TABLE III

LM Grain Counts of Myelin Sheaths

Time of chase	Days in vitro		
	35	70	94
hr	%	%	%
0	30 (27–34)	24 (22–26)	30 (24–34)
6	64 (46–77)	46 (43–49)	47 (44–52)
48	62 (46–86)	67 (66–74)	72 (64–82)

These data are from LM radioautograms of cultures 35, 70, and 94 DIV exposed to choline-^{3}H for 30 min and chased as indicated. The same batch of Ilford L-4 emulsion and similar preparative techniques were used in all instances. The figures were derived from direct counts, at the LM level, of the percentage of myelin sheaths viewed in cross-section which were directly overlain by silver grains. Several hundred sheaths were included for each interval at each age.

lyzed in the younger cultures were in fact already fully formed. These fully formed sheaths cannot be easily distinguished microscopically from those sheaths in which additional membrane formation is still occurring, after compaction has begun. This ambiguity does not detract, however, from the observations on the surprising degree of labeling that occurred in relation to sheaths considered fully mature. This observation is discussed in more detail below.

Kodak NTE Emulsion

Initially, it was hoped that the present study would allow a precise localization of sites in which components are added to forming myelin. Because of the continuity of the collar of cytoplasm internal and external to the myelin lamellae (62), it might be predicted that initial incorporation could occur on either or both aspects of the compact myelin. Because the average thickness of the myelin in these cultures was only approximately 20 lamellae (about 2300 A), and because of the resolution considerations discussed earlier, it was not possible, with Ilford L-4 emulsion, to localize grains to specific regions of the myelin lamellae. In an attempt to improve resolution, a fine-grained emulsion—Kodak NTE—

with a predicted resolution of less than 1000 A was employed.

These preparations showed developed grains directly over myelin, and thus the results were consistent with those obtained with Ilford L-4 emulsion. Unfortunately, when used as described, the NTE emulsion proved not sufficiently sensitive to yield grain counts which differed significantly from background levels. With Dektol development, there was approximately a 3:1 ratio of tissue to background grains. With gold latensification and Elon ascorbic acid development, a procedure recommended for the greatest resolution (6), the ratio was approximately 1:1. In addition, there was a large amount of variability, from one grid to another, in the absolute number of grains present. Therefore, the data obtained are not considered reliable, and it is not possible to comment on the exact location of the addition of the choline phospholipids to the developing and the mature myelin sheath. According to Bachmann and Salpter (7), recent batches of NTE emulsion have, in fact, been less sensitive than those originally tested. Efforts are continuing with other fine-grained emulsions in an attempt to resolve this question.

Scintillation Counting

In radioautographic analysis of lipid compounds, such as the choline phospholipids studied in the present experiments, one must be concerned with the loss of labeled material which may occur during the fixation and dehydration procedures. In studies of choline-labeled lung tissue, there was a substantial loss (approximately 67%) of radioactivity during tissue preparative procedures with osmium tetroxide fixation (35). About 38% of this loss of radioactivity was lipid in the nature of lecithin. Studies of amoeba, on the other hand, indicated that most of the phospholipids (87%) are retained when tissues are processed for electron microscopy after osmium tetroxide fixation (28).

