ULTRASTRUCTURAL LOCALIZATION OF PHOSPHOLIPID SYNTHESIS IN THE RAT TRIGEMINAL NERVE DURING MYELINATION

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ABSTRACT

A method for the ultrastructural localization of acyltransferase enzymes involved in phospholipid metabolism has been applied to the developing rat trigeminal nerve. Determination of acyltransferase levels in the nerve indicated that a peak of activity occurs at the 8th day after birth with gradual declines of activity up to 15 days. Morphological surveys and determinations of cholesterol levels suggested that heavy myelin formation occurs in the nerve during this latter period. Fixed nerves incubated in a medium for localization of acyltransferases indicated deposition of reaction product associated with Golgi cisternae, intracellular smooth vesicles, and the plasma membrane of the Schwann cell in the incipient stages of myelin formation. Golgi-derived vesicles appeared to move toward the Schwann cell surface and fuse with the plasma membrane. Activity continued to be detectable in the plasma membrane of the internal mesaxon as long as cytoplasm was evident and mature myelin membrane was not yet formed. Cells in which myelin formation appeared advanced showed little or no enzyme marker. Consistent with cytochemical observations were biochemical determinations of acyltransferases which showed high levels of the enzymes in microsomes, while no activity could be detected in the myelin fraction. Acyltransferase reaction product was also observed in the Golgi apparatus of ganglion cell bodies, axoplasmic smooth vesicles, and the axolemma. Localization of acyltransferase enzymes in Schwann cells, ganglion cell bodies, and axons during development of the nerve is discussed in relation to membrane biogenesis in the nervous system.

The appearance of differentiated function in the nervous system is associated with the growth of axons and dendrites, and the appearance of myelin, both in vivo (30, 42, 5) and in vitro (12, 13, 11, 9). The events underlying the synthesis of membrane precursors and their assembly into nervous system membranes are largely unknown, and present themselves as challenging problems. The use of myelin for X-ray diffraction studies (48, 10), its easy isolation in pure form from whole tissue (14), and its relatively uniform composition in various parts of the nervous system of most vertebrates (17) have resulted in the frequent use of this membrane as a model for morphological and biochemical studies. Particular emphasis in recent years has been given to the biogenesis of myelin during development. Morphometric analysis has suggested that a 100-fold increase of Schwann cell surface membrane and a 500-fold increase of myelin surface membrane occurs during development of the sciatic nerve (52). Myelin has been shown

to have a characteristically high content of lipid relative to protein (33), though, as in plasma membrane of many mammalian cell types, cholesterol and phospholipid appear to be the predominant lipid classes (2, 14). Previous biochemical (44, 45, 16, 31) and autoradiographic studies at the ultrastructural level (24) have attempted to investigate synthesis of myelin lipid during development. However, neither of the latter two approaches has been able to distinguish between sites of synthesis of myelin components and their points of assembly into the mature structure. Cytochemical localizations of enzymes involved in synthesis of myelin precursors, therefore, seemed to be a useful approach to this problem. Methods for the ultrastructural localization of acyltransferases, which synthesize the intermediates of phospholipid, have been developed in this laboratory using various eucaryotic cell types (26, 27, 34, 3). This present study applies one of these methods, along with correlative biochemical studies, to the trigeminal nerve of the neonatal rat in order to gain some understanding of the cellular events underlying membrane proliferation during myelin formation by Schwann cells.

MATERIALS AND METHODS

Animals and Tissues

Neonatal albino rats were used between days 5 and 15 after birth (the day of birth was denoted as day 1). All animals were sacrificed by decapitation, and their trigeminal nerves were removed bilaterally from the basal fossae and immersed in ice-cold cacodylate buffer (0.025 M), containing 4.5% dextrose, pH 7.4. For studies of fixed nerves, tissues were immersed in a variety of ice-cold fixatives, to be described later, for 30 min, and washed in several changes of ice-cold buffer before subsequent processing.

Preparation of Microsomal and Myelin Fractions

10% (wt/vol) homogenates of unfixed trigeminal nerves in 0.32 M sucrose-1 mM EDTA were used for the separation of subcellular fractions by a modification of the method reported by Cuzner and Davison (14). A heavy fraction was obtained by an initial centrifugation of the homogenate at 5,000 rpm in a type 50 rotor in a Beckman Model L Ultracentrifuge. This fraction was washed once by resuspension in 0.32 M sucrose-1 mM EDTA, and recentrifuged at 5,000 rpm. The combined super-

natants from the initial centrifugation steps were centrifuged at 12,500 rpm for 15 min in the type 50 rotor to yield a "crude mitochondrial fraction," containing mitochondria, lysosomes, myelin, and some nerve-ending material. The supernatant from this latter spin was centrifuged at 29,000 rpm for 30 min, yielding a microsomal preparation and a nonopalescent supernatant.

Myelin was isolated from the crude mitochondrial fraction by use of a discontinuous gradient in a SW50.1 swing-out rotor. The crude mitochondrial fraction in 0.75 M sucrose was sandwiched between a 2.25 ml layer of 1.2 M sucrose and a 1.25 ml layer of 0.32 M sucrose and centrifuged for 60 min at 36,000 rpm at 2°C in the Beckman model L ultracentrifuge. As indicated by previous studies (14), a fluffy white fraction formed a band at the first interface (myelin), a homogeneous white band at the second interface (nerve endings), and a brown pellet appeared at the bottom of the tube (mitochondria). All three fractions were removed and pelleted by recentrifugation in the type 50 rotor at 15,000 rpm for 10 min.

All pellets and fractions obtained were resuspended in 0.32 M sucrose and recentrifuged at the speed used for isolation to remove the lower density contaminants. In some cases, the final pellets were fixed by immersion in 5% glutaraldehyde, refixed in 1% osmium tetroxide, and processed for electron microscopy in the manner described later for cytochemical studies.

Biochemical Studies

Microsomal and myelin suspension, homogenates of unfixed or fixed trigeminal nerves, or blocks of fixed nerves were incubated in media containing L-[^{14}C]α-glycerol (UL) phosphate (International Chemical and Nuclear Corporation, Irvine, Calif.) or unlabeled α-glycerophosphate, [1-^{14}C]-S-palmityl CoA (International Chemical and Nuclear Corporation) or unlabeled palmityl-CoA (Sigma Chemical Co., St. Louis, Mo.) in cacodylate buffer (0.025 M) containing 4.5% dextrose at 37°C to determine acyltransferase activity. Control experiments were performed as indicated in the text. At the end of the incubation period the reaction was stopped by addition of ice-cold chloroform-methanol (2:1) to extract lipids (18) for determination of incorporation of radioactive label. Portions of the total lipid extracts were separated on thin layers of silica gel into the major lipid classes, using the solvent system isopropyl ether-heptane-glacial acetic acid (40:60:2, vol/vol/vol) as previously described (28). The lipids were visualized with iodine vapor, which was allowed to sublime before the lipid-containing bands were scraped directly into counting vials. Portions of the total

lipid extract as well as of the lipid fractions were counted in a Packard Tricarb scintillation counter.

Protein was determined according to the method of Lowry et al. (1951), and cholesterol according to the method of Bowman and Wolf (1967).

