Organization of Membrane Proteins in the Intact Myelin Sheath

PYRIDOXAL PHOSPHATE AND SALICYLALDEHYDE AS PROBES OF MYELIN STRUCTURE*

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ELAINE E. GOLDS AND PETER E. BRAUN†

From the Department of Biochemistry, McGill University, Montreal, Quebec, Canada

Pyridoxal phosphate and salicylaldehyde were used as protein-labeling probes to study the organization of membrane proteins in the intact myelin sheath of the cat dorsal column. Both reagents react with protein amino groups to form Schiff's bases which can be reduced with NaBH₄. The relatively membrane-impermeant pyridoxal phosphate labels all proteins of the intact myelin except basic protein. This major protein of myelin is labeled only after loss of membrane integrity. The relatively membrane-permeant probe, salicylaldehyde, was then used to establish that the basic protein is truly located on the cytoplasmic side of the myelin bilayer, and not merely sequestered within the multiple lamellar structure of the sheath. All proteins in the intact myelin are readily labeled by this reagent, with the label distribution pattern identical to that of disrupted myelin fragments. These data suggest a model for myelin structure in which the basic protein is the only major protein component located exclusively on the cytoplasmic side of the membrane (the major period zone of the sheath), with the other major proteins disposed wholly, or in part, in the extracellular half of the myelin bilayer (the intraperiod zone). All proteins, although asymmetrically disposed with respect to membrane sidedness, appear to be randomly distributed throughout the lamellae which comprise the sheath.

Myelin is a specialized plasma membrane with a relatively simple protein composition. It is a multilamellar structure that is spirally disposed around an axon, having both extracellular and cytoplasmic surfaces of the membrane in close apposition (1). The solution properties of the major protein components of myelin, the proteolipid protein and the basic encephalitogenic protein have been well characterized; as yet no enzymatic activity has been ascribed to either of them. It seems likely that their function in the membrane is to structurally maintain the unique morphology of myelin.

Our first objective in delineating the role of myelin proteins was to determine their location within the membrane by using membrane-impermeant surface probes. Poduslo and Braun (2) used lactoperoxidase to catalyze the iodination of accessible tyrosine residues of myelin proteins in the intact membrane. The extensive iodination of myelin basic protein only when the structural integrity of the membrane was mechanically disrupted indicated its location to be the cytoplasmic surface of myelin. In this communication we confirm and extend these observations.

Pyridoxal phosphate was first used as an impermeant membrane probe by Rifkin et al. (3). Subsequently, this reagent has been used as a surface probe of several membrane systems with varying degrees of success (4–8). Pyridoxal phosphate forms Schiff's bases with protein amino groups at physiological pH. The reduction of these complexes with sodium borotritide serves to introduce a tritium label of high specific radioactivity. The membrane impermeancy of pyridoxal phosphate lies in its highly hydrophilic nature and its negative charge. Because its carbonyl group reacts principally with the ε-amino group of lysines, this reagent provides information on the surface accessibility of an amino acid residue other than those residues (tyrosine and histidine) which are labeled by lactoperoxidase-catalyzed iodination. More importantly with regard to a study of the molecular architecture of myelin, pyridoxal phosphate is a much smaller molecule than lactoperoxidase, a protein of molecular weight 78,600. The extracellular spacing between adjacent lamellae of myelin, often referred to as the intraperiod zone, has been experimentally estimated to be 20 to 30 Å in width (9). The diffusion of a molecule the size of lactoperoxidase is known to be very limited in such a 20 to 30 Å space (10). The effect of this restricted diffusion of lactoperoxidase should be to iodinate tyrosine residues on mainly the very outer surfaces of myelin sheaths surrounding individual nerve fibers. Since pyridoxal phosphate is a much smaller molecule, its diffusion in the intraperiod zone should not be as severely restricted; thus, it should penetrate deeper into the myelin sheath where it can label sites not accessible to the larger enzyme.
We selected salicylaldehyde as a membrane-permeant probe because it is highly lipophilic, is similar in size to pyridoxal phosphate, and reacts with amino groups in an analogous manner under the same conditions. Although it has not previously been used as a membrane probe, as far as we are aware, its reaction with lysine residues of cytochrome c has been reported (11).