In order to assay this factor in the present study and to determine possible reasons for the heavier labeling of the tissue seen after the chase intervals, scintillation counting was done on the various solutions used during feeding, fixation, and dehydration. This analysis was performed only for the series of mature cultures. The results are summarized in Table IV. After an initial large loss of free unincorporated label, the BSS
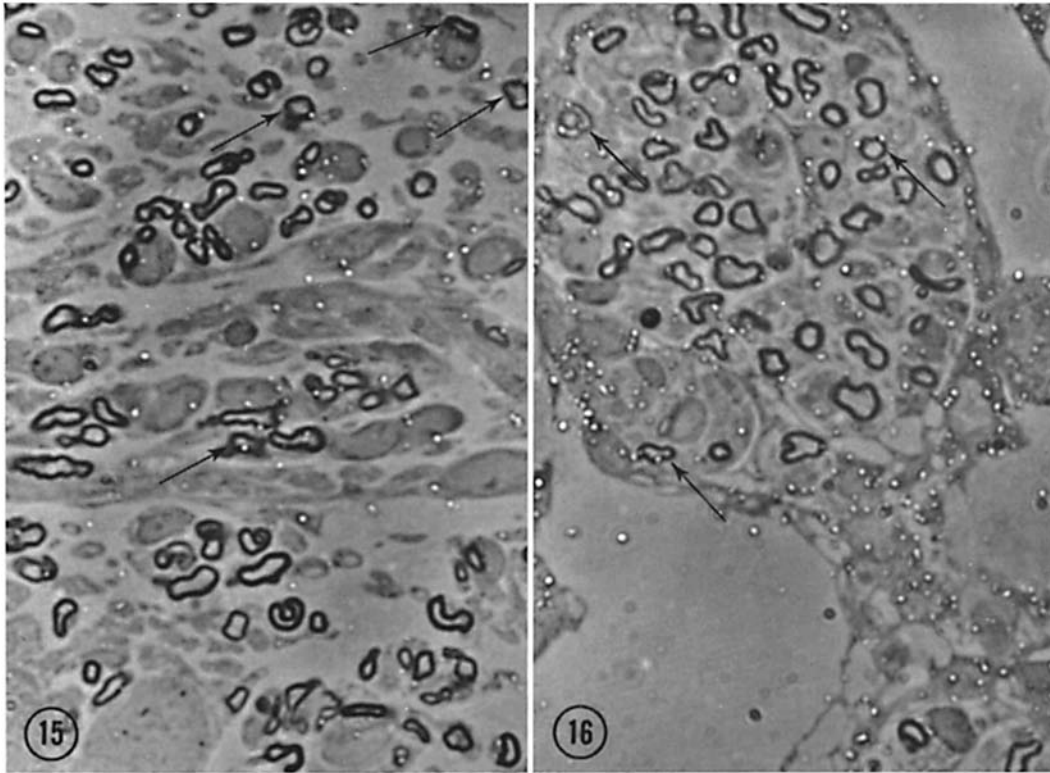
FIGURE 15   Light microscopic radioautogram of a culture 70 DIV exposed to choline-^{3}H for 30 min and fixed immediately. Because of the focal level used in this phase micrograph, the silver grains appear as white dots. Although this technique produces a superior photographic image, not every grain present is recorded in the picture obtained. Grains directly overlie a number of myelin sheaths (arrows); approximately one-fourth of the sheaths are so labeled. This proportion is approximately the same as that seen in Fig. 1, which is a culture of 35 DIV. The noncellular spaces have a rare grain, indicating a low level of background. Additional grains overlie the other cell elements. Semithin Epon section exposed to Ilford L-4 emulsion as in Fig. 1. × 1,300.

FIGURE 16   Light microscopic radioautogram of a culture 70 DIV exposed to choline-^{3}H for 30 min followed by a chase incubation of 6 hr. Approximately one-half of the myelin profiles are overlain with grains, often with more than one grain (arrows). This labeling, which is to be contrasted to the labeling in Fig. 15, is comparable to the labeling in Fig. 2 of a culture 35 DIV. Prepared as in Fig. 15. × 1,300.

washes continued to extract a small quantity of radioactivity, which may represent water-soluble intermediaries of choline metabolism. During the period of chase incubation, label was lost into the "cold" feeding medium. Therefore, the radioactive choline pool in the tissue should have been diluted. Nevertheless, the radioautograms were consistently heavier after the chase incubation. If the tissue was fixed without further incubation, labeled material was lost into the osmium tetroxide fixative. In comparison, cultures incubated in the chase medium showed very little loss of radioactivity during fixation in the osmium tetroxide. This could be interpreted as indicating that an intermediary compound (see Table I) was lost into the fixative after the pulse-labeling experiment. Further incubation allowed these intermediary compounds to metabolize into more permanent tissue components (for example, sphingomyelin) which were not extracted. In all series, a small amount of labeled material was lost into the ethanol solutions.