Cytochemical Studies

The method used for the localization of acyltransferases was as previously described for liver (3). Before incubation in the cytochemical medium, tissue blocks were washed 30 min in ice-cold buffer after fixation and then preincubated for 30 min in ice-cold 0.5 mM potassium ferricyanide to oxidize endogenous reducing groups in the tissue (25). After this last procedure, the tissues were washed with several changes of ice-cold buffer for approximately 5 h. In preliminary experiments, specimens which were washed for shorter periods of time before incubation in the cytochemical medium showed some deposition of reaction product in the tissues with a localization similar to that observed in tissues incubated in the presence of both substrates and capture reagents. With the assumption that this localization in controls was due to the persistence of endogenous substrates in fixed tissues, the wash period was extended to remove this background localization, 5 h being adequate for this purpose. Tissues were then incubated for 60 min at 37°C in a medium containing copper sulfate (3.0 mM), potassium ferricyanide (0.5 mM), sodium citrate (5.0 mM), α-glycerophosphate (2.5 mM), and palmityl-CoA (0.18 mM) in cacodylate buffer (0.025 M), containing 4.5% dextrose, pH 7.4. All reagents were made fresh immediately before use. Retention of acyltransferase activity in fixed tissues incubated in the medium described was indicated by biochemical experiments with labeled substrates to be described later.

In control experiments, other fixed tissues were heated at 60°C for 4 h in buffer before incubation in cytochemical media containing both substrates and capture reagents. An additional control consisted of fixed tissues incubated in 0.5 mM potassium ferrocyanide, the reduction product of potassium ferricyanide, for 30 min at 37°C, at which time 3.0 mM copper sulfate-5.0 mM sodium citrate was added and the tissues were incubated for an additional 30 min. In this latter experiment, the characteristic red-brown copper ferrocyanide precipitate appeared throughout the medium.

After incubation in the cytochemical media, all tissues were washed several times with ice-cold buffer, refixed in 1% osmium tetroxide for 60 min, dehydrated in a graded series of ethyl alcohol and propylene oxide, and embedded in Epon. Thin sections were cut on a Porter-Blum MT-1 ultramicrotome and viewed, either unstained or after staining with lead citrate for 30 s, in an Hitachi 11B electron microscope.

RESULTS

ACYLTRANSFERASE ACTIVITY DURING DEVELOPMENT OF THE TRIGEMINAL NERVE WHOLE TISSUE HOMOGENATES: Studies of the levels of acyltransferases in the trigeminal nerve during development indicated that a sharp increase in the specific activity of these enzymes occurred at the 8th day after birth, followed by a gradual decline of activity up to 15 days (Fig. 1). The rise and fall in enzyme levels were not due to variable protein concentration during this period, since homogenates used for the assays were made with a constant dilution factor (10%, wt/vol) and, under the conditions used, the enzyme activity was linear with respect to protein. While the total wet weight of the nerve increases during this developmental interval, the relative concentration of protein in the nerve did not vary to any significant degree, so that the range of protein concentration was the same at each time point assayed.

To correlate these results with myelin formation, cholesterol levels for single nerves at various intervals after birth were assayed, since previous studies have indicated that rises in this lipid reflect myelin formation in the cortex (14). The cholesterol content showed a linear rise beginning at day 7 and extending through day 15 (Fig. 1). The increases of cholesterol showed an inverse relationship to the specific activity of acyltransferases during this interval.

SUBCELLULAR FRACTIONS: Determinations of the acyltransferase activity of microsomal and myelin fractions from homogenates of the trigeminal nerve showed that this activity was associated with the former, but not the latter (Table I). The specific activity of the enzymes in the microsomal preparation at various developmental intervals roughly paralleled that of whole homogenates. The myelin fraction showed no incorporation of radioactive α-glycerophosphate into esterified lipids at any stage of development.

Retention of Acyltransferase Activity under Cytochemical Conditions

The results of biochemical studies performed to ascertain retention of acyltransferase activity in blocks of trigeminal nerve after fixation by a
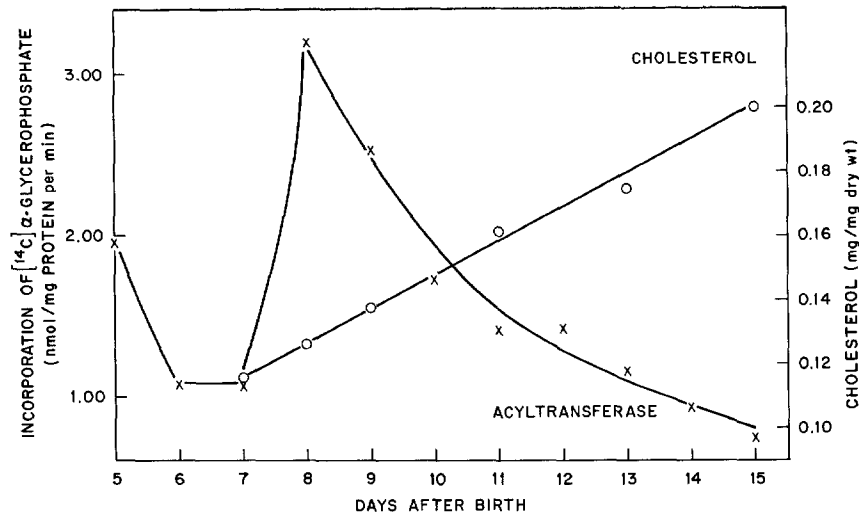
FIGURE 1 Incorporation of [^{14}C]α-glycerophosphate into total esterified lipids of the neonate rat trigeminal nerve. 10% homogenates (wt/vol) of unfixed tissue were incubated 30 min at 37°C in a medium containing [^{14}C]α-glycerophosphate (2.5 mM) and palmityl-CoA (0.18 mM) in cacodylate buffer (0.025 M), pH 7.4. Lipids were extracted and radioactivity was counted as described in the Materials and Methods section.

TABLE I

Acyltransferase Activity in Microsomal and Myelin Fractions of the Neonatal Trigeminal Nerve

Development intervals (days)	Incorporation of [^{14}C]α-glycerophosphate into total esterified lipids (nmol/mg protein per min)				
	5	8	13	15	25
Microsomes	3.40	4.80	1.49	0.90	0.18
Myelin	0.00	0.00	0.00	0.00	0.00

Microsomal and myelin fractions were suspended in cacodylate buffer (0.025 M), containing 4.5% dextrose, pH 7.4, and portions incubated for varying intervals of time at 37°C in a medium containing [^{14}C]α-glycerophosphate (2.5 mM) and palmityl-CoA (0.18 mM). Reactions were stopped with ice-cold chloroform-methanol (2:1), and lipids extracted and counted as described in Materials and Methods. Protein was determined for each fraction according to the method of Lowry et al. (1951) and results expressed as nmol incorporation of [^{14}C]α-glycerophosphate into total esterified lipids per mg protein per minute.

variety of procedures and incubation in capture reagents are indicated in Table II. A comparison of various fixation procedures showed that 2% formaldehyde or the combination of 1% formaldehyde-0.25% glutaraldehyde is preferable

TABLE II

Incorporation of [^{14}C]α-glycerophosphate into Total Esterified Lipids by Blocks of Trigeminal Nerve Subjected to Various Methods of Fixation

Fixation	Incorporation of [^{14}C]α-glycerophosphate into total esterified lipids (nmol/mg dry weight nonlipid material)
1% Glutaraldehyde	0.625
0.5% Glutaraldehyde	1.782
2% Formaldehyde	3.780
1% Formaldehyde-0.25% Glutaraldehyde	3.025

Blocks of 8-day trigeminal nerves were fixed by immersion for 30 min in the various solutions indicated above. Samples of each were then incubated at 37°C for 60 min in a medium containing [^{14}C]α-glycerophosphate, palmityl-CoA, copper sulfate, sodium citrate, and potassium ferricyanide in cacodylate buffer. Lipids were extracted and counted for incorporation of radioactivity as described in Materials and Methods. Results are expressed as nmol incorporation per mg dry nonlipid material.

to glutaraldehyde alone. Though not indicated, 0.25% glutaraldehyde alone was more inhibitory than the fixative combination above. Despite the preservation of high levels of acyltransferases

observed with formaldehyde alone, it was not used because of poor preservation of ultrastructural detail.

