INCORPORATION OF GLYCOPROTEINS INTO PERIPHERAL NERVE MYELIN

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ABSTRACT

Peripheral nerve myelin contains a dominant low molecular weight glycoprotein (12, 17, 22, 25, 32) called Po (17). To study the metabolism of this glycoprotein, tritiated fucose was injected into the peripheral nerves of adult mice and developing rats, and the temporal distribution of label was examined by autoradiography and gel electrophoresis.

Mice and rat pups, injected with fucose, were sacrificed from 1 h to 98 days later. Series of autoradiographs were prepared. At the shortest labeling periods, newly formed product was confined to juxtanuclear Schwann cell cytoplasm, in association with regions rich in Golgi apparatus. After longer labeling periods, silver grain levels in Schwann cell cytoplasm decreased; concomitantly, there was an increase of silver grains associated with myelin. In adult animals, label associated with myelin was concentrated over outer layers of thickly myelinated fibers. Even at the longest time intervals examined (72 and 98 days), this distribution of label was largely retained. In contrast, in developing animals, label became associated with inner layers of the thicker sheaths. At no time was label observed over axons.

Gel electrophoresis revealed that tritiated fucose was a suitable precursor for the faster migrating peripheral nerve glycoprotein(s). At all times examined, there was a single major peak of radioactivity that co-migrated on sodium dodecyl sulfate (SDS) acrylamide gels with the Po protein (17). Sometimes, a faster migrating shoulder of radioactivity was noted. With increased labeling periods, there was an enrichment of radioactivity associated with Po, indicative of a relatively slow turnover rate.

KEY WORDS peripheral nerve · myelin · glycoprotein metabolism · Po protein · lateral diffusion · EM autoradiography

Metabolism of choline- and inositol-labeled lipids of peripheral nerve myelin has recently been studied by quantitative autoradiography (10, 15, 16, and Gould, unpublished observations). It was demonstrated that these phospholipids migrate from synthetic sites in Schwann cell cytoplasm surrounding the myelin sheath into and through the myelin. Several days are needed for the innermost lamellae of thickly myelinated fibers to become as thoroughly labeled as the outer lamellae. From such a time-course and knowledge of the surface dimensions over which the phospholipids would travel, diffusion constants of between 10^{-7} and 10^{-8} cm^2/s were determined.

Peripheral nerve myelin contains two dominant glycoproteins having molecular weights of 23,000

and 28,000 daltons (36). These constitute 60–80% of the peripheral nerve myelin protein (17, 36, 40). Cytochemical investigations suggest that the carbohydrate portions of peripheral nerve glycoproteins are exposed at the intraperiod lines of the myelin membrane (43). Recent freeze-fracture studies indicate that some peripheral myelin proteins intercalate across the bilayer structure (31). As major peripheral nerve myelin constituents which are difficult to solubilize from isolated myelin (6, 22), it would seem plausible that these glycoproteins span the myelin bilayer.

In the present study, tritiated fucose has been used to localize sites of synthesis of glycoproteins and to follow their subsequent incorporation into myelin. Temporal changes in the distribution of label have been measured in developing, younger and older animals. Results from these studies and those of other investigations will be used to construct a model for structural properties and metabolism observed in peripheral nervous system myelin.

MATERIALS AND METHODS

Animals and Materials

Male mice (COBS-CD-1 [ICR]BR, Charles River Breeding Laboratories, Wilmington, Mass.) between 2 and 10 mo of age and rat pups (CDF strain, Charles River Laboratories) 8 days old were used. Two or three animals were injected for each biochemical and autoradiographic determination. Radioactive L-[6-^{3}H]fucose (sp act 12 Ci/nmol) was purchased from New England Nuclear Corp. (Boston, Mass.). Ilford L4 and K2 nuclear emulsions and Phen-X developer were purchased from Polysciences, Inc. (Warrington, Pa.). All other chemicals were of analytical reagent grade. Polyacrylamide slab gel electrophoresis was performed on model 220 system supplied by Bio-Rad Laboratories (Rockville Centre, New York).

Direct Labeling of Animals

Procedures for labeling the animals and embedding the nerve are those described previously (15), with slight modification. Radioactive fucose stored in 50% ethanol was dried under a stream of nitrogen and redissolved at a concentration of 100 μCi per μl in sterilized saline. Mice anesthetized with halothane gas, or rat pups rendered insensitive to the surgery by chilling in ice for several minutes, were each injected with 0.2–0.3 μl of radioactive solution directly into exposed right sciatic nerve.

At sacrifice, 3- to 4-mm lengths of nerve were removed and immersed in Millonig's buffered 3% glutaraldehyde (15). Fixative and buffer washes contained 2–10 mM L-fucose. Soaking the nerve for several days in buffer reduced the level of trichloroacetic acid (TCA)-soluble radioactivity to <1% of that present in TCA-insoluble residue. After washing, nerves were postfixed in an unbuffered 1% osmium tetroxide, 1.5% potassium ferrocyanide solution (21) before dehydration and embedding (15).

Identification of Radioactivity in Fixed Nerve

1- to 2-mm lengths of the fixed, washed nerve were homogenized in 0.1 N H_2SO_4 and hydrolyzed for 8 h at 100°C (37). The hydrolysates were applied to ion exchange resins (12, 37) and the uncharged eluates were separated by paper chromatography (44). The chromatogram was scanned for radioactivity with a Berthold LB60 chart scanner (Shandon Southern Instruments Inc., Sewickley, Pa.).