An important feature of these studies is that we maintain structural integrity of myelin sheaths by carrying out labeling experiments directly on carefully excised segments of the dorsal column from cat spinal cord. In such intact, myelinated nerve preparations only myelin proteins which are located on the extracellular membrane surface, corresponding to the intraperiod zone of the sheath, are accessible to the pyridoxal phosphate. The cytoplasmic membrane surfaces, corresponding to the major period zone become exposed to this nonpermeant reagent when structural integrity is destroyed by mechanical shearing of the dorsal column.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Pyridoxal phosphate was obtained from Sigma Chemical Co., and salicylaldehyde from Aldrich Chemical Co. Trinitiated sodium borohydride was obtained from either Amersham/Searle Corp. or New England Nuclear with a specific activity of 5 to 15 Ci/mmol. All other chemicals were of reagent grade.

**Dorsal Column Preparation and Myelin Isolation**—The dissection of the dorsal column and use of the incubation chamber for labeling intact myelin have been described previously (2). Myelin was isolated by the procedure of Norton and Poduslo (12).

**Membrane Preparations, Intact and Disrupted**—Three different preparations were studied with respect to the labeling of their constituent myelin proteins by pyridoxal phosphate and salicylaldehyde.

1. The freshly dissected dorsal column of the cat spinal cord served as a preparation of intact myelin. The dissection procedure and the use of the incubation chamber for labeling intact myelin have been described previously (2). A 2-cm segment of the cord was normally used; about 1 mg of myelin protein could be isolated from this amount.

2. Disrupted membrane preparations were obtained by mechanically shearing the freshly dissected dorsal column in isotonic Krebs-Ringer bicarbonate buffer (pH 7.6) using light strokes of a loosely fitting Teflon pestle in a hand-held glass homogenizer.

3. Isolated myelin fragments free from other cellular components were prepared by standard myelin isolation procedures (12). The final fraction was suspended in isotonic Krebs-Ringer bicarbonate buffer, pH 7.6.

**Labeling Procedures**—Labeling was carried out in isotonic Krebs-Ringer bicarbonate buffer, pH 7.6. Schiff's basic formation, which is pH-dependent, proceeded better at this pH than in the slightly lower physiological pH range. Both pyridoxal phosphate and salicylaldehyde were prepared in this buffer as a 20 mM stock solution just before use. Salicylaldehyde is not completely soluble in this medium, and must be finely dispersed either by vortex mixing or by sonication of the stock solution. Usually 800 nmol of reagent, a 2-fold molar excess with respect to the amount of lysine in 1 mg of myelin protein, was used. The reaction was allowed to proceed for 10 min at room temperature before reducing agent was added. In the case of the experiments with intact myelin, the incubation mixture containing unreacted reagent was removed from the chamber and replaced with fresh buffer prior to reduction. The disrupted membrane fragments and the purified myelin were reduced by the addition of the borohydride directly to the incubation mixture (1 ml).

Reduction was achieved by adding 10 µl of trinitiated sodium borohydride, freshly prepared in 0.01 N NaOH, to the membrane preparations. A 3-fold excess, with respect to the aldehyde reagent, was found to be adequate. The reaction was complete in 3 min. The labeled dorsal column was immediately rinsed in fresh buffer and myelin was isolated (12). This step served to remove extraneous radioactivity.

**Preparation of Myelin Proteins for Polyacrylamide Gel Electrophoresis**—The myelin isolated from the three experimental preparations was always lysophilized to reduce the loss of protein. Partial delipidation was performed by three extractions with diethyl ether/absolute ethanol (3/2, v/v), using 5 µl/mg of myelin protein. The myelin was suspended in the ether/ethanol by a 30-s sonication in an ultrasonic cleaning bath. This partially delipidated myelin was then solubilized with a 7% concentration of sodium dodecyl sulfate (1% sodium borohydride was found to vary considerably from one commercial preparation to another. It was, therefore, routinely assayed spectrophotometrically. The decrease in optical density at 388 nm, caused by reduction of pyridoxal phosphate, was used to determine the reducing activity. When concentrations identical to those used to label intact myelin preparations were employed, reduction of the carbonyl group was complete in 2 min. The amount of nonvolatile triutum remaining in solution after acidification of a sodium borohydride preparation was also used as a measure of purity.