TABLE IV

Liquid Scintillation Counts

Sample	Volume	Chase period		
		0 hr	6 hr	48 hr
Used medium—30 min	50 μl	7.8×10^6	9.4×10^6	4.8×10^6
BSS Wash No. 1	10 ml	547,180	455,360	326,580
No. 2	"	18,600	15,880	12,340
No. 3	"	19,380	9,920	7,560
Used medium after Chase period	50 μl	—	4.9×10^5	2×10^6
Prefixation BSS No. 1	10 ml	13,580	38,640	94,560
No. 2	"	3,060	9,880	3,740
No. 3	"	15,580	4,440	260
No. 4	"	1,460	1,660	680
Fixative 2% OsO_4—VA*	"	69,960	17,280	16,320
ETOH‡ dehydration 50%	"	6,760	2,320	2,600
75%	"	4,940	10,980	10,680
95%	"	3,700	9,140	9,100
100%	"	620	1,880	2,480
Propylene oxide§ No. 1	"	148	170	138
No. 2	"	58	96	104
Radioautographic (LM) density		+	+++	++(+)

* Veronal acetate.
‡ Ethanol.
§ R/G ratios not significant.
This table presents liquid scintillation counts of the feeding, washing, and fixation solutions used in the present experiments. The figures are presented as counts per minute (cpm) and represent the averages of several counting runs. The counts were adjusted to represent the total amount of measured radioactivity in each of the solutions. The measured radioactivity of the original feeding medium before use was 1×10^9 cpm per 50 μl. Relative LM radioautographic density was estimated on a 3+ scale by visual comparison.

DISCUSSION

The experiments described illustrate one approach to the study of the metabolism of the myelin-Schwann cell unit. Choline was found to be a suitable precursor for studies of this type. By the use of parallel experiments and exactly parallel radioautographic preparative techniques, it has been possible to follow the incorporation of precursor at different times after application, as well as to compare amounts of incorporation into tissues of different ages. These data are meaningful for comparative purposes, but not in absolute quantitative terms. Without a companion biochemical analysis it is not possible, by the methods employed, to determine with certainty which compounds have been labeled and to what degree.

In the present study, the pattern of precursor incorporation into the myelin-related cell and the myelin suggests that myelin membrane is constructed all along the internode, rather than being initially assembled in one area and then dispatched to more remote regions. An increased volume of ribosome-rich Schwann cell cytoplasm appears to be distributed all along the internode during the period of active myelin formation. In the mature state, the volume of Schwann cell cytoplasm is not always sufficient to form a complete collar around the external myelin surface. And yet the incorporation of choline into both the maturing and mature myelin-Schwann cell unit was of the same order of magnitude. In order to explain the mechanism for this latter incorporation, considering the decreased amount of cell machinery, one might suggest that cellular metabolism is focused on myelin-related activities during maturity. Further evidence for ongoing synthesis of myelin components in adult animals is available from the work of Cuzner et al. (19),

among others. Their data from neonatal and adult brain myelin fractions after labeled phosphate injections might be interpreted as indicating that phospholipids are synthesized at similar rates throughout life in the rat brain. Studies of the surface membranes of L cells grown in tissue culture indicate that the constituent molecules of this membrane are synthesized at the same rate in a dividing as well as a stable cell population (69). During growth, the membrane components accumulate in new membrane material, but during maturity, when the amount of cell membrane is presumed constant, the rate of synthesis is balanced by degradative processes (69). If these same considerations apply to myelin membrane, then a constant rate of synthesis throughout life would not be inconsistent with the general biochemical observation (19, 21, 22) that more total myelin precursor incorporation occurs during initial myelin formation in young animals. More precursor is retained during neonatal life because new myelin construction requires the accumulation of myelin components. Later, during the phase of maintenance and repair, components being synthesized and added to the myelin structure would be balanced by components being lost and degraded.

Additional parallels between our observations on mature PNS myelin and recent biochemical experiments on CNS myelin are worthy of note. In the CNS, as in the PNS, the myelin-forming cell (in this case the oligodendrocyte) considerably atrophies with maturity, with respect to the amount of cytoplasm related to the compact myelin (12). Nevertheless, when certain lipid precursors (e.g. ethanolamine [2]) are injected directly into adult brain, a considerable amount is incorporated into the myelin subfraction. These observations suggest that, when barriers between precursor and the myelin-related cell are circumvented, the degree of labeling of the mature myelin is substantial.