Comparisons of incorporation of [^{14}C]α-glycerophosphate into total esterified lipids by unfixed nerves and fixed nerves incubated with capture reagents are indicated in Fig. 2. These data were expressed as incorporation of radioactivity per nerve, since the effects of fixation on the retention or loss of material which would affect the dry weight was not understood. During these studies, it was observed that gentle homogenization of fixed, but not unfixed, tissues sometimes resulted in severe reductions of enzyme levels. For this reason, comparative studies between unfixed and fixed tissues were performed using tissue blocks (1 mm^3). The results of these studies indicated that 28.7% of the activity of unfixed tissue was retained under the condition of fixation and incubation in capture reagents employed for cytochemistry.

Additional studies were performed in which [^{14}C]palmityl-CoA was substituted for [^{14}C]α-glycerophosphate as a radioactively tagged sub-strate to determine incorporation of exogenous palmitate into total esterified lipids and the extent of hydrolysis of the thioester during incubation (Table III). The results indicate that incorporation of palmitate into total lipids of the fixed trigeminal nerve occurred. Thin-layer chromatographic separation of these lipids showed that radioactivity appeared in the various fractions of lipids at the zero time interval. These values for esterified lipids may reflect exchange reactions occurring during the lipid extraction procedures. Appearance of radioactivity in free fatty acid in the zero time controls was particularly high and probably reflects palmityl-CoA hydrolysis which occurs during storage or lipid extraction. On this basis, the values obtained for the zero time interval were subtracted from values obtained after 30 min of incubation. During the incubation, more than 80% of the radioactivity appeared in the phospholipid fraction, while less than 20% was recovered as free fatty acid. No incorporation of radioactivity into diglyceride, triglyceride, or cholesterol esters was detectable above the blank value.
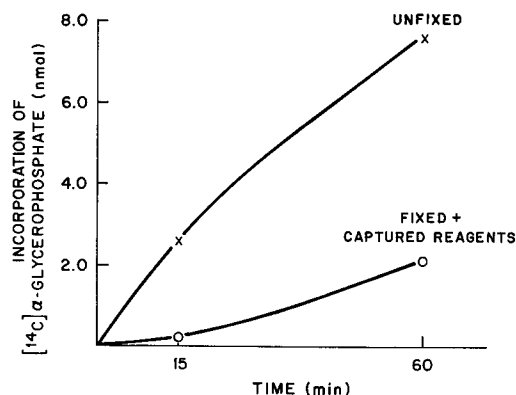

FIGURE 2 Comparison of incorporation of [^{14}C]α-glycerophosphate into total esterified lipids by blocks of unfixed nerves and fixed nerves incubated in the presence of capture reagents. 8-day trigeminal nerves were cut into 1 mm^3 blocks and incubated at 37°C for various periods of time in media containing [^{14}C]α-glycerophosphate (17 mM), palmityl-CoA (0.12 mM), and, where indicated, copper sulfate (3.0 mM), sodium citrate (5.0 mM), and potassium ferricyanide (0.50 mM) in cacodylate buffer (0.025 M), containing 4.5% dextrose, pH 7.4. Lipids were extracted and counted as described in Materials and Methods. Results are expressed as nmol incorporation of [^{14}C]α-glycerophosphate by five trigeminal nerves.

Cytochemical Studies

Tissue blocks incubated in the cytochemical assay system described in Materials and Methods and subsequently processed for electron microscopy showed the appearance of reaction product associated with various membranous elements of Schwann and ganglion cells. Several control studies were performed to evaluate the cytochemical reaction. The results of these studies indicated that the reaction product observed was substrate-dependent, requiring both α-glycerophosphate and palmityl-CoA. The deposition of heavy metal precipitate also appeared to be attributable to acyltransferase activity in the presence of substrates, and capture reagents, since treatments which partially or completely destroy biochemically detectable acyltransferase activity also result in the reduction or elimination of reaction product in tissues incubated in the complete cytochemical medium. For example, tissues fixed with 1% glutaraldehyde (Table II) indicated very small amounts of reaction product, having the same localization as that observed under optimal conditions, while fixed trigeminal nerves which were heated showed neither biochemical nor cytochemical activity. Further studies were

Table III

Incorporation of [^{14}C]palmitate into Lipid Fractions by Fixed Trigeminal Nerves

Time of incubation	Incorporation of [^{14}C]palmitate nmol					
	Total	Phospholipid	Diglyceride	Free fatty acid	Triglyceride	Cholesterol esters
min						
0	26.10	5.20	7.91	10.96	1.33	0.70
30	31.00	11.12	6.34	12.22	0.88	0.37

10% (wt.vol) homogenates were incubated at 37°C in a medium containing L-α-glycerophosphate [^{14}C]palmityl-CoA, and the copper sulfate-potassium ferricyanide capture system, as described in Materials and Methods. Lipids were extracted, separated, and counted for incorporation of radioactivity into total lipid and fractions as described in Materials and Methods. Results are expressed as nmol incorporation by 20 mg wet weight of tissue.

performed to investigate the possibility of false localization of copper ferrocyanide formed as a result of diffusion of free CoA (before reaction with ferricyanide) or ferrocyanide ions (before precipitation with copper ions) away from the active site of enzymes, or nonenzymatic hydrolysis of palmityl-CoA. Fixed trigeminal nerves incubated in copper ferrocyanide followed by the addition of copper ions showed no deposition of heavy metal precipitate in the tissue.

Electron Microscope Observations

SCHWANN CELLS: Most observations were made on blocks of nerve cut in cross section to allow a broader sampling of cytochemical reaction in each specimen. Such sections indicated profiles in a variety of stages of myelination, ranging from Schwann cells beginning to extend a cell process toward an axon, to cells in which well developed myelin profiles were already present. Although most of these observations were confined to the trigeminal nerve at 8 days

post partum (the peak of acyltransferase activity), some observations extended from the 5th day nerve to the 10th day nerve. Although the process of fixation and cytochemical incubation provided conditions that were less than optimal for adequate morphological preservation, particularly of myelin, fine structural detail was, nevertheless, recognizable.

Reaction product was observed in Schwann cell processes, apparently in the process of surrounding and rotating about axons (Figs. 3, 4, 9, and 10). Here, acyltransferase activity was associated with the plasma membrane of these cell extensions, as well as with intracellular vesicular elements. At these sites, the deposits of final product were either linear or more commonly occurred as large aggregates that usually protruded toward the cytoplasm (Figs. 3, 4, and 9).

Cells in the early stages of myelination of axons showed reaction product most extensively associated with some cisternae and small vesicles of the Golgi apparatus (Figs. 5 and 6). The Golgi

FIGURE 3 A section of fixed trigeminal nerve (day 8) incubated in the complete medium for localization of acyltransferases as described in Materials and Methods. Electron opacities are associated with the plasma membrane of Schwann cell processes surrounding two axons (*A*) and with small vesicles in the cytoplasm of the Schwann cell (arrow). X 50,000.

FIGURE 4 A Schwann cell process is enclosing an axon (*A*). Acyltransferase reaction product is associated with the Schwann cell plasma membrane, particularly that of the rolling pseudopodium surrounding the axon. X 50,000.