Distribution of Label in Different Proteins

Nerve segments, proximal and distal to that immersed in fixative, were removed and homogenized in 1 ml of ether:ethanol (3:2). Water-soluble radioactivity and lipid were removed in this and two subsequent ether:ethanol washings. The dried protein residues were dissolved in a 1% SDS solution (42, 43). Solubilization was augmented by sonication. Electrophoresis was performed in a slab gel apparatus (17 × 14 cm × 1.5 mm with 10 wells/slab) with either 15% polyacrylamide or a gradient or 5–18% polyacrylamide in buffer systems as described by Greenfield et al. (18). After electrophoresis, slabs containing 50–150 μg of protein per sample well were stained with Coomassie Blue, and myelin proteins were identified by their migrations relative to that of horse heart cytochrome *c* (Sigma Chem. Co., St. Louis, Mo. mol wt = 12,400) and tubulin (mol wt = 54,000) standards. The distribution of radioactivity was determined after the gels were sliced into 3-mm segments and solubilized by heating in 30% (vol/vol) H_2O_2 at 50°C for 6 h (39), followed by liquid scintillation spectrometry with 10 ml of Aquasol (New England Nuclear).

Light and Electron Microscope Autoradiography

Transverse sections were cut from labeled nerves embedded in TAAB resin and prepared for both light and EM autoradiography (15). Light microscope autoradiographs were stained with methylene blue-azure II (20). Procedures for quantitation of grain distributions in the electron microscope autoradiographs have been described elsewhere (10, 15).

Micrographs (× 15,000) were also used for a second type of analysis as described by Rawlins (33). Myelinated nerves were classified according to the thickness of their sheaths. The classification was based on the nomenclature of Salpeter et al. (34). Sheaths used were of thicknesses from 3 to 8 HD units; 165 nm was taken as 1 HD unit. A sheath of cross-sectional thickness of 330-

500 nm was classified as 3 HD, 500-670 nm as 4 HD, and so forth. Measurements were made across the narrowest sheath cross section in the profile.

Regions of thicker cross section were assumed to result from oblique cutting. Grain distributions across these were made after they were proportionally subdivided according to the determined HD thickness. Myelinated fibers with poor morphology were not used in the quantitation. Total numbers of grains and circles measured for each analysis are included in the appropriate Figure or Table. In general, 15 photographs (11 × 14 inches) were used for each time point in the × 10,000 magnification analyses (Tables I and II) and 30 photographs (11 × 14 inches) were used for the × 15,000 magnification analyses (Fig. 4).

Junctional items in Table I and Fig. 4 are derived from grains and circles overlapping the boundaries of the myelin sheath and external Schwann cell basal lamina. For the grains, these were fitted into circles of 1 HD unit diam before being classed. In the table, all junctional categories are listed separately, whereas in the figure these are grouped into two categories, M/S and M/A, marking the outer and inner edges of the sheath. Standard deviations (Fig. 4) were determined according to Rawlins (33).

RESULTS

Biochemical Studies of Fucose Metabolism in Nerve

Tritiated fucose injected into mouse and rat pup sciatic nerves was rapidly incorporated into TCA-insoluble material. Label in this precipitate was not extracted with chloroform:methanol or chloroform:methanol:water mixtures designed to remove glycolipids and glycosylated isoprenoid lipid intermediates (24). This result indicates that the label resided in glycoprotein.

Further characterizations were made with both fixed and fresh tissue segments. Pieces of fixed nerve from animals sacrificed 1, 8, and 72 days after labeling with [^{3}H]fucose, were hydrolyzed in acid. The solubilized label (~80%) was purified by ion exchange chromatography, and the eluted neutral sugars were separated by paper chromatography. Essentially all eluted radioactivity co-migrated with a fucose standard. This result demonstrated that the radioactive fucose injected into peripheral nerve remains incorporated into glycoprotein without modification.

Labeled nerve segments were solubilized in 1% SDS solutions and subjected to gel electrophoresis. At 1 h, 1, 8, and 72 days (Fig. 1 A-D), the dominant peak of radioactivity co-migrated with the Po myelin glycoprotein. Sometimes a shoulder of radioactivity migrating slightly faster than the major Po protein peak could be distinguished (8 day). Some radioactivity (20–50%) was always present in the high molecular weight region of the gels. In the developing rat, after a 2-h labeling period (Fig. 1 E), fucose glycoproteins were found throughout the gel with a small peak corresponding to the Po protein. After 5 wk of labeling, the peak of label co-migrating with Po became dominant (Fig. 1 F).

Labeling Patterns Seen in Light and EM Autoradiographs

SYNTHETIC SITES: The silver grain distribution in transverse sections of adult mouse peripheral nerve was qualitatively similar to that previously described for choline-lipid distribution (15). At the earliest times examined (1 and 4 h), label associated with myelinated fibers was concentrated over the juxtanuclear region of the adaxonal Schwann cell cytoplasm (Fig. 2*A* and *B*). At high resolution, the silver grains appeared to be confined to specific regions of some of the Schwann cell profiles. It was apparent that the silver grains were accumulated over regions of cytoplasm containing Golgi apparatus (Fig. 2*C*).