Thus, the discussion returns to the problem of barriers mentioned earlier. A consideration of the effects of barriers, such as the blood-brain barrier (BBB), might alter the interpretation of some previous myelin labeling experiments. These barrier effects could be manifested in a number of ways in studies concerned with incorporation and retention of radioactive substances. First, if the diffusion of precursors into the brain parenchyma is inhibited, only a small quantity of sys-

temically injected precursor may reach brain tissue. Experiments employing intracisternal injections or injections into brain parenchyma have consistently demonstrated that more precursor incorporation occurs after injection by these routes than occurs after systemic injection (33, 37). In a recent study, it was found that sulfate-^{35}S incorporation into adult rat brain increased 100-fold if intracerebral rather than intraperitoneal injection was employed (22); in this case, the myelin subfraction accounted for 50–60% of the labeled whole brain sulfolipids 4 hr after the injection. Furthermore, this regulation of precursor access to brain tissue may vary with age. For example, the BBB is permeable to Na acetate during the developmental period, but a progressive decrease in permeability occurs with age (37, 38).

It has been demonstrated that tight junctions between adjacent overlapping endothelial cells of the brain capillaries constitute the effective blood-brain barrier to certain molecules (48), and furthermore, that these same areas block the movement of certain substances from the brain parenchyma into the capillary lumen (10). This latter factor—the limitation to the egress of material from the brain parenchyma—suggests a second manner in which barriers could distort the results of metabolic studies. A study of sulfate metabolism has indicated that there was a constancy in the percentage distribution of sulfolipids-^{35}S in four brain regions examined; it was concluded that most sulfolipids once formed in an area remained there (47). The evidence presented in this as well as other studies (22, 33) could be interpreted as indicating a local reutilization of metabolites. If local recycling does occur, then an estimate of metabolic turnover, defined as the loss of radioactivity with time, may yield an erroneously low figure. Local recycling may be a normal part of the metabolic turnover occurring, perhaps at different rates (31), for various myelin components.

Considerations in regard to barrier phenomena would also apply to a histological approach to myelin metabolism. A light microscopic study of mouse brain after intraperitoneal injection of labeled precursors (65) demonstrated that the slowest incorporation and replacement of radioactivity in lipids occurred in the white matter and that the fastest incorporation and replacement of radioactivity occurred in the region of the nerve

cell bodies. There was a longer persistence of label in the white matter and in areas of neuropil. The cellular localization was not determined in these experiments.

In the peripheral nervous system, there seem to be two barriers present. The first is the blood-nerve barrier of the endoneurial blood vessels (42, 68). The second barrier to the diffusion of material is the perineurial layer of overlapping cell processes, separating the nerve elements from the surrounding tissue (67). Therefore, the same type of analysis concerning possible barrier effects must be applied to studies of PNS myelin metabolism. It should be noted that the blood-nerve barrier is not present in the culture system employed in the present study, and that perineurial ensheathment is not always complete (11).

There are few studies dealing directly with biochemical aspects of PNS myelin metabolism (see reference 21). One light microscopic radioautographic study of cholesterol incorporation into maturing mouse sciatic nerve has appeared (66). The data as presented are in preliminary form and do not yet permit an evaluation of dynamic versus stable aspects of myelin components. Singer and collaborators have presented a series of radioautographic studies of newt peripheral nerve following the systemic injection of protein and nucleic acid precursors. These studies provided EM radioautographic evidence of labeling over Schwann cells, myelin sheaths, and axons after the injection of histidine (61) and lysine (59), and similar observations at the light microscopic level were made after the injection of uridine (60). Labeling occurred indiscriminately along the length of the internode. The parallels between these observations on nucleic acid and protein precursors and the present data on lipid precursor are striking. The crucial question is how to interpret the "labeling" of the myelin–Schwann cell unit. Singer and collaborators cast this unit in the role of an intermediary for the supply of the nutritional requirements of the axon. In fact, there is evidence that axons (for example, 44) and neurons (29) can survive (sometimes for considerable periods) without myelin or any other type of ensheathment. In addition, labeling seen after the administration of some amino acids may sometimes be attributable to nonspecific ionic binding and may not represent incorporation (59). Because there are two cellular systems involved in this analysis (both the Schwann cell–

myelin unit and the axon), each of which probably anabolizes and catabolizes materials at various rates for its own internal needs, a determination of material interchange between them becomes difficult, if not impossible, by the use of radioautographic studies of whole tissues.

