Examination of brain myelin proteins by sodium dodecyl sulfate-gel electrophoresis followed by fluorography clearly showed that both proteolipid protein (PLP) and DM-20 were acylated 24 h after the intracerebral injection of 30-day-old rats with \(^{3}H\)palmitic acid. The radioactivity associated with PLP remained after purification, re-electrophoresis, and fluorography. Most of the radioactivity associated with PLP was removed when the gels were treated with hydroxylamine and then fluorographed, indicating that fatty acids were bound to PLP by ester linkage. Cleavage of purified PLP with methanolic sodium hydroxide readily released almost all protein-bound radioactivity. Thin layer chromatography of this material on both silver nitrate and reverse-phase plates provided evidence that most of the radioactivity co-migrated with methyl palmitate (77%) and methyl stearate (19%); however, some radioactivity was associated with methyl oleate (4%). Gas-liquid chromatography of the fatty acids associated with PLP distinctly revealed the presence of methyl palmitate and a detectable peak of methyl stearate.

Folch and Lees (1) first described a chloroform:methanol (2:1, v/v)-soluble protein fraction in bovine white matter which was designated proteolipid protein. Subsequently, Stoffyn and Folch-Pi (2) converted PLP into a water-soluble form of protein called apoprotein, which was virtually devoid of lipids. The apoprotein was shown to contain 2-3% fatty acids which were released only after treatment of the apoprotein with alkali, suggesting that the fatty acids were linked to the apoprotein by ester linkages (3). Gagnon et al. (4) purified a protein from human brain myelin by chromatography on Sephadex LH-20 resin and designated this protein N2. The amino acid composition of N2 was virtually identical to that of apoprotein, and N2 was also found to contain 2% fatty acids. Nussbaum et al. (5) isolated PLP from rat brain myelin by gel electrophoresis in sodium dodecyl sulfate according to the technique of Waehneldt (6), and determined the N-terminal sequence as well as the sequence of 11 tryptic peptides of PLP (7). One of the tryptic peptides was suggested to contain bound fatty acids (7). Subsequently, a homogeneous preparation of PLP from rat brain myelin was isolated by SDS-gel electrophoresis, chemically characterized, and used for the production of precipitating antibodies (8). Myelin PLP was shown by immunofluorescence to be exclusively localized to oligodendrogial cells and the myelin sheath in the central nervous system (8). In addition, the same preparation of PLP was also found to contain a small amount of bound fatty acids (8). In this context, we can now report that both the PLP and DM-20 (9) of rat brain myelin are readily labeled 24 h after intracerebral injection of \(^{3}H\)palmitic acid. Proteolipid protein was purified to homogeneity and chemically characterized, and the linkage of \(^{3}H\)palmitic acid to PLP by an ester bond was established.

### MATERIALS AND METHODS

The reagents used for SDS-polyacrylamide gel electrophoresis and fluorography were obtained from Eastman and Bio-Rad. Soluene-350, Insta-Gel, and 2,5-diphenyloxazole were purchased from Packard Instrument Co., 2’7’-dichlorofluorescein and fatty acid-free bovine serum albumin from Sigma, precoated Silica G plates from Fisher, and sialinized reverse-phase precoated plates from Brinkmann Instruments. MDPF was provided by Dr. M. Weigele. The 6-foot glass liquid chromatography column (2-mm internal diameter) containing 10% diethyleneglycol succinate on 80/100 Supelcoport and fatty acid methyl esters were purchased from Supelco, Inc., Bellefonte, PA. Omnifluor and [9,10-\(^{3}H\)]palmitic acid (11.8 Ci/mmol) were purchased from New England Nuclear. After evaporation of radioactivity under nitrogen, \(^{3}H\)palmitic acid (25 mCi) was suspended in 3% (w/v) fatty acid-free bovine serum albumin (1 ml). The animals (60-day-old Sprague-Dawley rats) were lightly anesthetized with ether, injected intracerebrally with 2 mCi of \(^{3}H\)palmitic acid, and killed 24 h later, and the myelin was isolated as described previously (10). The yield of dry myelin was 30 ± 0.7 mg (S.D.)/brain. The results presented in this paper are the means of at least 4 separate experiments.

**Removal of Lipids from Myelin**—Lipids were removed by lyophilization of myelin (50 mg) by successive extractions at 4°C with ether:ethanol (3:2, v/v) and ether (10). The resulting pellet was dissolved at a concentration of 2.5 mg of protein/ml, in 10 mM borate buffer (pH 9.0) containing 1% (w/v) SDS, 1.5% (w/v) dithiothreitol, and 8% (w/v) sucrose. The protein solution was heated for 2 min in a boiling water bath and stored in small aliquots at ~80°C. Protein concentration in all cases was determined by the method of Lowry et al. (12) using bovine serum albumin as a standard. The radioactivity was determined by liquid scintillation spectrometry and the counting efficiency was determined by internal standardization.