**Quantification of Myelin Proteins**—The quantification of myelin proteins by spectrophotometric scanning of polyacrylamide gels at 280 nm, using published extinction coefficients for basic protein and proteolipid protein has already been described (2). The results, showing the protein composition of cat spinal cord myelin to be 31% proteolipid protein, 32% basic protein, and 37% remaining proteins, were used to calculate specific activities. It was found by dry weight analysis that 92 µg of cat spinal cord myelin protein are equivalent to 100 µg of bovine serum albumin by the Lowry protein determination. An appropriate correction factor was, therefore, applied to the calculations of specific activity. The specific activities of the myelin proteins could not be averaged from one experiment to another because of variables such as the size of the dorsal column and the reducing activity of the sodium borohydride. Data pooled from several experiments are, therefore, shown as a percentage distribution of radioactive activity for each protein group. This method of data presentation permitted us to obtain some information about the labeling of minor protein

*The abbreviations used are: SDS, sodium dodecyl sulfate; PAS, periodic acid-Schiff.
components for which specific activities could not readily be determined.

RESULTS

Characteristics of Labeling Reaction—Our first objective was to characterize the reaction of myelin proteins with pyridoxal phosphate. The possibility that the reactivities of myelin proteins depend on the concentration of pyridoxal phosphate or on the reaction time had to be examined before meaningful comparisons could be made between myelin with one side and both sides of the membrane exposed. One of the characteristic properties of myelin is that it does not form perfectly resealed vesicles when isolated. Consequently, in isolated myelin fragments proteins on either side of the membrane are able to react with pyridoxal phosphate. Therefore, we determined reaction conditions on isolated myelin fragments in order to establish the extent to which all myelin proteins could be pyridoxylated.

The distribution of pyridoxal phosphate amongst the protein groups should correspond approximately to the lysine distribution in these groups, as shown in Table I. Also shown is the distribution of the salicylaldehyde label amongst the myelin protein groups. This distribution is not identical to that of pyridoxal phosphate, which may reflect the hydrophobic character of salicylaldehyde and possibly a greater affinity for proteins which are in a more hydrophobic environment than the basic protein.

The actual amount of pyridoxal phosphate (13 nmol) which is covalently bound to proteins in myelin fragments after reduction with borotritide, in a typical experiment, corresponds to about 3% of the total lysines (440 nmol) present in 1 mg of total protein. Similarly, 1% of the lysines are modified by salicylaldehyde. The extent of incorporation, measured in terms of specific radioactivities, can be increased when the ratio of pyridoxal phosphate to protein is increased (Fig. 1). However, the relative distribution of label amongst the three groups of proteins remains constant over the complete range of concentrations tested (see also Fig. 2, Bars 1, 3, and 4). For most experiments 800 nmol of pyridoxal phosphate/mg of protein was an adequate ratio.

The membrane surface area in the dorsal column which is accessible to pyridoxal phosphate was estimated to be about 6% of that area which is accessible in isolated myelin fragments. This was done by comparing the specific radioactivities of the labeled proteins in each experiment (Fig. 6a). Thus, the possibility had to be considered that the effective ratio of pyridoxal phosphate to accessible sites in the intact myelin sheath was as much as 15 times greater than that in fragmented myelin. In order to demonstrate that comparisons between the two preparations are valid, we subjected myelin fragments to a 30-fold greater amount of pyridoxal phosphate. A comparison of Bars 4 and 5 in Fig. 2 illustrates that the distribution of the label among six categories of myelin proteins remains unaltered under these conditions. Bars 1 and 6 establish that a large increase in the borohydride concentration has no effect on label distribution.

A time course study of the labeling reaction with pyridoxal phosphate revealed that myelin proteins are maximally labeled in 10 min. A comparison of Bars 1 and 2 in Fig. 2 illustrates that the extent of labeling of myelin fragments with pyridoxal phosphate at 10 and 60 min is identical and that the distribution of label among the different categories of proteins is also identical.

The isolation procedure of purified myelin fragments normally requires about 5 hours to complete. This relatively long period of time, as well as the severely hypotonic conditions during part of the procedure could conceivably result in distortions of the molecular structure of the membrane. We attempted to assess this by comparing the pyridoxylation of the various myelin protein before and after the myelin fragments were isolated. Freshly dissected dorsal column, crudely fragmented by shearing for several seconds in a homogenizer under isotonic conditions represented the zero time fraction; myelin isolated from a portion of this fraction represented the final preparation. A comparison of Bars 5 and 7 in Fig. 2 indicates that all categories of myelin proteins are similarly labeled.