The conclusion reached from this discussion of possible barrier effects and recent biochemical and cytological experiments is that the concept of the metabolic stability of myelin components may not be tenable. It seems reasonable to view the myelin sheath not as an exceptionally stable structure, but as a structure containing components which are continually being exchanged with component pools from outside the myelin itself. Activities related to this component pool would involve both synthesis and degradation, at a rate which is perhaps more rapid than has generally been acknowledged. A reasonable site to localize this myelin-related metabolism is the cytoplasm of the associated cell. In this regard, current evidence indicates that the oligodendrocyte, despite its small size, is a metabolically active cell; and it has recently been pointed out that the synthetic machinery of the oligodendrocyte seems especially equipped for lipid synthesis (reviewed in 12; see also 63).

A clearer understanding of these dynamic events in relation to myelin can be expected to be most useful in delineating the mechanism of some demyelinating diseases. Diphtheritic neuropathy is a classical example of segmental PNS demyelination. Morphologically, the process seems to develop in the paranodal areas (71), and smaller diameter fibers and the myelin of younger animals seem to be more affected (27). Metabolic studies have indicated that diphtherial toxin interferes with protein metabolism (for example, 32, 34) and thus an anabolic block may explain the mechanism of demyelination. An alternative explanation, however, is that increased levels of acid phosphatase enzymes as well as the presence of histochemically demonstrable secondary lysosomes in the Schwann cells in diphtheritic nerves lead to a primarily catabolic process of demyelination (24, 72).

Metachromatic leukodystrophy is another disease process which involves myelin primarily, affecting both the CNS (for example, 3) and the PNS (for example, 16). Various inclusions in the cytoplasm of the Schwann cells and oligodendrocytes are part of the pathological picture. It has

been demonstrated that the accumulation of sulfatides (e.g. 17) results from the deficiency of sulfatase enzymes (5). The occasional onset of this disease in adulthood presents a serious challenge to the concept of the metabolic stability of myelin (4). It is difficult to explain how this type of catabolic enzyme deficiency could result in the breakdown of myelin. A possible explanation that has been presented (4) implicates the maintenance process of the myelin-glial unit. Thus, an interference with the functioning of the cell responsible for myelin maintenance (because of metabolite accumulation) would result in a failure of the resynthesis constantly necessary to maintain normal myelin.

REFERENCES

1. ADAMS, C. W. M., and A. N. DAVISON. 1965. The myelin sheath. *In* Neurohistochemistry. C. W. M. Adams, editor. Elsevier, N. Y. 332.

2. ANSELL, G. B., and S. SPANNER. 1967. The metabolism of labelled ethanolamine in the brain of the rat *in vivo*. *J. Neurochem.* 14:873.

3. AUREBECK, G., K. OSTERBERG, M. BLAW, S. CHOU, and E. NELSON. 1964. Electron microscopic observations on metachromatic leukodystrophy. *Arch. Neurol.* 11:273.

4. AUSTIN, J., D. ARMSTRONG, S. FOUCH, C. MITCHELL, D. STUMPF, L. SHEARER, and O. BRINER. 1968. Metachromatic leukodystrophy (MLD). VIII. MLD in adults; diagnosis and pathogenesis. *Arch. Neurol.* 18:225.

5. AUSTIN, J., D. MCAFEE, D. ARMSTRONG, M. O'ROURKE, L. SHEARER, and B. BACHHAWAT. 1964. Abnormal sulphatase activities in two human diseases (Metachromatic Leucodystrophy and Gargoylism). *Biochem. J.* 93:15C.

6. BACHMANN, L., and M. M. SALPETER. 1965. Autoradiography with the electron microscope. A quantitative evaluation. *Lab. Invest.* 14:1041.

7. BACHMANN, L., and M. M. SALPETER. 1967. Absolute sensitivity of electron microscope radioautography. *J. Cell Biol.* 33:299.

8. BASERGA, R. 1967. Autoradiographic methods. *In* Methods in Cancer Research. H. Busch, editor. Academic Press, N. Y. 1:45.