FIGURE 5 A Schwann cell in which myelin formation is just beginning. Acyltransferase reaction product is associated with Golgi cisternae (*G*) and small cytoplasmic veiscles (arrow). X 23,000.
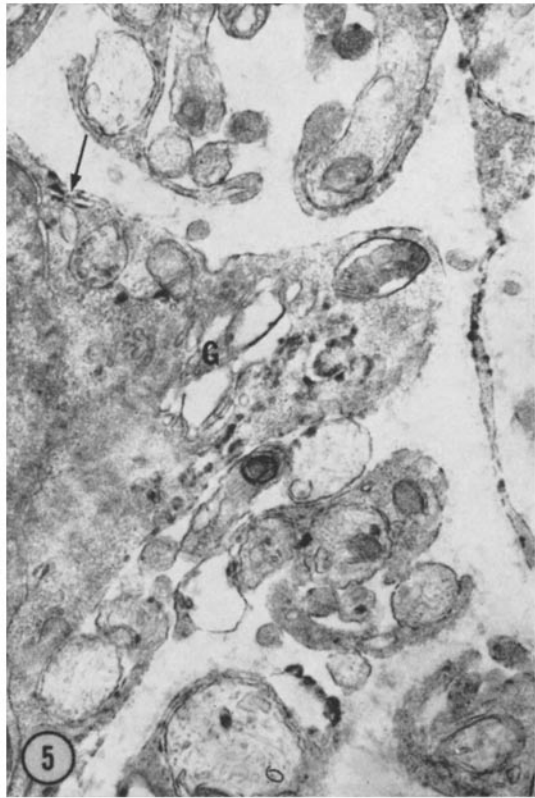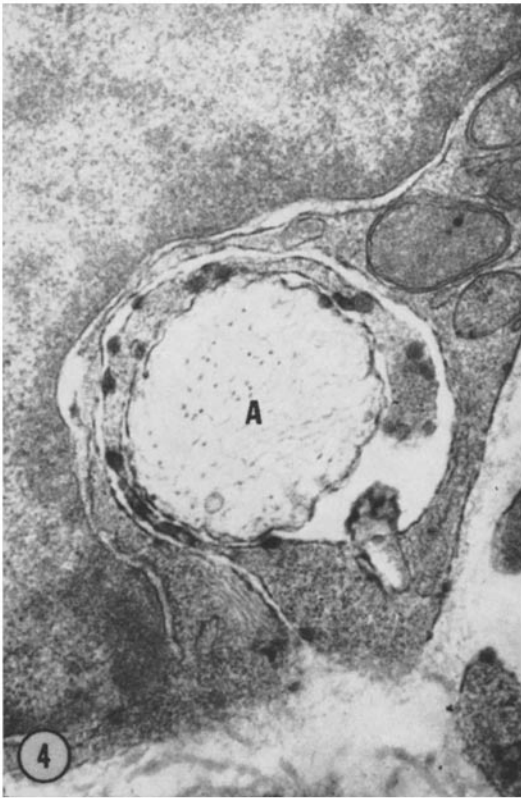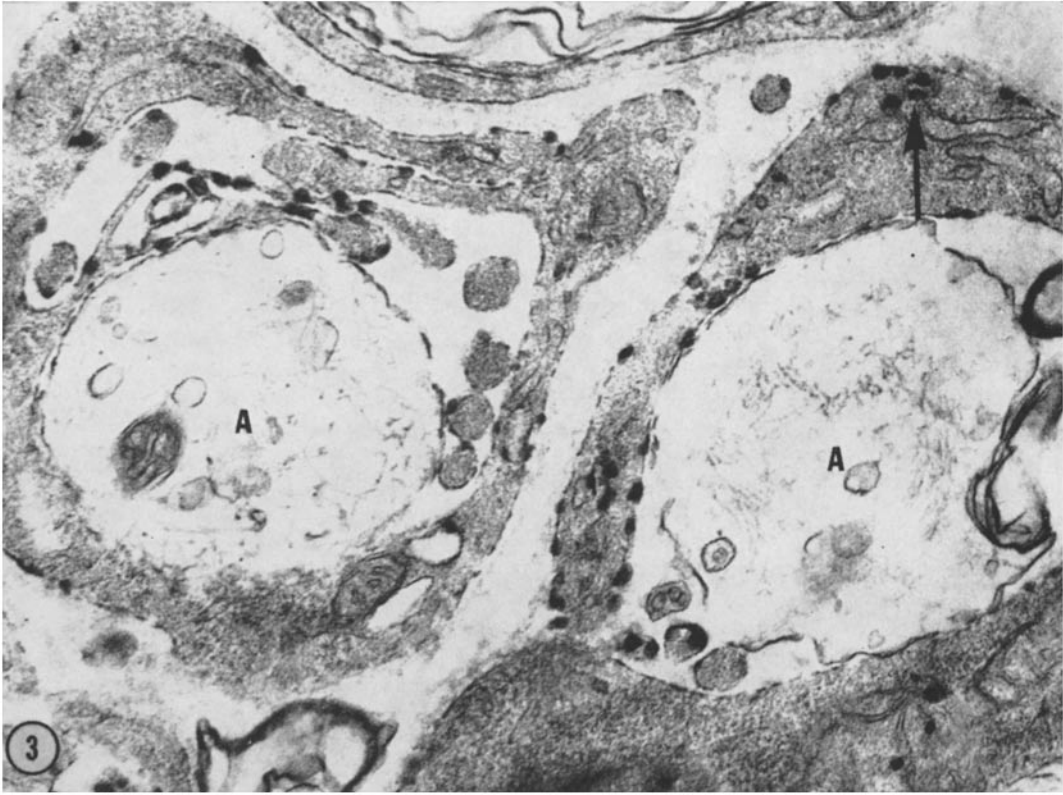
apparatus at this stage was sufficiently large and irregular that no consistent pattern of activity was noted within the cisternae. Some small vesicles showing activity extended from the region of the Golgi complex, especially the lateral surfaces of cisternae (Fig. 5), through the cytoplasm toward the plasma membrane (Figs. 6, 7, and 8). Some small reactive vesicles were closely approximated to the plasma membrane (Figs. 3, 4, 7, and 8) and frequently were seen in omega forms, presumably due to fusion of vesicles with membrane (Figs. 7 and 8). The reaction product associated with vesicles and cisternae of the Golgi complex was located on the cytoplasmic aspect of their limiting membrane (Figs. 5-8). In no case was the deposition of the reaction product on the plasma membrane confluent, but rather occurred in a spotty fashion so that irregular stretches of membrane showed no reaction product. During the process of myelination, the latter structure showed activity as long as Schwann cell cytoplasm could be identified within the turns of the internal mesaxon. In cell commencing myelination, the plasma membrane showed activity at various points around the cell body, as well as in the cell process itself (Figs. 3, 4, and 9). Where myelination was more complete, and the concentric rings of membrane about the axon tighter and appearing as complete myelin membrane, no activity was found (Fig. 6). Thus, completely myelinated profiles were unreactive, and the Schwann cell associated with these forms showed little or no activity at its surface. Partially myelinated forms showed activity only in relation to the plasma membrane forming the outer two to four concentric rings; the inner more compacted rings were unreactive.

GANGLION CELLS: Reaction product marking acyltransferase activity was observed in elements of rough endoplasmic reticulum and the Golgi complex (Figs. 11-13). In the case of the rough endoplasmic reticulum, reaction product was sparse and the product was located intracisternally (Fig. 12), while that of the Golgi complex appeared on the cytoplasmic aspect of membranes (Fig. 13). Acyltransferase marker was most frequently associated with the innermost cisternae of the Golgi stacks (Figs. 11-13). In most cases, the plasma membrane of the ganglion cell body was unreactive (Fig. 12), although small amounts of reaction product were occasionally seen (Fig. 11).

AXONS: Axons in the process of being myelinated showed acyltransferase reaction product associated with the smooth membranous vesicles within the axoplasm (Figs. 5 and 14). Axons which were more completely myelinated also showed similar localizations; but, in addition, electron opacities were observed in association with the axolemma (Fig. 14). In some cases, axoplasmic vesicles with reaction product could be seen in apposition to the axolemma.