Problems Encountered With Borotritide Treatment of Myelin Proteins—The use of tritiated sodium borohydride was always resulted in some incorporation of radioactivity into the myelin proteins in the absence of pyridoxal phosphate or any other labeling agent. Consequently, a control sample to which no pyridoxal phosphate had been added was always carried through the experimental procedure. Calculations of specific activities and percentage distribution of radioactivity were done after the background due to borotritide alone had been subtracted. The amount of background radioactivity varied between zero and 25% among the different lots of borotritide.

![Graph](http://www.jbc.org/)

**Fig. 1.** Labeling of myelin proteins in isolated myelin fragments as a function of the pyridoxal phosphate concentration. ◆, basic protein; ○, proteolipid protein; ▲, remaining protein.
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The distribution of radioactivity due to pyridoxal phosphate under a variety of conditions. PM, proteolipid protein; BP, basic protein; the remaining proteins are subdivided arbitrarily according to molecular size as shown in Fig. 3. H, the highest molecular weight group (75,000 to 90,000); W, the Wolfgram fraction (60,000 to 70,000); M, the medium size group (40,000 to 55,000); IP, the two protein bands (24,000) observed between the proteolipid and basic proteins. These molecular weights are only approximate, and were determined by polyacrylamide gel electrophoresis. Bar 1, isolated myelin fragments labeled with 0.8 amol of pyridoxal phosphate for 10 min, followed by a 3-min reduction with a 3-fold molar excess of NaB$^3$H$_4$; Bar 2, same as Bar 1, but with 24 min incubation with pyridoxal phosphate; Bar 3, same as Bar 1 except that the pyridoxal phosphate is 2.4 amol; Bar 4, same as Bar 1, but with 24 amol of pyridoxal phosphate; Bar 5, same as Bar 1; Bar 6, same as Bar 1, but with a 100-fold molar excess of NaB$^3$H$_4$; Bar 7, same as Bar 1 except that a dorsal column homogenate was used as the starting material.

The distribution of radioactivity due to pyridoxal phosphate labeling of myelin proteins, however, was constant after the background had been subtracted. A pretreatment of isolated myelin fragments with unlabeled borohydride, followed by washing to remove unreacted borohydride, and then normal reaction with tritiated borohydride resulted in a 45% reduction of background radioactivity and no change in pyridoxal phosphate labeling patterns.

Some incorporation of tritium from sodium borotritide into myelin proteins is possible by reduction of disulfide bonds, aldehydes, or carbohydrate moieties; however, none of these reactions should be extensive because of the low concentration of borohydride employed (2.4 mM). Most of the background radioactivity, we believe, is due, not to sodium borotritide, but to tritium incorporation from impurities present in the commercial preparations. This conclusion is supported by the following observations. (a) A pretreatment with unlabeled borohydride did not entirely eliminate the background; (b) background levels were the highest when sodium borotritide with the lowest reducing activity and highest acid-soluble blanks was used; conversely, when borotritide with the highest reducing activity and lowest acid-soluble blank was used, the background was negligible; (c) background radioactivity was not due to labeling of myelin proteins by endogenous carbonyl compounds, as the addition of acetone to controls and to pyridoxylated samples not only increased the "background," but also changed the percentage distribution of radioactivity due to pyridoxal phosphate incorporation into the various myelin proteins.

Comparison of Intact Myelin with Myelin Fragments Labeled by Pyridoxylation—Once we had established standard conditions for pyridoxylation of myelin proteins and had shown that the variations in the procedure introduced by the use of intact myelin would not result in a change of labeling patterns, we could then attribute differences between intact myelin and isolated myelin fragments to the asymmetric distribution of proteins within the membrane. A protein located on the cytoplasmic side of the membrane would not be labeled in the intact myelin sheath but would be labeled in either isolated myelin fragments or in the dorsal column homogenate. A protein located on the extracellular surface of the membrane, or one which spanned the bilayer would be labeled under all three sets of conditions.