9. BRAY, G. A. 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. *Anal. Biochem.* 1: 279.

10. BRIGHTMAN, M. W. 1967. Intracerebral movement of proteins injected into blood and cerebrospinal fluid. *Anat. Rec.* 157:219.

11. BUNGE, M. B., R. P. BUNGE, E. R. PETERSON, and M. R. MURRAY. 1967. A light and electron microscope study of long-term organized cultures of rat dorsal root ganglia. *J. Cell Biol.* 32:439.

12. BUNGE, R. P. 1968. Glial cells and the central myelin sheath. *Physiol. Rev.* 48:197.

13. BUNGE, R., and M. BUNGE. 1966. An evaluation of some determinants of neuronal size and internode length in peripheral nerve. *Anat. Rec.* 154:324.

14. BUNGE, R. P., M. B. BUNGE, and E. R. PETERSON. 1965. An electron microscope study of cultured rat spinal cord. *J. Cell Biol.* 24:163.

15. CARO, L. G. 1964. High-resolution autoradiography. *In* Methods in Cell Physiology. D. M. Prescott, editor. Academic Press Inc., N. Y. 1:327.

16. CRAVIOTO, H., J. S. O'BRIEN, B. H. LANDING, and B. FINCK. 1966. Ultrastructure of peripheral nerve in metachromatic leukodystrophy. *Acta Neuropathol.* 7:111.

17. CUMINGS, J. N., E. J. THOMPSON, and H. GOODWIN. 1968. Sphingolipids and phospholipids in microsomes and myelin from normal and pathological brains. *J. Neurochem.* 15:243.

18. CUZNER, M. L., A. N. DAVISON, and N. A. GREGSON. 1965. The chemical composition of vertebrate myelin and microsomes. *J. Neurochem.* 12:469.

19. CUZNER, M. L., A. N. DAVISON, and N. A. GREGSON. 1965. Chemical and metabolic studies of rat myelin of the central nervous system. *Ann. N. Y. Acad. Sci.* 122:86.

20. DAVISON, A. N. 1964. Myelin metabolism. *In*

Metabolism and Physiological Significance of Lipids. R. M. C. Dawson and D. N. Rhodes, editors. J. Wiley, N. Y. 527.

21. DAVISON, A. N., and N. A. GREGSON. 1962. The physiological role of cerebron sulphuric acid (sulphatide) in the brain. *Biochem. J.* 85:558.

22. DAVISON, A. N., and N. A. GREGSON. 1966. Metabolism of cellular membrane sulpholipids in the rat brain. *Biochem. J.* 98:915.

23. EICHBERG, J., JUN., V. P. WHITTAKER, and R. M. C. DAWSON. 1964. Distribution of lipids in subcellular particles of guinea-pig brain. *Biochem. J.* 92:91.

24. FOWLER, S. D. 1967. Phospholipid digestion by hepatic lysosomes. *J. Cell Biol.* 35:41A. (Abstr.)

25. GEREN, B. B. 1954. The formation from the Schwann cell surface of myelin in the peripheral nerves of chick embryos. *Exp. Cell Res.* 7:558.

26. HENDELMAN, W. 1967. Incorporation of H^3-choline in myelinating nerve tissue cultures. *Anat. Rec.* 157:259.

27. JACOBS, J. M. 1967. Experimental diphtheritic neuropathy in the rat. *Brit. J. Exp. Pathol.* 48:204.

28. KORN, E. D., and R. A. WEISMAN. 1966. I. Loss of lipids during preparation of amoebae for electron microscopy. *Biochim. Biophys. Acta.* 116:309.

29. KUFFLER, S. W., and J. G. NICHOLLS. 1966. The physiology of neuroglial cells. *Ergeb. Physiol.* 57:1.

30. LUCK, D. J. L. 1963. Formation of mitochondria in *Neurospora Crassa*. A quantitative radio-autographic study. *J. Cell Biol.* 16:483.

31. MANDEL, P., and J. L. NUSSBAUM. 1966. Incorporation of ^{32}P into the phosphatides of myelin sheaths and of intracellular membranes. *J. Neurochem.* 13:629.