Sites of fucosylation were also determined in developing rat sciatic nerve. After a 2-h labeling interval, silver grains were prominent in the extensive areas of Schwann cell cytoplasm surrounding the emerging myelin, but not in the axons (Fig. 2*D*). With electron microscope autoradiography (Fig. 2*E*), label was found over regions rich in Golgi cisternae and, to a lesser extent, over Schwann cell plasma membrane and outer regions of the myelin sheath. Some label was also associated with unmyelinated fibers. At these early times in both developing and adult nerve, perineurial, endoneurial, and endothelial cells were also labeled.

MOVEMENT INTO MYELIN: In the adult mouse after 1 day of labeling, fucose-tagged protein appeared more widely distributed than at 1 and 4 h (Fig. 3*A*). Silver grains were present over many organelles within the Schwann cell cytoplasm, and along the plasma membrane/basal lamina border. Silver grains were found over outer regions of the myelin sheath and over unmyelinated fibers. At 8 and 18 days after labeling (Fig. 3*B* and *C*), many of the silver grains were associated with the outermost edges of the myelin sheaths. Label associated with the Schwann cell cytoplasm was reduced and was spread over vari-

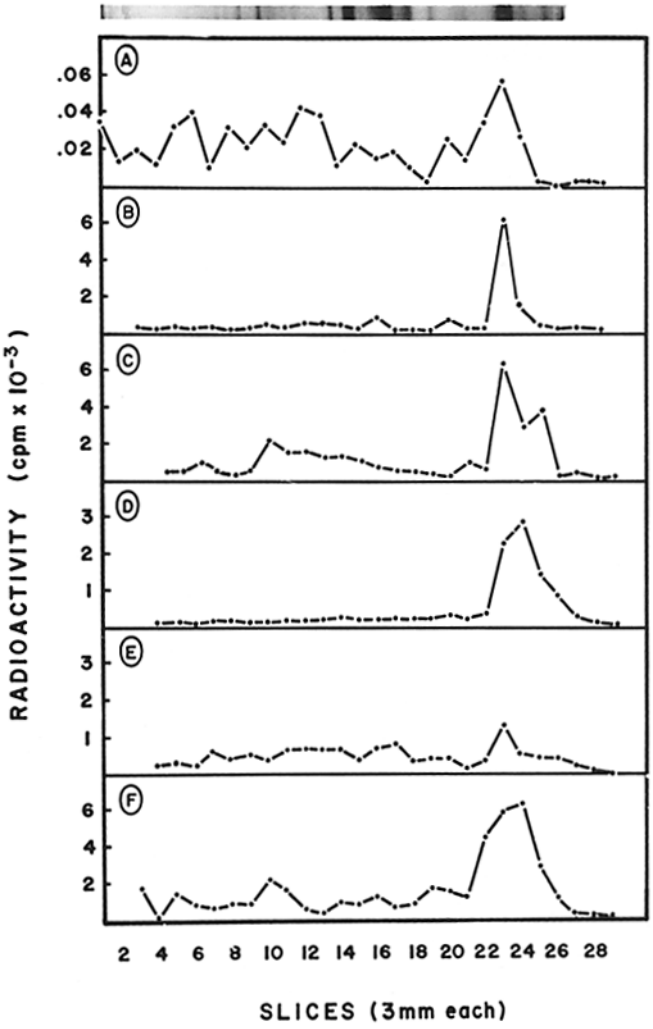

FIGURE 1 SDS gel electrophoresis of proteins prepared after labeling mouse (A-D) and developing rat (E and F) nerves with [^{3}H]fucose in vivo for various times: (A) 1 h, (B) 1 day, (C) 8 days, (D) 72 days, (E) 2 h, and (F) 35 days. Above the graphs showing the distribution of radioactivity is a sample gel electrophoresis pattern run with a 5–18% polyacrylamide gradient, showing the migration of the heavily stained Po protein. The distribution of label in the gels was quantitated after slicing the gels (see Materials and Methods).

ous internal organelles and along the plasma membrane and nuclear envelope. At no time were silver grains found in the Schwann cell nucleus.

After much longer labeling times, 72 days (Fig. 3*E*) and 98 days (Fig. 3*F*), fucose glycoprotein appeared somewhat more diffusely distributed over the myelin. Silver grain deposits associated with other cellular features were further diminished. Careful examination of the distribution of silver grains associated with the larger caliber fibers at 72 days revealed that it was not uniform; fewer grains appeared over innermost portions of the thick myelin sheaths. This observation was more evident in animals labeled later in life (e.g., Fig. 3*F*), where grains remain concentrated over the outer myelin perimeter.

A different pattern of labeling was seen in developing rat peripheral nerve. When a rat pup, 8 days of age, was injected with [^{3}H]fucose and allowed to survive for 5 wk, silver grains repre-