DISCUSSION

Evaluation of Cytochemical Method

The problems associated with the interpretation of localization of acyltransferases by cytochemical methods have been discussed elsewhere (3, 25–27). A major problem in the present study is the possibility that an absence of reaction product could mean no enzyme, low enzyme, or inactivated enzyme activities. However, similar localizations of reaction product, but fewer deposits, were found

FIGURE 6 Two Schwann cells at different stages of myelination illustrate that reaction product marking acyltransferase activity is detectable in the early stages of myelin formation (left), but is not apparent as the mature myelin sheath is evident (right). Where an axon (A) is surrounded by the Schwann cell process, reaction product is seen in the Golgi complex (G), in small cytoplasmic vesicles (arrows), and associated with the Schwann cell plasma membrane, particularly where it is surrounding the axon. X 17,000.

FIGURE 7 Vesicles with acyltransferase reaction product are seen in close relation to the plasma membrane. Some vesicles form an omega profile with the plasma membrane (V_1) suggesting a sequence of events in which the intravesicular space does not at first communicate with the extracellular space, then the neck of the flask-shaped vesicles becomes continuous with the plasma membrane (V_2), and gradually expands (V_3). X 69,000.

FIGURE 8 Acyltransferase reaction product is associated with spherical vesicles within the cytoplasm, or in the process of fusing with the plasma membrane of the Schwann cell. X 44,000.
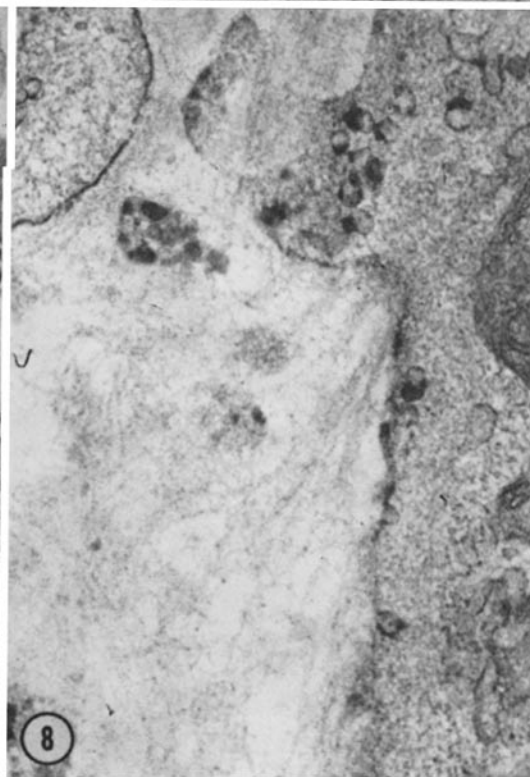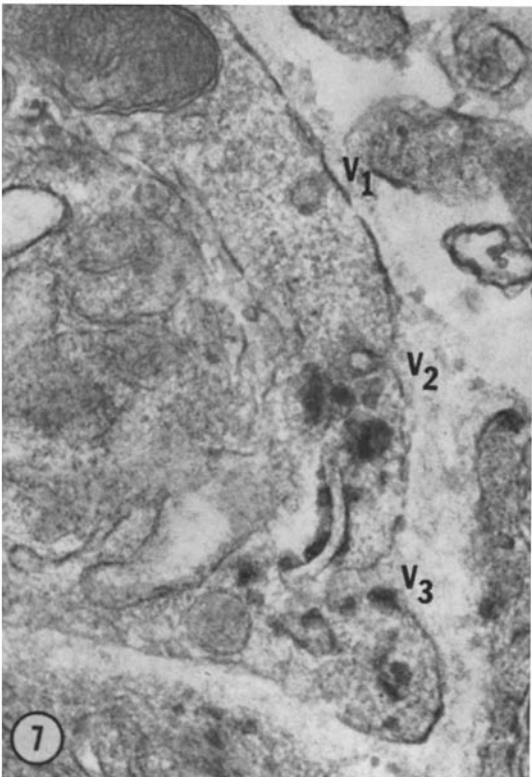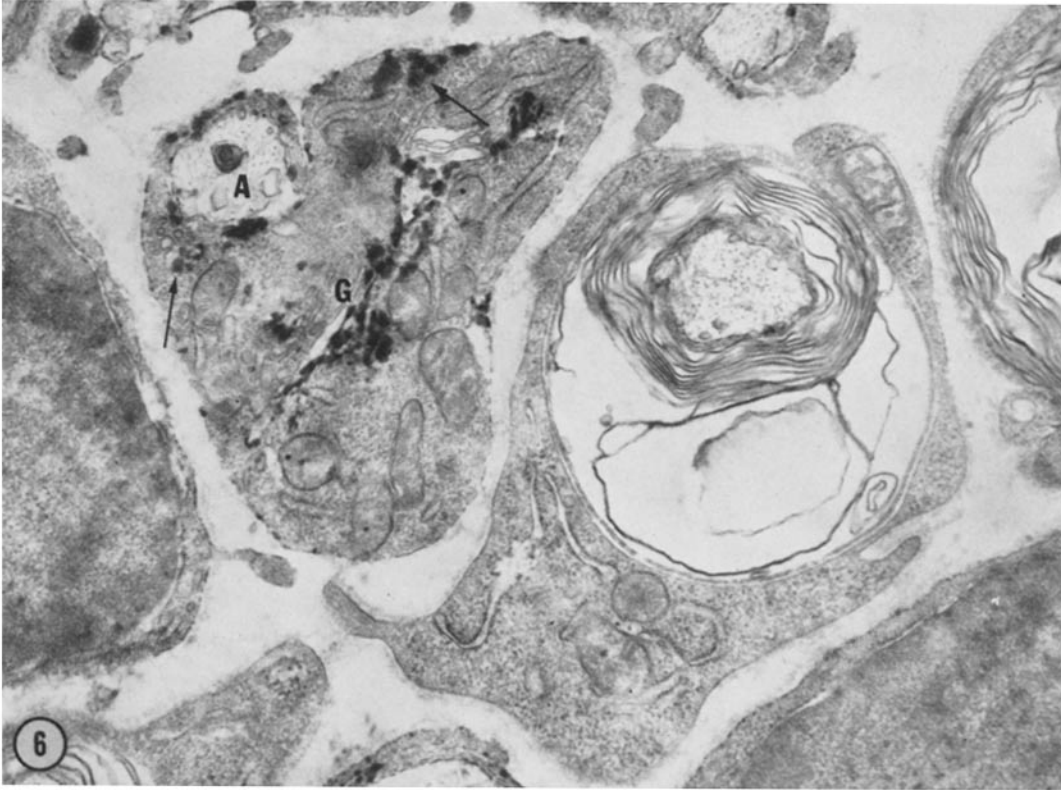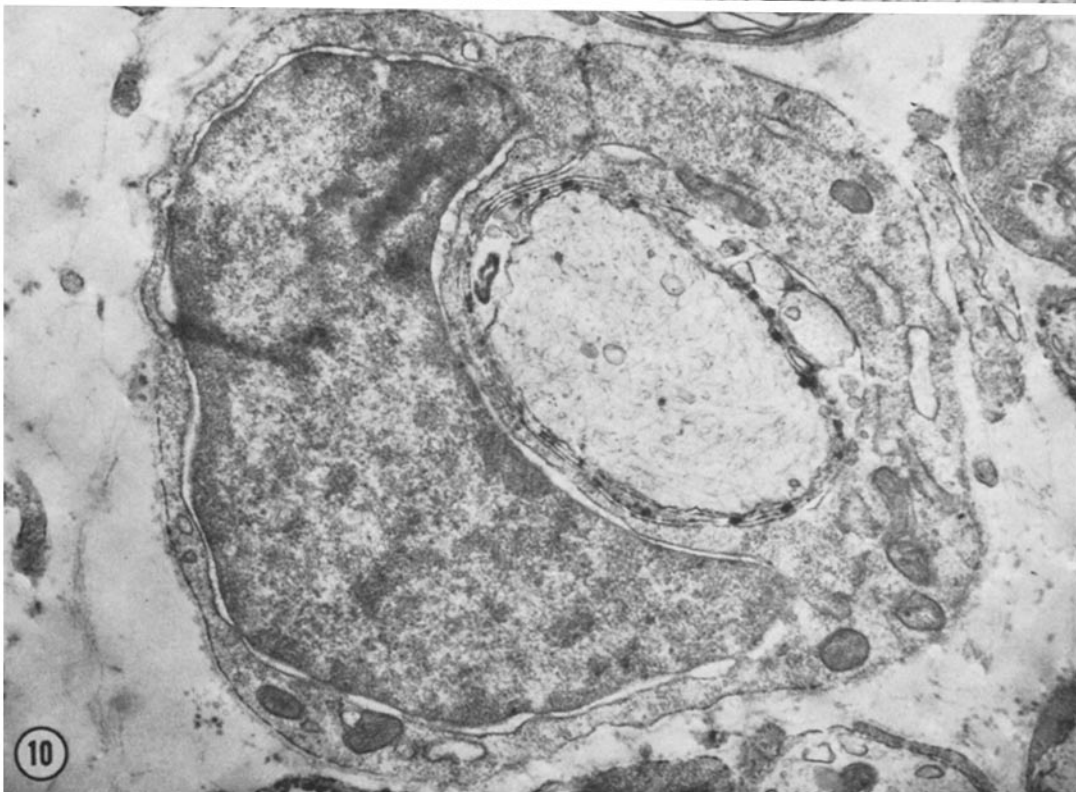
FIGURE 9   A Schwann cell process is enclosing an axon. Acyltransferase reaction product is associated with the plasma membrane, cytoplasmic vesicles (arrows), and the plasma membrane lining the pseudopod which becomes inner (IM) and outer (OM) mesaxon. × 56,000.