32. MATHESON, D. F. 1968. Incorporation of [^{14}C] glycine into protein of the adult rat peripheral nerve: Effects of inhibitors. *J. Neurochem.* 15:179.

33. McMILLAN, P. J., G. W. DOUGLAS, and R. A. MORTENSEN. 1957. Incorporation of C^{14} of acetate-1-C^{14} and pyruvate-2-C^{14} into brain cholesterol in the intact rat. *Proc. Soc. Exp. Biol. Med.* 96:738.

34. MOEHRING, T. J., J. M. MOEHRING, R. J. KUCHLER, and M. SOLOTOROVSKY. 1967. The response of cultured mammalian cells to diphtheria toxin. I. Amino acid transport, accumulation, and incorporation in normal and intoxicated sensitive cells. *J. Exp. Med.* 126:407.

35. MORGAN, T. E., and G. L. HUBER. 1967. Loss of lipid during fixation for electron microscopy. *J. Cell Biol.* 32:757.

36. MOSES, M. J. 1964. Application of autoradiography to electron microscopy. *J. Histochem. Cytochem.* 12:115.

37. NICHOLAS, H. J., and B. E. THOMAS. 1958. The metabolism of cholesterol and fatty acids in the central nervous system. *J. Neurochem.* 4:42.

38. NICHOLAS, H. J., and B. E. THOMAS. 1961. Cholesterol metabolism and the blood-brain barrier: An experimental study with 2-C^{14}-sodium acetate. *Brain.* 84:320.

39. NORTON, W. T., and L. A. AUTILIO. 1965. The chemical composition of bovine CNS myelin. *Ann. N. Y. Acad. Sci.* 122:77.

40. NORTON, W. T., and L. A. AUTILIO. 1966. The lipid composition of purified bovine brain myelin. *J. Neurochem.* 13:213.

41. O'BRIEN, J. S., E. L. SAMPSON, and M. B. STERN. 1967. Lipid composition of myelin from peripheral nervous system. Intradural spinal roots. *J. Neurochem.* 14:357.

42. OLSSON, Y. 1968. Topographical differences in the vascular permeability of the peripheral nervous system. *Acta Neuropathol.* 10:26.

43. PELC, S. R. 1963. Theory of electron autoradiography. *J. Roy. Microsc. Soc.* 81:131.

44. PÉRIER, O., and A. GREGOIRE. 1965. Electron microscopic features of multiple sclerosis lesions. *Brain.* 88:937.

45. PETERSON, E. R., and M. R. MURRAY. 1965. Patterns of peripheral demyelination *in vitro*. *Ann. N. Y. Acad. Sci.* 122:39.

46. POMERAT, C. M., W. J. HENDELMAN, C. W. RAIBORN, JR., and J. F. MASSEY. 1967. Dynamic activities of nervous tissue *in vitro*. In The Neuron. H. Hydén, editor. Elsevier, N. Y. 119.

47. PRITCHARD, E. T. 1966. *In vivo* labelling of sulphatides from [^{35}S] sulphate in rat brain during early growth. *J. Neurochem.* 13:13.

48. REESE, T. S., and M. J. KARNOVSKY. 1967. Fine structural localization of a blood-brain barrier to exogenous peroxidase. *J. Cell Biol.* 34:207.

49. REVEL, J. P., and E. D. HAY. 1963. An autoradiographic and electron microscopic study of collagen synthesis in differentiating cartilage. *Z. Zellforsch. Mikrosk. Anat.* 61:110.

50. REYNOLDS, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* 17:208.

51. ROBERTSON, J. D. 1955. The ultrastructure of adult vertebrate peripheral myelinated nerve fibers in relation to myelinogenesis. *J. Biophys. Biochem. Cytol.* 1:271.

52. ROGERS, A. W. 1967. Techniques of Autoradiography. Elsevier, N. Y.

53. ROSENBLUTH, J. 1966. Redundant myelin sheaths

and other ultrastructural features of the toad cerebellum. *J. Cell Biol.* **28**:73.

54. ROSSITER, R. J. 1967. Biochemistry of phosphatides. *In* Lipids and Lipidoses. G. Schettler, editor. Springer-Verlag, N. Y. 93.

55. SALPETER, M. M., and L. BACHMANN. 1964. Autoradiography with the electron microscope. A procedure for improving resolution, sensitivity, and contrast. *J. Cell Biol.* **22**:469.