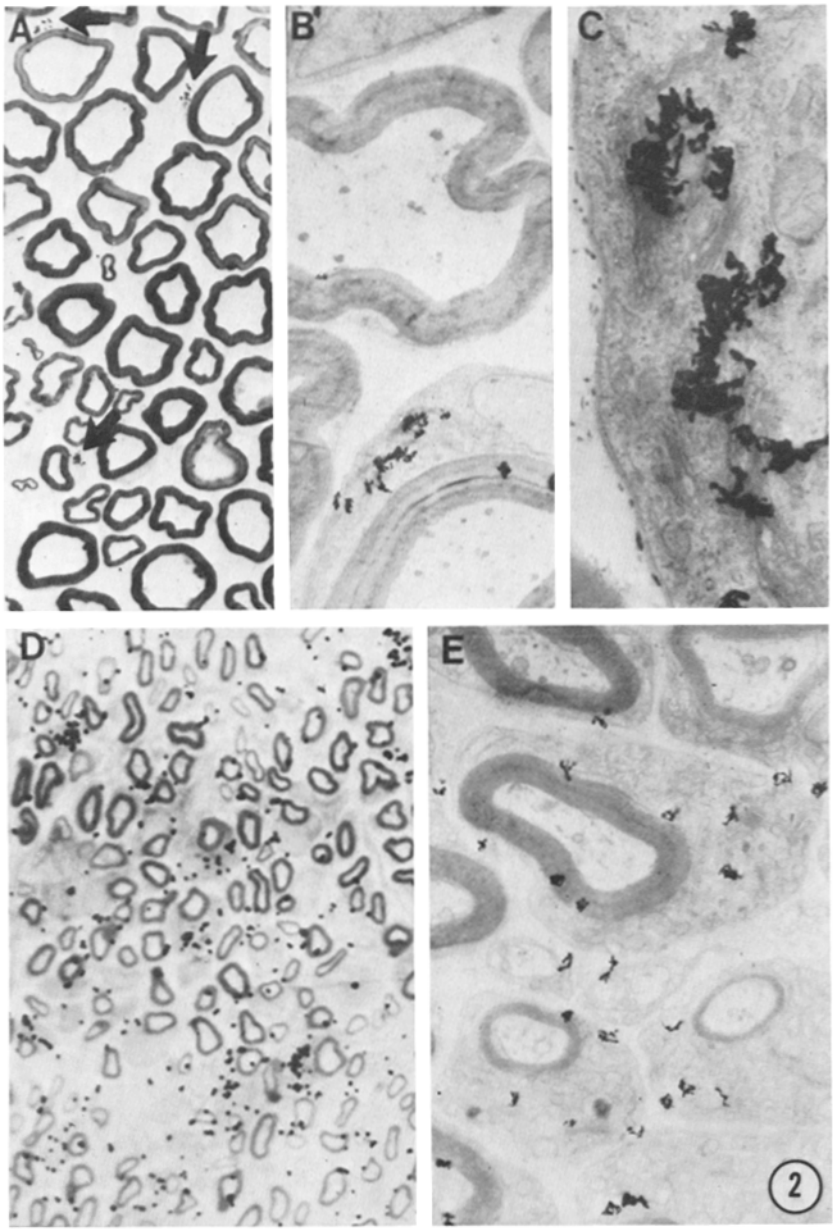

FIGURE 2 Light and electron microscope autoradiographs illustrating sites of incorporation of fucose into glycoprotein after short labeling periods. (*A–C*) Light and electron micrographs of transverse section from nerve of adult mouse, 6 mo, fixed 1 h after injection. (*A*) Grains are present only in three tightly packed regions over Schwann cell cytoplasm (arrowheads). × 1,250. (*B*) Grains are concentrated in juxtanuclear regions. × 5,400. (*C*) These regions contain extensive elaborations of Golgi complex. × 12,500. (*D* and *E*) Light and EM autoradiographs from 8-day rat pup sciatic nerve fixed 2 h after fucose injection. (*D*) Grains are largely clustered over extensive Schwann cell cytoplasm. × 1,875. (*E*) Grains are associated with Golgi complex, plasma membrane, and external myelin. × 8,400.