Spectrophotometric scans of polyacrylamide gels containing myelin proteins with superimposed distribution profiles of tritium incorporation into the three different preparations are shown in Figs. 3, 4, and 5. In both the homogenate of the dorsal column and isolated myelin fragments (Figs. 3 and 4), all protein groups are labeled, but the predominant peak of radioactivity corresponds to the basic protein band. Although the large number of minor components in the high molecular weight area of the gel are not completely resolved, most, if not all, of the protein bands are labeled. Included in this group of labeled, high molecular weight proteins is a major peak of radioactivity which is coincident with a positive PAS-stained zone on the gel, and which corresponds in its electrophoretic mobility to the glycoprotein which has been reported by Quarles et al. (18) and Poduslo et al. (19). However, because of the multiplicity of Coomassie blue staining bands in this region of the gel it is not possible to state with certainty that it is the glycoprotein which is so prominently labeled. Preceding the basic protein band in the gel is a shoulder of unknown origin which also has some tritium associated with it. This component is not always seen, and it could be a lipid-SDS complex which migrates close to the gel front, or it could be one of the lower molecular weight forms of the proteolipid protein as suggested by Chan and Lees (20).

When a comparison is made between intact myelin (Fig. 5) and either the isolated myelin fragments (Fig. 4) or the dorsal column homogenate (Fig. 3), the obvious and only major difference is the absence of label from the basic protein band of intact myelin. This is also readily apparent in Fig. 6a, in which the specific activities of the pyridoxal phosphate-labeled basic protein, proteolipid protein, and remaining proteins (as a group) of intact myelin and of isolated myelin fragments are shown. The lower specific radioactivity of the intact myelin (about 6% of that in myelin fragments) is a reflection of the smaller surface area available to the label in the dorsal column.

Although no distinct peak of radioactivity is associated with the basic protein of intact myelin (Fig. 5), some tritium is distributed over a broad zone in this region of the gel. We surmised that pyridoxal phosphate is not completely impermeant to myelin, and tested this by incubating a dorsal column with pyridoxal phosphate for 1 hour, instead of the usual 10 min. This resulted in a greatly enhanced labeling of basic...
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Fig. 3. Densitometric scan at 555 nm of Coomassie blue-stained myelin proteins on a polyacrylamide gel, showing the distribution of tritium (●—●) due to labeling by pyridoxal phosphate. A homogenate of the dorsal column was the starting material for the labeling reaction. PLP, proteolipid protein; BP, basic protein; the remaining proteins are divided into subgroups according to molecular size, H, W, M, and IP (see Fig. 2).

Fig. 4. Densitometric scan at 555 nm of Coomassie blue-stained myelin proteins separated on a polyacrylamide gel after labeling myelin fragments with pyridoxal phosphate. The distribution of tritium is shown (●—●). PLP, proteolipid protein; BP, basic protein.

Fig. 5. Densitometric scan at 555 nm of Coomassie blue-stained myelin proteins on a polyacrylamide gel after labeling a dorsal column (intact myelin) with pyridoxal phosphate. ●—●, tritium distribution; PLP, proteolipid protein; BP, basic protein.

protein, approaching 50% of that in the readily labeled proteolipid protein. However, preincubation of another dorsal column for 50 min in buffer, followed by the usual 10 min incubation with pyridoxal phosphate, produced results which were identical to the labeling experiment with pyridoxal phosphate present for the full 60 min. This strongly suggested that the labeling of basic protein during the longer incubations is mostly due to the deterioration of the myelin sheath and possibly only to a small extent to the permeancy of the reagent. This observation is strengthened by the appearance in the gels of low molecular weight peptides which arise from proteolysis of the myelin proteins, products which are not observed when the labeling experiment is restricted to 10 min.

Labeling of Intact Myelin and Myelin Fragments with Salicylaldehyde—The negligible labeling by pyridoxal phosphate of the basic protein in intact myelin can be explained in two ways: (a) the protein is located on the cytoplasmic surface of the membrane bilayer matrix, or (b) the protein is located only in the innermost lamellae of the multilamellar myelin sheath, regardless of its disposition in the bilayer. Pyridoxal phosphate can diffuse in the intact dorsal column not only around the bundles of myelinated axons but also between adjacent lamellae of each individual myelin sheath. i.e., in the intraperiod zone. Since short incubation periods (10 min) with intact myelin must be used to avoid the proteolysis that occurs over longer periods of time, the diffusion of pyridoxal phosphate in the intraperiod zone, between the lamellae of the myelin sheath, will necessarily be limited to the outermost layers. Thus, short labeling periods will result in preferential labeling of only the outermost layers of the intraperiod zone.