56. SALPETER, M. M., and L. BACHMANN. 1965. Assessment of technical steps in electron microscope autoradiography. *In* The Use of Radioautography in Investigating Protein Synthesis. C. P. Leblond and K. B. Warren, editors. Academic Press Inc., N. Y. 23.

57. SALPETER, M. M., L. BACHMANN, and E. E. SALPETER. 1967. Resolution in electron microscope radioautography. *J. Cell Biol.* **35**:119A. (Abstr.)

58. SCHNEIDER, W. C. 1963. Intracellular distribution of enzymes. XIII. Enzymatic synthesis of deoxycytidine diphosphate choline and lecithin in rat liver. *J. Biol. Chem.* **238**:3572.

59. SINGER, M. 1968. Penetration of labelled amino acids into the peripheral nerve fiber from surrounding body fluids. *In* Growth of the Nervous System. G. E. Wolstenholme and M. O'Connor, editors. Churchill Press, London. 200.

60. SINGER, M., and M. R. GREEN. 1968. Autoradiographic studies of uridine incorporation in peripheral nerve of the newt, *Triturus. J. Morphol.* **124**:321.

61. SINGER, M., and M. M. SALPETER. 1966. The transport of H^3-L-histidine through the Schwann and myelin sheath into the axon, including a reevaluation of myelin function. *J. Morphol.* **120**:281.

62. SMART, I. 1965. Reconstruction of myelinated Schwann cells by "unrolling." *J. Anat.* **99**:212.

63. SOTELO, C. 1967. Cerebellar neuroglia: Morphological and histochemical aspects. *In* Progress in Brain Research. The Cerebellum. C. A. Fox and R. S. Snider, editors. Elsevier, N. Y. **25**:226.

64. STEVENS, A. R. 1966. High resolution autoradiography. *In* Methods in Cell Physiology. D. M. Prescott, editor. Academic Press Inc., N. Y. **2**:255.

65. TORVIK, A., and R. L. SIDMAN. 1965. Autoradiographic studies on lipid synthesis in the mouse brain during postnatal development. *J. Neurochem.* **12**:555.

66. UZMAN, B. G., and E. T. HEDLEY-WHYTE. 1968. Myelin: dynamic or stable? *J. Gen. Physiol.* $51:8_s-18_s$.

67. WAGGENER, J. D., S. M. BUNN, and J. BEGGS. 1965. The diffusion of ferritin within the peripheral nerve sheath: An electron microscopy study. *J. Neuropathol. Exp. Neurol.* **24**:430.

68. WAKSMAN, B. H. 1961. Experimental study of diphtheritic polyneuritis in the rabbit and guinea pig. LII. The blood-nerve barrier in the rabbit. *J. Neuropathol. Exp. Neurol.* **20**:35.

69. WARREN, L., and M. C. GLICK. 1968. Membranes of animal cells. II. The metabolism and turnover of the surface membrane. *J. Cell Biol.* **37**:729.

70. WEBSTER, H. DE F., and D. SPIRO. 1960. Phase and electron microscopic studies of experimental demyelination. I. Variations in myelin sheath contour in normal guinea pig sciatic nerve. *J. Neuropathol. Exp. Neurol.* **19**:42.

71. WEBSTER, H. DE F., D. SPIRO, B. WAKSMAN, and R. D. ADAMS. 1961. Phase and electron microscopic studies of experimental demyelination. II. Schwann cell changes in guinea pig sciatic nerves during experimental diphtheritic neuritis. *J. Neuropathol. Exp. Neurol.* **20**:5.

72. WELLER, R. O., and R. S. MELLICK. 1966. Acid phosphatase and lysosome activity in diphtheritic neuropathy and Wallerian degeneration. *Brit. J. Exp. Pathol.* **47**:425.

73. WILGRAM, G. F., and E. P. KENNEDY. 1963. Intracellular distribution of some enzymes catalyzing reactions in the biosynthesis of complex lipids. *J. Biol. Chem.* **238**:2615.

74. WRIGHT, P. G. 1961. The metabolism of myelin. *Proc. Roy. Soc. Med.* **54**:26.