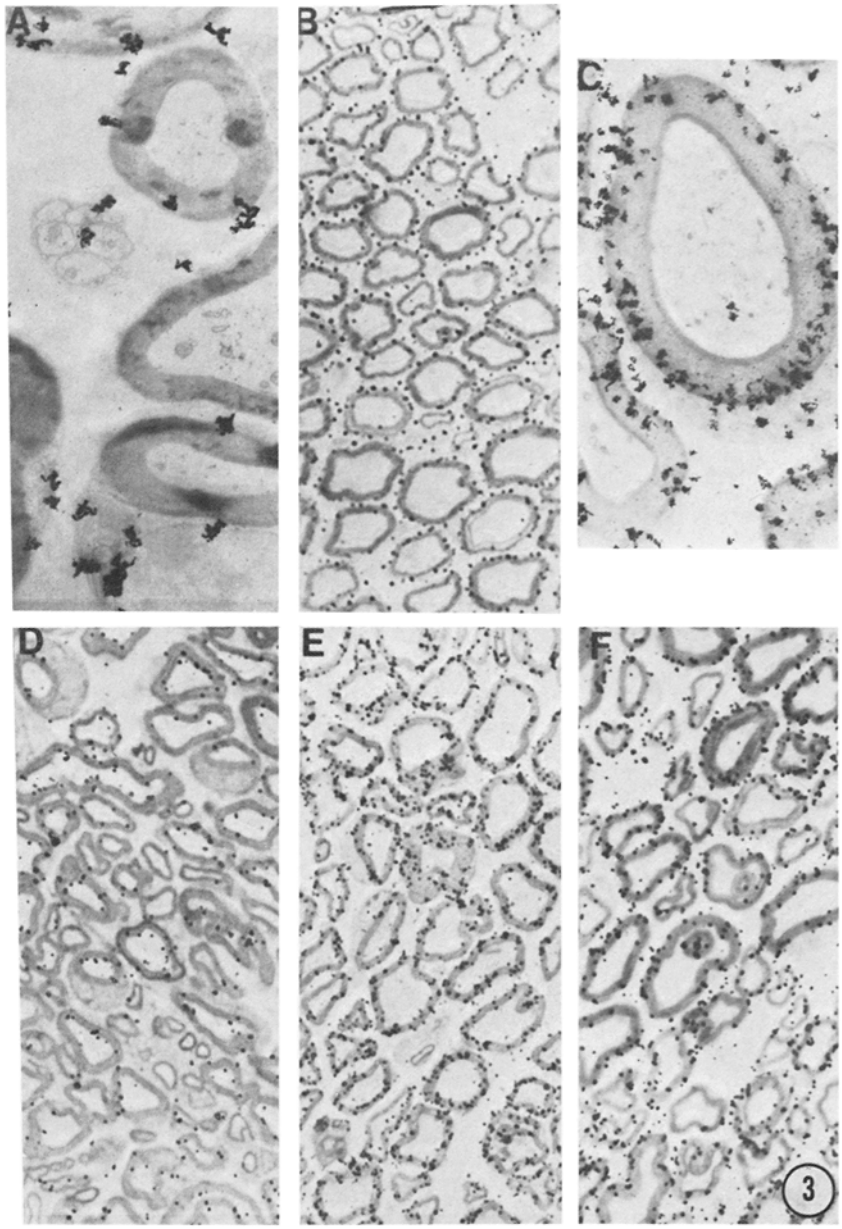

FIGURE 3 Light and EM autoradiographs of mouse and rat sciatic nerve fixed after fucose glycoprotein has moved into the myelin. (*A*) With a 1-day labeling period, adult mouse, 6 mo, grains are over plasma and nuclear membranes, organelles within Schwann cell cytoplasm and unmyelinated fibers. × 6,200. (*B*) With an 8-day labeling period, adult mouse, 4 mo, grains are largely over the outer myelin perimeter. × 1,250. (*C*) With an 18-day labeling period, adult mouse, 4 mo, grains are concentrated over outer myelin layers. × 6,200. (*D*) With a 35-day labeling period, young rat, 6 wk, grains are mainly along the inner myelin perimeter. × 1,250. (*E* and *F*) With 72-day (*E*) and 98-day (*F*) labeling periods, adult mice, 4 and 13 mo, grains are over myelin. × 1,250.

senting glycoprotein were located largely over the innermost portions of the myelin sheath (Fig. 2*D*).

Quantitation of the Movement of Glycoprotein into Myelin

Measurements of silver grain densities over major morphological entities and their junctions were made on animals labeled between 1 and 72 days (Table I). To limit age-related considerations, all of these mice were sacrificed between 10 and 16 wk of age. With a 1-day labeling period, the density of label in Schwann cell cytoplasm was high compared with other major structural features. Junctions of Schwann cell cytoplasm with either extracellular space or myelin were also densely labeled. Very few grains were associated with the myelinated axons and the myelin/axon interface.

After longer labeling periods, especially 18 and 72 days, significant augmentation of the density of silver grains overlying myelin was evident. In contrast, levels associated with Schwann cell cytoplasm and unmyelinated fibers were reduced. Label associated with borders between major structures was likewise reduced.

To study the temporal distribution of labeled glycoprotein across the myelin sheath, grain density levels were computed over discrete bands of 250 nm thickness; subdivision 1 represents the outermost band (10, 15). The results are presented in Table II. At all the times examined, most of the silver grains were accumulated over the outer myelin layers (subdivisions 1 and 2). There was, however, a temporal decrease of label in the outermost lamellae (subdivision 1) and a rise in label over the more inner layers.

Further quantitation was carried out as suggested by Rawlins (33). Histograms illustrating some of the most pertinent findings are presented in Fig. 4. For a young mouse sacrificed 8 days after fucose injection (Fig. 4 A and D), label was concentrated in Schwann cytoplasm and, to an even greater extent, in the outer interfaces of different-sized sheaths. Grains associated with myelin inside this border can be accounted for as being due to scatter from the border. This conclusion, based upon the use of universal curves (34),

TABLE I

Radioactive Glycoprotein Distribution within the Mouse Nerves

	Time after injection			
	1 day	8 days	18 days	72 days
Major Features				
Myelin	1.00 (21%)	0.99 (26%)	1.72 (56%)	2.26 (67%)
Axon	0.12 (2%)	0.10 (2%)	0.13 (2%)	0.22 (5%)
Schwann cell cytoplasm	4.73 (8%)	1.85 (5%)	0.83 (2%)	0.41 (1%)
Unmyelinated fiber	1.71 (2%)	1.41 (1%)	0.78 (1%)	0.18 (0.5%)
Extracellular space	0.66 (24%)	0.66 (20%)	0.38 (11%)	0.29 (6%)
Junctional				
Schwann/myelin	5.62 (11%)	4.27 (13%)	2.20 (10%)	1.30 (5%)
Myelin/extracell	2.54 (17%)	3.40 (16%)	1.98 (10%)	1.30 (6%)
Schwann/extracell	4.14 (10%)	2.05 (13%)	1.07 (6%)	1.03 (4%)
Myelin/axon	0.28 (1.5%)	0.27 (1.5%)	0.42 (2.5%)	0.79 (5%)
UMF/extracell	3.62 (1%)	1.53 (1.5%)	1.00 (<0.5%)	0.42 (0.5%)
Total grains	2,381	2,165	2,053	1,748
Total circles	2,453	1,249	4,213	2,953
Significance	0.001	0.001	0.001	0.001

Analyses of electron microscope autoradiographs are presented for mice sacrificed at the times indicated. Results for each time are from one animal sacrificed at roughly 3 mo of age. All categories except those containing Schwann cell nucleus and other cell types are included. Results are expressed in terms of normalized grain density (15) and percentage distribution of grains, the latter in parentheses. Under junctions, Schwann refers to Schwann cell cytoplasm, extracell to extracellular space, and UMF to unmyelinated fibers. Total grain and total circle refer to the number of silver grains and random circles counted for each time interval. Random circles counts refer to counts made after a grid of 2.5-mm diameter circles, with centers 2.5 cm apart, was placed over the photograph. Significance was based on chi-square analyses.