FIGURE 10   A Schwann cell in the process of myelinating an axon is seen at the level of the Schwann cell nucleus. Acyltransferase activity is primarily associated with the internal spinals of the processes. × 19,000.

in tissues in which enzymatic activity was inhibited to a greater extent by fixation (0.5% or 1% glutaraldehyde), compared with that reported here. For these reasons, the conclusions we have made are based on positive results which were consistent.

The results of this study have indicated that acyltransferase activity is retained in fixed trigeminal nerves incubated in the presence of the cupric sulfate-potassium ferricyanide capture system. Incorporation of both radioactively labeled α-glycerophosphate and palmityl-CoA into total esterified lipids occurred. The amount of acyltransferase activity retained in the trigeminal nerve under the conditions employed for cytochemical localization of the enzymes (29%) is greater than that observed in the adult liver reported in earlier studies (10%) (3). Chromatographic separation of total lipids indicated that phospholipid was the major labeled fraction. Some hydrolysis of palmityl-CoA producing free fatty acid also occurred; however, this was small in comparison to that observed in similar studies of liver in which it was shown that hydrolase activity does not contribute to the final product observed (3). Moreover, the glycerophosphate dependence of the reaction product further suggests that the localization does not arise from hydrolytic cleavage of palmityl-CoA. Correlative biochemical and cytochemical studies have shown that treatments which produce severe decrements in levels of enzyme activity of tissues also result in the reduction (1% glutaraldehyde) or absence (heating) of reaction product in similar tissues prepared for electron microscopy.

The product observed in these studies did not arise by adhesion of suspended copper ferrocyanide, since fixed tissue blocks incubated in the presence of copper sulfate and potassium ferrocyanide showed no electron opacities. Therefore, localization of reaction product apparently relies upon a membrane-bound enzyme to serve as a focal point for formation and growth of the cytochemical end product. Copper ferrocyanide, therefore, may not form in response to diffuse or low concentrations of CoA or ferrocyanide. It should also be noted that copper ferrocyanide is insoluble in lipid solvents as well as in phospholipid micelles.

Schwann Cells and Myelin Formation

The present studies have indicated that between the 7th and 8th day after birth, a 3-fold increase in acyltransferase specific activity occurred in the rat trigeminal nerve. Morphological surveys and determinations of cholesterol levels performed in this study suggest that heavy myelination in the trigeminal nerve may begin around the 8th day and extend to about 15 days of age. On this basis, it was assumed that cytochemical localizations of acyltransferases in Schwann cells of this nerve during this period of development are related to the synthesis of myelin phospholipid. Although the acyltransferases catalyze the first two steps in the synthesis of phospholipid, recent reports indicate that choline phosphotransferase, which further metabolizes the intermediates to phosphatidylcholine, have a parallel distribution with acyltransferases in various subcellular fractions of the liver (51). The intermediates, therefore, probably are not transferred to different compartments during phospholipid synthesis, and the site of acyltransferase activity probably is the site of synthesis of the complete phospholipid molecules, at least at an organelle level.