In order to discriminate between these two possibilities, we sought an analog of pyridoxal phosphate, of similar molecular dimensions, which would be membrane-permeant and would label the basic protein in intact myelin if this protein is on the cytoplasmic surface of the membrane. On the other hand, if the protein is located only in the innermost lamellae of the sheath, on the extracellular membrane surface, then it should be labeled no more extensively by the analog than by pyridoxal phosphate in short incubations because of the similar diffusion properties of the two reagents. Furthermore, if the myelin proteins are uniformly distributed throughout the lamellae (although asymmetrically distributed with respect to membrane sidedness), then labeling of intact myelin and isolated myelin fragments with a membrane permeant probe should produce identical labeling patterns.

Pyridoxal seemed, at first, to be an ideal choice for a membrane-permeant probe of similar size and specificity to pyridoxal phosphate. However, like pyridoxal phosphate, it is still a fairly hydrophilic reagent which is only slightly soluble in apolar solvents such as ether and chloroform/methanol. A much more suitable reagent is salicylaldehyde, which also forms Schiff's bases with protein amino groups. Although it is very soluble in ether and benzene and only sparingly soluble in water, it is still reactive with polar lysine residues as evidenced by its complete reaction with all the lysine residues in cytochrome c (11). Accordingly, the labeling pattern was
established by incubating isolated myelin fragments with salicylaldehyde under the standard conditions used for pyridoxal phosphate labeling. As expected, the amount of salicylaldehyde incorporated into total myelin proteins was approximately one-third the amount of pyridoxal phosphate incorporated under identical conditions because salicylaldehyde is less reactive than pyridoxal phosphate in Schiff's base formation. Profiles of radioactivity associated with proteins in the densitometric scans of gels (not shown) are very similar to those in Fig. 4, and the percentage distribution of label among the various protein groups is only slightly different. Proteins labeled with salicylaldehyde in the intact myelin have an average specific activity 12% of that found in myelin fragments (Fig. 6b). This value differs only by a factor of 2 from that determined by comparing pyridoxal phosphate labeling of intact myelin and isolated myelin fragments, and the specific activity distribution of these three protein groups is nearly the same for both membrane preparations. Figure 7b illustrates this comparative profile, expressed as percentage distribution of specific radioactivities for the two major proteins, as well as for subgroups of the remaining proteins. Thus, the similar labeling of the basic protein in the intact as well as the fragmented myelin, taken with the pyridoxylaytion experiments described above, is strong evidence for the presence of this protein on the cytoplasmic surface of the membrane, and for its distribution throughout the myelin sheath.

**DISCUSSION**

The fairly good agreement between the pyridoxal phosphate labeling pattern of myelin proteins with the distribution of lysines among these proteins (Table I) is a good indication that all of these proteins, or portions of them, are about equally accessible to the reagent in myelin fragments. Therefore, the fragmented membrane preparation serves as a control in which a representative labeling of all protein types occurs. Small observed differences can be accounted for by the anticipated differences in reactivity among lysine side chains. Salicylaldehyde, too, labels all proteins in myelin fragments (Figs. 6b and 7b; Table I); however, the distribution of label among these proteins suggests that, perhaps because of its greater lipophilic character, it favors the integral (intrinsic) membrane proteins to the peripheral (extrinsic) basic myelin protein.

The labeling by pyridoxal phosphate of all proteins except the basic protein in the intact myelin sheath of the dorsal column clearly suggests two possible sites at which the basic protein might be located: (a) it is present at the inner, cytoplasmic surface of the membrane where it is not accessible to the impermeant pyridoxal phosphate; or (b) it is present on only the innermost lamellae of the lamellar myelin sheath, relatively inaccessible to pyridoxal phosphate because of the time required for the reagent to diffuse in the intraperiod zone. Our earlier experiments with lactoperoxidase-catalyzed iodination (2) suggested the first of these two possibilities but did not completely eliminate the second, since lactoperoxidase is excluded from the intraperiod space of the sheath because of its comparatively large size.