TABLE II

Radioactive Glycoprotein Distribution across Myelin

	Time after injection			
	1 day	8 days	18 days	72 days
Myelin Subdivisions				
1	2.66	2.58	1.64	1.00
2	0.79	0.83	1.17	1.18
3	0.38	0.40	0.57	1.08
4	0.28	0.19	0.19	0.71
5	0.23	0.05	0.14	0.42
Total Grains	499	543	1,118	1,175
Total Circles	513	309	1,308	880
Significance	0.001	0.001	0.001	0.001

Silver grains and circles overlying myelin sheaths were classed according to the distances of their centers from the outer myelin border (10, 15). Results from fibers of different caliber are grouped together. Values are expressed as a normalized grain density calculated from the totals of grains and circles counted over the myelin. Explanations of total grain, total circle, and significance are as in Table I.

would suggest that, 8 days after the injection, fucose-glycoprotein remains concentrated in the outermost myelin lamellae.

If the mouse, injected at 2 mo, is allowed to survive for 72 days (Fig. 4 B and E), the distribution of grains is changed. Label is concentrated in more inner layers, between the second and fourth HD width from the outer myelin perimeter. When universal curves for solid bands of 4 HD width are superimposed over the histogram (34), it becomes apparent that the label is largely confined to a 500-nm band (3–4 HD) centered about 500 nm from the outer myelin interface. A small but significant labeling occurs over the inner myelin layers and the myelin/axon interface.

If an older mouse, 10 mo, is injected and allowed to survive for 72 days (Fig. 4 C and F), the distribution of grains resembles somewhat that of an animal after an 8-day labeling period. Grains are, however, more concentrated over myelin, although still largely within 330 nm of the outer myelin perimeter. These results suggest that differences in the localization of the label are dependent upon the stage of myelination of the nerve, and upon the time in which the nerve has been labeled. With the smaller caliber fibers (3 + 4 HD) after 72 days, but not 8 days, label appears rather uniformly distributed over the HD bands (results not included).

DISCUSSION

The following discussion will consider the various stages in glycoprotein incorporation into peripheral nerve myelin and relate these to the results on the incorporation of phospholipids (15, 16, and Gould and Buskirk, unpublished observations) and cholesterol. As myelin has in the past served so well as a model for the structure of the plasma membrane, so too here these results on its metabolism are probably important in the light of present knowledge of plasma membrane metabolism. In brief, these results demonstrate that glycoproteins and phospholipid are added into the membrane at distinct sites, and once added, diffuse within the plane of the membrane largely independently of one another. Cholesterol (33) movement within this plasma membrane appears to be too rapid to be ascribed to simple diffusion.

In the present investigation, tritiated fucose was used as a selective label to follow synthesis and subsequent metabolism of the major peripheral nerve protein, referred to as Po (17). Both from counts obtained after slicing the SDS gels and from a fluorographic method (Gould and Singh, unpublished observations), the single major protein labeled by fucose injection into adult mouse and developing rat sciatic nerve was indeed the Po protein. Between 40 and 70% of the radioactive label in the gels (Fig. 1) co-migrated with the Po band. Other higher molecular weight glycoproteins were also labeled. However, there was a temporal increase in radioactivity associated with Po which indicates that it undergoes a somewhat slower turnover than the bulk of these glycoproteins.

Although there is some suggestive evidence to the contrary (14, 28), it is generally believed that myelin proteins are a product of supportive glial cells. Glycoproteins made in the neuronal perikar-