SYNTHESIS OF ACYLTRANSFERASES: It has recently been suggested that nascent polypeptides synthesized by bound ribosomes enter the cisternal space of the rough endoplasmic reticulum (46). The absence of acyltransferase marker in the cisternae of the rough endoplasmic reticulum of Schwann cells may suggest that these enzymes are not made by bound ribosomes, or more likely, were not in an active conformation. However, it was noted that the cytoplasm of Schwann cells of the neonate trigeminal nerve contained many free ribosomes, but only a few profiles of rough endoplasmic reticulum. Since it is known that free polysomes synthesize protein (6), acyltransferase could be made by free ribosomes, released into the hyaloplasm, and inserted into Golgi membranes. The location of the enzyme marker on the cytoplasmic surface of Golgi cisternae is consistent with this concept. If the enzymes were synthesized in the rough endoplasmic reticulum and transported to the Golgi complex by transitional vesicles (32, 41) which bud off the ends of rough endoplasmic reticulum cisternae, they would presumably line the inner surface of Golgi membranes or be stored intracisternally. However, rearrangement of protein could occur during packaging within Golgi membranes. Synthesis of Golgi-associated acyltransferases by free or bound ribosomes is presently being studied by the differential effects
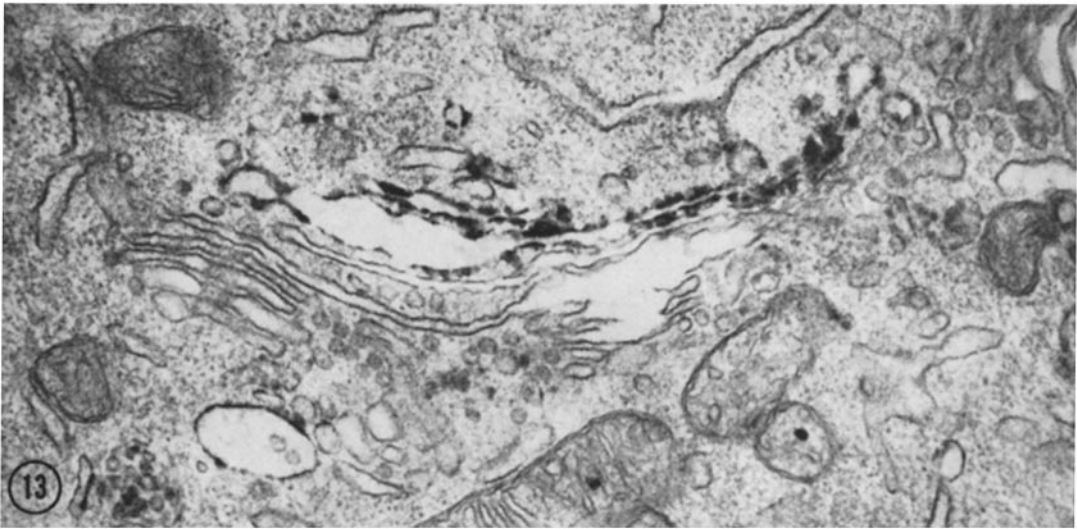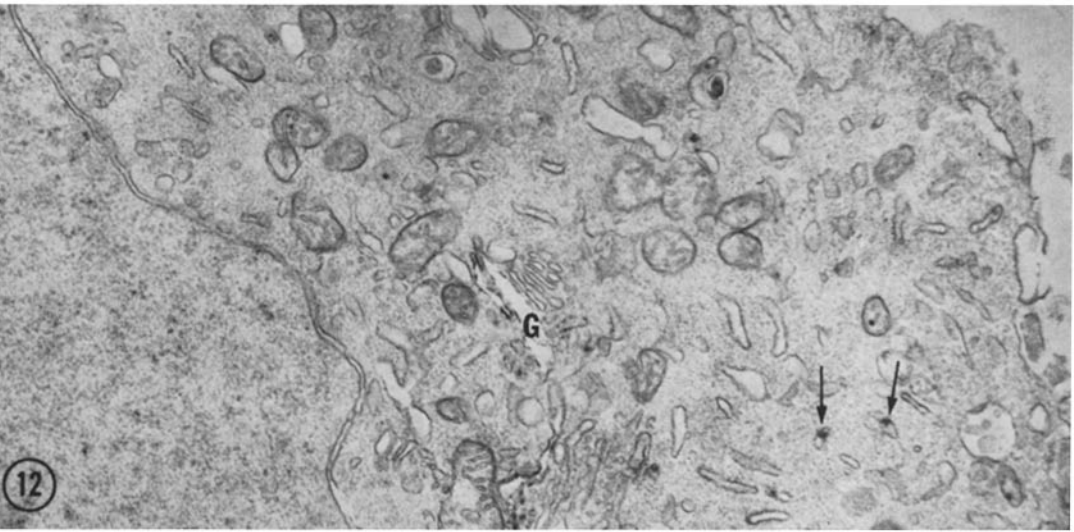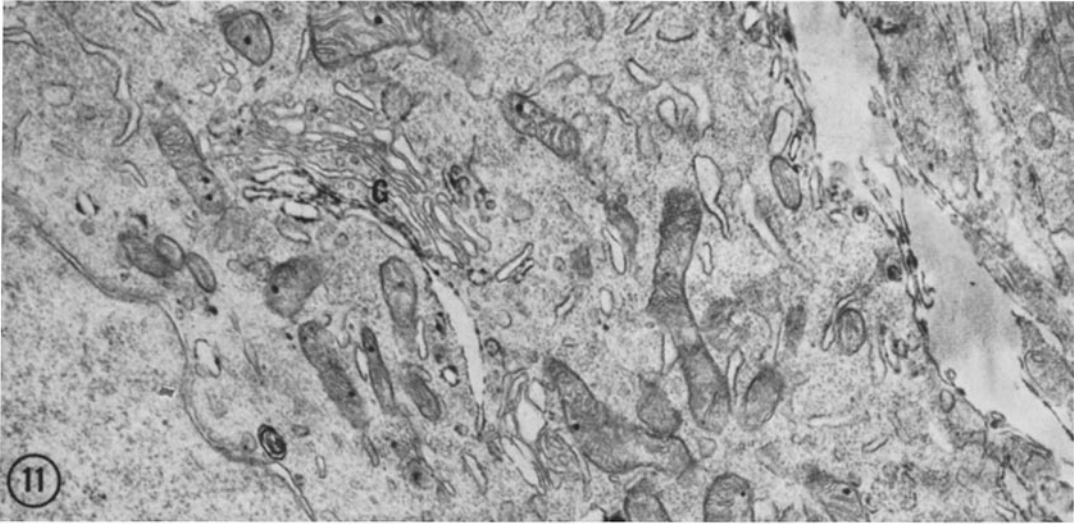
of various inhibitors of protein synthesis: cyclo-heximide, for example, has a more potent in-hibitory effect on bound, than free, ribosomes (22).

ACYLTRANSFERASES AND THE GOLGI COM-PLEX: The observation of reaction product marking acyltransferase activity associated with phospholipid synthesis in the Golgi complex of Schwann cells during myelinogenesis suggests the participation of this organelle in plasma mem-brane growth associated with myelin formation. Previous studies at the electron microscope level using morphological (39, 23, 54, 20), cytochemi-cal (43, 34), and autoradiographic (32, 4, 40) approaches have suggested the movement of ma-terial from Golgi cisternae to the plasma mem-brane by means of vesicles budding off the Golgi cisternae in a variety of cell types. The limiting membranes of Golgi-derived vesicles have been shown to fuse with the plasma membrane during secretion (32); thus, the participation of the Golgi complex in the elaboration of plasma membrane during myelinogenesis appears to be similar to that in the secretory process. In myelin formation, however, preformed membrane arising from Golgi elements appears to carry packets of enzyme to the cell surface for synthesis of additional phos-pholipid for plasma membrane growth. The syn-thesizing enzymes themselves, and whatever phospholipid is made in the Golgi complex, are thus analogous to the secretory products of other cell types, though in the mechanism suggested above they are probably part of the structure of the limiting membrane and are not actually re-leased outside the cell.

Vesicles, apparently derived from the Golgi complex, which demonstrated acyltransferase activity were observed in various stages of fusion with the plasma membrane. Consistent with the observations was the location of the enzyme marker on the cytoplasmic surface of both vesicle and plasma membrane. Thus, the disposition of acyltransferase enzymes in the membranes of endoplasmic retciulum and Golgi complex in different cell types may reflect different roles for these enzymes, the former subserving turnover of phospholipid of the endoplasmic reticulum and possibly other intracellular membranes, and the latter, phospholipid synthesis for growth of Golgi membranes and/or plasma membrane. The absence of these enzymes in sufficient concentra-tion to be detectable by cytochemical methods in the Golgi complex of Schwann cells in which myelin formation was complete suggests that they are not fixed or obligatory components of this organelle; the appearance of their activity in the Golgi complex may be a function of control factors which stimulate or repress plasma mem-brane biogenesis. It should be noted that in the duck salt gland stimulated to synthesize plasma membrane as a result of salt stress, acyl-transferases were also found to be associated with the Golgi membrane by cytochemical experiment, but not with the rough endoplasmic reticulum. However, in this instance no enzymatic activity occurred in the plasma membrane (34).

MYELIN FORMATION: The earliest phase of myelin formation involves the rotation of the Schwann cell process about the axon (21). The present studies support an early suggestion, based solely on morphological grounds, that en-zymes of phospholipid synthesis may be located in the plasma membrane of the Schwann cell

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FIGURE 11   The ganglion cell of an 8-day trigeminal nerve incubated in the complete cytochemical medium described in Materials and Methods. Electron opacities indicating sites of acyltransferase enzymes are associated with the innermost cisternae of the Golgi complex (G). Vesicles near the cis-ternae also show reaction product. In both instances, the electron opacities appear on the cytoplasmic aspect of the membranes. Traces of reaction product are associated with the plasma membrane of the ganglion cell, and some vesicles in close proximity to the plasma membrane contain electron opacities. Cell processes in the neuropil also appear to contain acyltransferase activity. × 17,000.