This problem was resolved in the present experiments with salicylaldehyde as probe. In addition to labeling all myelin protein in much the same way as pyridoxal phosphate, salicylaldehyde, because of its permeancy to the membrane also labels the myelin basic protein in the intact membrane (Figs. 6b and 7b). If the basic protein molecules were located on only the innermost lamellae, deep in the intraperiod zone of the sheath, the salicylaldehyde should prove to be no more effective than pyridoxal phosphate in labeling them since both reagents are of comparable molecular dimensions and should diffuse between the lamellae at comparable rates. Because salicylaldehyde extensively labels the basic protein in the intact myelinated dorsal column, we conclude that this protein is, indeed, located on the cytoplasmic surface of the membrane which constitutes the major period zone of the lamellar sheath. In this regard, it is of interest that Herndon et al. (21), using immunoelectron microscopic techniques, have reported that intact myelin is unable to react with antibodies to the basic protein and that disruption of the sheath was necessary to expose this protein. In addition, Wood and Moscarello (22) using 4,4'-disothiocyano-2,2'-dinitrostilbene disulfonic acid as a probe for the molecular organization of myelin fragments, reported that the basic protein is less reactive than the proteolipid protein to this reagent.

**Fig. 6 (left).** A comparison of the specific radioactivities of myelin proteins labeled by pyridoxal phosphate (a) and salicylaldehyde (b) in isolated myelin fragments (open bars) and in the intact myelin sheath (hatched bars). PLP, proteolipid protein; BP, basic protein; Rest, the remaining proteins.

**Fig. 7 (right).** Histogram showing the distribution of tritium among myelin proteins after labeling isolated myelin fragments (open bars) and the intact myelin sheath (hatched bars) with pyridoxal phosphate (a) and salicylaldehyde (b). PLP, proteolipid protein; BP, basic protein. The subdivision of myelin proteins is described in Fig. 2.
localization of these proteins, however, could not be determined from these experiments.

The disposition in the membrane of proteins other than the basic protein is less clearly defined. Some observations, however, can be made. Fig. 7a represents an amalgamation of data from four experiments of the type shown in Fig. 6a, presented as a distribution profile of label incorporated into six categories of myelin proteins based on molecular size. Predicated on the premise that all proteins which are labeled in the intact membrane by pyridoxal phosphate must possess some reactive sites at the extracellular surface, we reasoned that the distribution profile should be quite different for the fragmented membrane if some proteins span the bilayer and have at least as many reactive sites at the cytoplasmic surface as well. On the other hand, if these various proteins are accessible only at the extracellular surface of the bilayer, exposure of cytoplasmic surfaces to the labeling reagent will have little or no effect on the distribution profile. In Fig. 7a, the label in the basic protein accounts for approximately 50% of the total label incorporated into myelin fragments. Thus, elimination of this protein from the histogram (because it is not significantly labeled in the intact membrane) produces a 2-fold upward shift of label distribution amongst proteins of myelin fragments, resulting in a histogram (not shown) which differs less than 30% from the histogram for the intact membrane. Although this may be a significant difference, there is no evidence for the 100% or greater difference we anticipated if these proteins possess equivalent numbers of reactive sites on both sides of the bilayer. We conclude from this that these proteins may span the membrane, but if they do, they possess fewer reactive sites at the cytoplasmic surface than at the extracellular surface.

The accessibility to membrane surfaces in the intraperiod zone of intact myelin is limited by the diffusion rate of the reagent; consequently labeling of proteins in this intact preparation during the relatively short incubation period occurs mainly in the outer lamellae of the sheath. In isolated myelin fragments, on the other hand, all regions of the sheath should mainly in the outer lamellae of the sheath. In isolated myelin fragments, on the other hand, all regions of the sheath should be more or less equally accessible to the labeling reagent. Therefore, in Fig. 7b we have presented the combined data from two experiments, illustrating that the salicylaldehyde labeling pattern of six protein groups is the same, within experimental error, for myelin fragments as for the intact membrane. Since only 12% of the available surface area of myelin is available in the intact sheath as compared to the isolated fragments in a 10-min incubation (Fig. 6b), the identical labeling patterns seen in Fig. 7b would not be expected if certain types of protein were located to a greater extent in the outer myelin lamellae and others were located to a greater extent in the inner lamellae. We conclude, therefore, that the proteins of myelin are distributed randomly throughout the sheath. This view is in accord with recent observations that the lipid matrix of myelin is fluid (23) and that at least some of the constituent proteins are capable of translational diffusion within this bilayer (24).

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