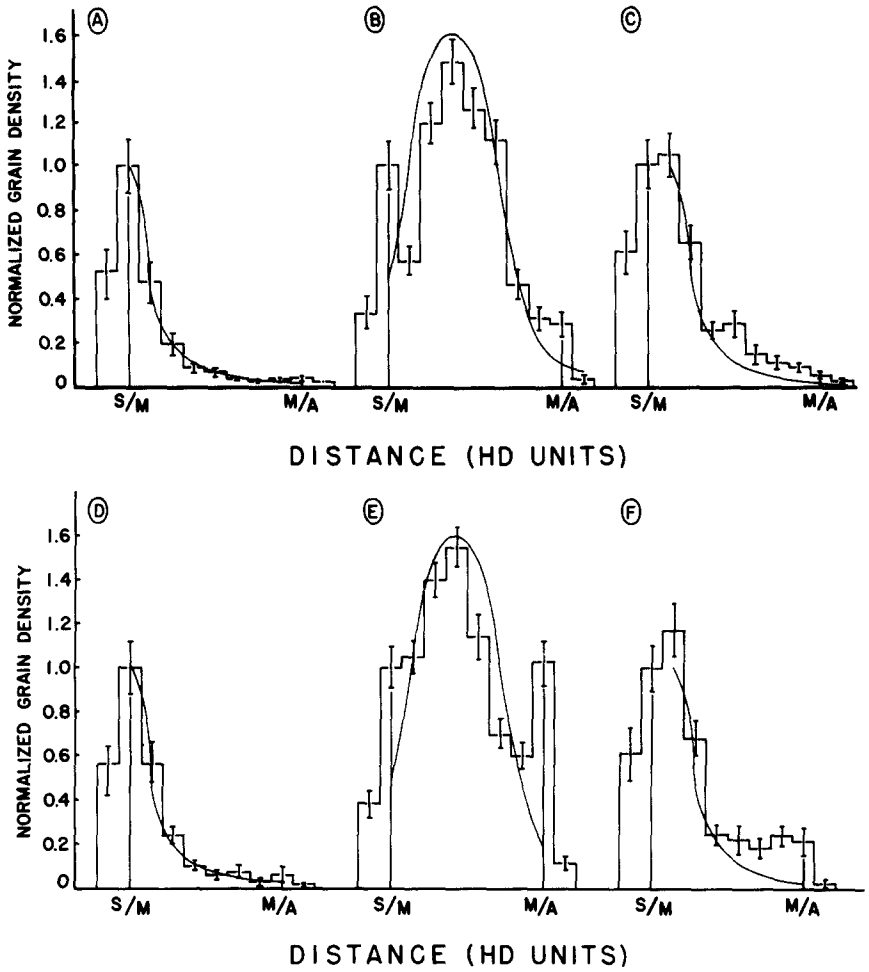

FIGURE 4 Histograms illustrating the density distribution of silver grains across the myelin sheaths of six (D, E, and F) and seven (A, B, and C) HD units width (1 HD unit = 165 nm). The results are shown for mice injected for 8 days (A and D) or 72 days (B, C, E, and F). The animals were sacrificed at 4 (A, B, D, and E) and 12 (C and F) mo of age. The histograms are calculated by a method similar to that described by Rawlins (33), except that the leftmost bar is cumulative for all grains and circles overlying Schwann cell cytoplasm and the right most bar is similarly cumulative for axons. Next to these are bars containing vertical slashes indicating the Schwann/myelin (*S/M*) and myelin/axon (*M/A*) junctions. These have been defined in Materials and Methods. The smooth curves superimposed over the histograms are universal curves taken from the paper of Salpeter et al. (34), and are included to better define the localization of silver grains. The standard deviations were estimated from a formula established by Rawlins (33).

yon are mainly carried down the axonal processes at a fast rate (13), and are delivered to nerve endings and to a lesser degree to the plasma membranes of the nerve processes (5). It would be a horrendous task for the neuron to also provide the vast expanses of internodal myelin sheaths with their proteins as well. Evidence that glial cells along the length of the optic tract of rabbit nerve supply central nerve myelin with major protein constituents has been forwarded by Autilio-Gambetti et al. (3).

In the present study, sites of glycoprotein fucosylation reactions have been localized to regions of juxtanuclear Schwann cell cytoplasm containing Golgi apparatus (Fig. 2*B*, *C*, and *D*), and other Golgi-containing cells seen far less frequently. A similar localization of glycosylation reaction sites has been documented for a variety of tissues by both autoradiographic (4, 27) and biochemical (29, 38) means. Since Schwann cell cytoplasm is the most prominent site of fucose incorporation (Fig. 2*A*), and since Po is the single most signifi-

cantly labeled protein, the Golgi complexes present in juxtanuclear Schwann cell cytoplasm are probably sites where Po is fucosylated (Fig. 5 A).

Other proteins glycosylated in the Schwann cell Golgi apparatus are probably transported to the carbohydrate-rich basal lamina. Evidence for this transfer may be gleaned from Table I, where it is apparent that there is a significant degree of label associated with the unmyelinated fiber/extracellular space (compared with the unmyelinated fiber) and the Schwann cell/extracellular junctions where the basal lamina is situated.

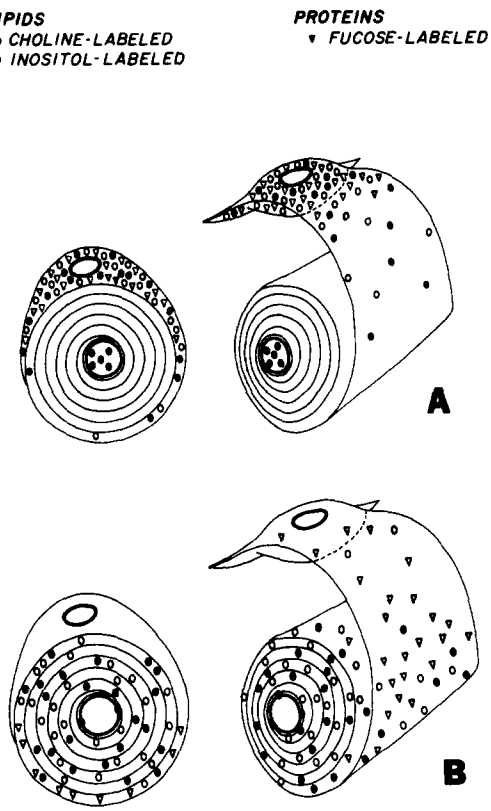

FIGURE 5 Artist's representation of cross-sectional and partly unfolded views of an internode of peripheral nerve myelin. (A) Synthesis: At short periods after injection of choline, inositol, and fucose precursors, lecithin, phosphatidylinositol, and the Po protein are formed in juxtanuclear Schwann cell cytoplasm. Phosphatidylinositol synthesis occurs in axons as well. Once transferred to the outermost myelin lamellae, lipids and proteins begin to diffuse inward in a spiraling path. (B) Movement: Rates of movement of phospholipids through the myelin are much more rapid than those of protein. At times when lipid becomes evenly distributed throughout the sheath, glycoprotein and proteins labeled with [^{3}H]lysine (Gould, unpublished observations) remain restricted in rather defined regions of the sheath.