FIGURE 12   The cytoplasm of a ganglion cell shows some reaction product associated with the innermost cisternae of the Golgi complex (G). Cisternae of rough endoplasmic reticulum show enzyme marker intracisternally (arrows). × 17,000,

FIGURE 13   A Golgi complex of an 8-day trigeminal ganglion cell showing acyltransferase reaction product associated with the innermost cisternae. A few smaller vesicles also contain reaction product. In both cases, electron opacities appear on the cytoplasmic aspect of the membrane. × 58,000.
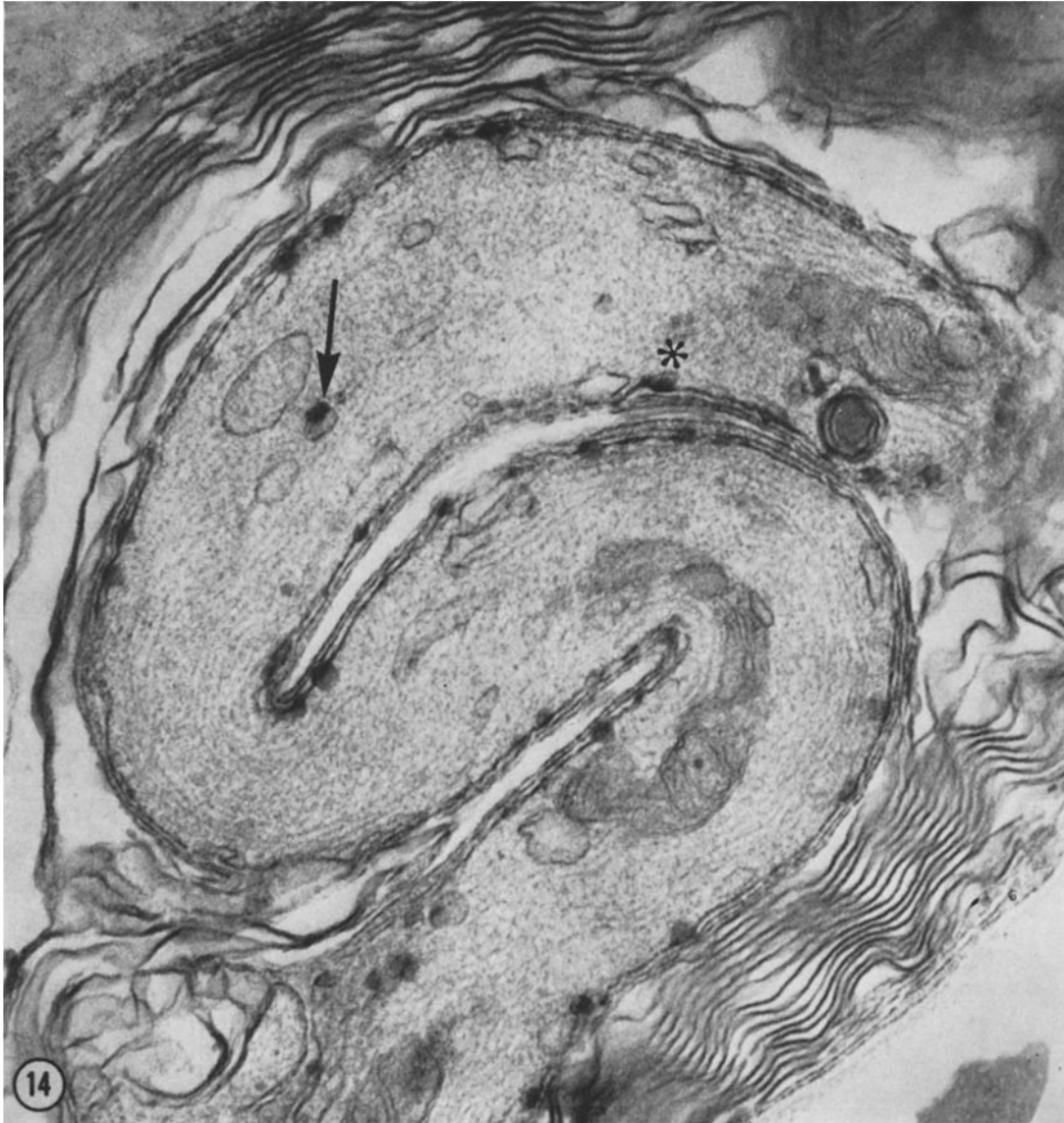
FIGURE 14 A longitudinal section of an axon separated from its well-developed myelin sheath. Acyl-transferase reaction product is associated with vesicles in the axoplasm (arrow) in close apposition with the axolemma (*), and with the axolemma itself, The electron opacities of both vesicles and axolemma appear on the axoplasmic aspect of these membranes, × 60,000.

process (49). As the Schwann cell pseudopodium spirals around the axon, actyltransferase activity continues to be detectable as long as the cytoplasm of the Schwann cell is discernible within the process. Determinations of the content of various lipids in myelin-pure fraction as a function of age have indicated that early myelin has a higher level of phospholipid relative to cholesterol than that of older animals, while cerebroside is found in appreciable amounts only in adult myelin (14). Phospholipid synthesis, therefore, appears to antecede that of some of the other myelin components, possibly producing a framework into which increasing amounts of other lipids are added (29).

The lack of acyltransferase activity in mature

myelin indicated by biochemical studies of myelin fractions and cytochemical observations of intact tissues is in accord with the concept of the enzymatic inertness of myelin suggested by previous histochemical (1) and biochemical (16, 31) studies of various enzyme activities. The disappearance of acyltransferase activity as mature myelin forms may relate to a "feed-back" phenomenon in which accumulations of phospholipids cause a reduction in activity of lipid-synthesizing enzymes, which has been proposed to explain higher synthetic activity in neonatal and demyelinating nerves than in fully myelinated systems (37, 38). It has been suggested that liver acyltransferases require acceptor protein for their products to maintain activity, and saturation of this protein may slow down or stop further synthesis (47). A characteristic of this kind for these enzymes could play a role in a myelinating system, where the final membrane has characteristically high levels of phospholipid relative to protein. A model may be envisioned in which packets of enzyme as well as phospholipid inserted from Golgi vesicles in the Schwann cell membrane at the earliest time interval would attain their maximum synthesis as phospholipid accumulates. A gradient within the spiraling pseudopod could be reflected by the activity of these enzymes as a function of time. As the membrane spins off with successive wrappings around the axon, the activity would plateau, slow, then shut off, for each successive acyltransferase inserted at various intervals of time after the process was initiated. After membrane formation during the process of myelin maturation, these enzymes either may become structural elements or may be removed or replaced by other myelin proteins which have been shown to turn over (50, 15, 53).

Axon Growth

The present studies have indicated the presence of acyltransferase enzymes in the axon of the trigeminal nerve. The localization occurred at two sites: axoplasmic smooth vesicles and the axolemma. Some correlation probably exists between the active sites of the Golgi apparatus of neurone perikarya and the smooth vesicles in the axon. It would not be unlikely that vesicles budding off the Golgi complex could migrate into the axons. It is difficult to explain the transfer of acyltransferases from an intracisternal position in the rough endoplasmic reticulum to line the cytoplasmic surface of Golgi cisternae and vesicles, unless the endoplasmic reticulum transferase subserves another function.

Some correlation also appeared to exist between the location of the enzyme and the extent to which the axon was myelinated. Early profiles of myelination indicated reaction product predominantly associated with axoplasmic vesicles, while more mature forms demonstrated activity associated with both smooth vesicles and the axolemma. It has been suggested that once an axon attains a critical diameter (36), Schwann cells may be stimulated to produce myelin, accelerated axon growth causing accelerated myelin formation (19). This latter report in which myelination distal to ligatures was studied indicated that axons can continue to increase in diameter, even when already myelinated. The results of this present study are consistent with this concept.

Little is known regarding the mechanism by which neuronal processes are elaborated. It appears that de novo extension of axons proceeds by insertion of new membrane components at points most distal to the perikarya (8). The rare appearance of reaction product marking acyltransferase activity in cell body plasma membrane and its presence in the growing axon are compatible with this observation.

The present cytochemical results have helped to clarify and expand the interpretation of biochemical determinations of acyltransferase activity in microsomes, obtained from the trigeminal nerve, probably containing fragments of endoplasmic reticulum, Golgi cisternae, and vesicles, and plasma membrane of Schwann cells, ganglion cell bodies, and axons. The present studies illustrate the advantages of enzyme cytochemical approaches correlated with biochemistry in studies of the developing nervous system, where heterogeneous cell types and single cell types in different stages of differentiation are present.

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