In the above model (Fig. 5 A), sites of phospholipid formation have also been placed within the juxtanuclear Schwann cell cytoplasm (15, 16). Organelles in which these compounds are made have not been so easily identified as those for glycoproteins, presumably because of the rapid transfer of phospholipids between cytoplasmic organelles (9, 41).

Major sites of synthesis of inositol lipids have been localized in axoplasm (16). Lipids formed at these sites, however, do not seem to penetrate into the myelin sheath (Gould and Buskirk, unpublished observations). It is apparent that little if any glycoprotein fucosylation occurs in the axon. At no time (1 h–98 days) was there any accumulation of silver grains over this structure. This result would extend other observations (8, 19, 23) suggesting that protein synthesis does not normally occur in axons.

Once formed internally, plasma membrane constituents are presumably brought to the surface by some exchange or transport mechanism. Likewise, with myelin, constituents synthesized in organelles in Schwann cell cytoplasm are transported to an exposed surface continuous with myelin. In plasma membrane metabolism, it appears that proteins and glycoproteins are added at different rates, and presumably by different mechanisms (1, 2). It is apparent from the present study that the myelin membrane is no exception. Choline lipids (10, and Fig. 4 A and Table V of reference 15) are certainly transferred into myelin faster than the Po glycoprotein. Cholesterol transfer to myelin also appears to be very rapid (33). If, as for other membrane glycoproteins (27) and secretory proteins (30), Po is indeed transported to the Schwann cell plasma membrane in the inside of small Golgi vesicles, the rate of movement of these vesicles may affect the rate at which glycoproteins are inserted into the plasma membrane. Fusion of these vesicles with plasma membrane would place the carbohydrate moiety of this protein on the outer surface (27). Subsequent diffusion along the plane of the membrane would yield an intraperiod line localization of this moiety, such as has been demonstrated by Wood and McLaughlin (43).

Once associated with the outermost layers of myelin, glycoprotein does not penetrate through the myelin sheath nearly as rapidly as lecithin or phosphatidylinositol. In peripheral nerve myelin, choline and inositol lipids, incorporated into the outer myelin layers, move inward at such a rate that by 4 days they are approximately evenly dis-

tributed throughout the myelin sheaths of even the largest-caliber fibers (15, Gould and Buskirk, unpublished observations). In contrast, fucose-labeled glycoprotein moves very slowly from regions of initial insertion. Even at 14 wk after fucose injection, glycoprotein remains concentrated in outer regions of the large-caliber myelin fibers (Fig. 3*F*).

Once lipid and protein constituents become incorporated into membranes continuous with myelin, they probably diffuse laterally inward through the extensive surface of the compacted myelin. It is well established that the lateral diffusion of phospholipids is in general several orders of magnitude faster than that of membrane proteins (7, 11). Such appears to be the case in myelin as lipids equilibrate over the large expanse of large-caliber myelin fibers much more rapidly than proteins. In the myelin model (Fig. 5 B), newly formed phospholipid molecules entering myelin are rapidly mixed throughout the complete surface of the myelin sheath. Fucose-labeled myelin proteins remain for much longer times concentrated in specific regions of the myelin membrane. Those proteins formed earliest in development are added to the outer layers of the emerging sheath. These are covered, as new membrane is laid down, more rapidly than they diffuse from regions where they were added (Fig. 3*D*). Glycoproteins inserted at a later stage, but while myelin is still actively growing, are concentrated in intermediate layers (Fig. 3*E* and Fig. 4 B and E). Those proteins added to the myelin of mature animals remain concentrated in outer regions of the sheaths of the large-caliber fibers (Fig. 3*F* and Fig. 4 C and F). Some diffusion is occurring because there is a spreading of labeled glycoprotein from a narrow margin after 8 days to more extensive surfaces of membrane by 72 days (Fig. 4). Lateral movement of central nervous system myelin glycoproteins over short distances has been detected by other methods (26).

From these results it can be stated that the myelin membrane acts as a classic fluid mosaic membrane according to the design of Singer and Nicolson (35). Lipid diffusion in the membrane bilayer appears to be a characteristic of lecithin and phosphatidylinositol, although perhaps not of cholesterol (33). Little of the lipid appears to be held back in a permanent interaction with slower moving protein. A fluid myelin membrane could undergo expansion to accommodate a growing axon. Such an elastic picture of myelin would replace the older less dynamic notions, where myelin expansion would involve the slippage of one rigid surface over another.

In summary, it has been demonstrated that the major peripheral protein of myelin, the Po protein (17), is formed in the Schwann cell cytoplasm with fucosylation reactions occurring in the Golgi apparatus. Newly formed glycoprotein transferred from the synthetic sites is added to the outermost regions of the myelin sheath in both developing and mature animals. Diffusion from the sites where glycoprotein is added into the myelin is extremely slow, in the range of one-hundredth the rate of phospholipid movement.

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