**Preparation of Proteolipid Protein-enriched Fractions**—The PLP-enriched fraction from dry myelin (200 mg) was prepared according to the procedure of Gonzales-Sastre (13) as modified by Nussbaum et al. (5). The proteolipid protein-enriched precipitate was suspended in 10 mM borate buffer (pH 9.0) containing 1% SDS and 1.5% dithiothreitol. The proteins were solubilized by homogenization in a Teflon-glass homogenizer, followed by heating in a boiling water bath for 2 min. The resulting opalescent solution was centrifuged at 10,000 x g for 30 min, and the clear supernatant was used to determine the protein concentration and the radioactivity.

**Conjugation of Myelin Proteins with MDPF and Purification of PLP**—Lipids were removed from dry myelin (225 mg) as described above. The pellet was dried under N\(_2\), and the proteins were immediately dissolved in a 30 mM borate buffer (pH 9.0) containing 5% (w/v) SDS at a concentration of 2.5 mg of protein/ml. Myelin proteins were coupled with MDPF (11, 15) and separated by semipreparative disc gel electrophoresis (1.25 mg of protein/gel), in a discontinuous SDS gel system (14, 15). The fluorescent bands corresponding to PLP

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\(^{1}\) H. C. Agrawal, unpublished observations.
were excised and eluted electrophoretically (6 gel sections/tube) into dialysis tubing (previously cleaned by boiling in 1% SDS and 1% 2-mercaptoethanol) until the gels were devoid of fluorescence. The PLP was dialyzed against 0.1% SDS containing 0.01% 2-mercaptoethanol (1 dialysis bag/500 ml of buffer, with 4 changes of buffer in 24 h). After the concentration of dithiothreitol was adjusted to 0.1%, an aliquot of the PLP was lyophilized for the determination of the purity of PLP by SDS-slab gel electrophoresis (11). In order to reduce the consumption of SDS, because it inhibited the cleavage of fatty acid from PLP with methanolic NaOH and interfered with the determination of amino acid composition and NH2-terminal sequence, the remaining retentate was dialyzed against 0.001% SDS (1000 changes in 24 h) and lyophilized. As a background control, eluates from lanes of protein were treated under identical conditions. These preparations were used for amino acid analysis, determination of NH2-terminal sequence, and cleavage of bound fatty acids from PLP.

**SDS-Slab Gel Electrophoresis—**Total myelin proteins (72-100 µg), proteins in the PLP-enriched fraction (100 µg), and purified PLP (40-73 µg) were separated in two discontinuous SDS systems (11, 14). Portions of the gels were infiltrated with 1 M hydroxylamine at pH 6.6 (200 ml/gel) with gentle shaking at room temperature for 24 h (17). These gels were thoroughly washed with water (200 ml/gel) 2 times for 1 h with shaking. They were then fluorographed using the techniques of Bonner and Laskey (18). Other portions of the gels (unstained as well as stained) (9) were infiltrated with dimethyl sulfoxide:2.5-diphenyloxazole and fluorographed as described (18). Similarly, portions of both the hydroxylamine-treated and untreated gels were infiltrated with an aqueous solution of 1 M sodium salicylate and fluorographed as described by Chamberlin (19). The protein bands corresponding to PLP and DM-20 were excised from the stained gels and radioactivity was determined by liquid scintillation spectrometry as described (20). The counting efficiency was 38%.

**Amino Acid Analysis and Determination of NH2-terminal Sequence of Purified Myelin PLP—**Three samples of myelin PLP (500 µg each) were hydrolyzed with 1 ml of constant boiling 6 N HCl in vacuo at 110 °C for 18 h. The amino acids in the hydrolysate were analyzed with a Beckman 119 CL automatic amino acid analyzer equipped with an integrator. Cystine was determined after performic acid oxidation of purified PLP (21). The NH2-terminal amino acids of purified PLP were determined by Edman degradation (22) using a Beckman automatic amino acid sequencer.

**Cleavage of Protein-bound Fatty Acid of Purified Myelin PLP—**PLP (2.5-3.0 mg) (virtually devoid of SDS) and control eluate were hydrolyzed with 2 ml of methanolic NaOH (0.2 n) at room temperature in the dark for 24 h. The hydrolysates were dried under N2, 2 ml of 1 N HCl was added, and the contents were vigorously shaken until they formed a homogeneous suspension. The suspensions were extracted with 4 ml of peroxide-free diethyl ether and centrifuged at 2000 rpm at 4 °C. This step was repeated 3 more times. The 4 ether extracts were combined, and the volume was reduced under N2, in subdued light, to 100 µl. A freshly prepared solution of diazomethane in ether (19.9 ml) was added to the released fatty acids until the yellow color persisted; this mixture was left at -20 °C for 16 h. The methylated fatty acids were dried under N2 and redissolved in 250 µl of chloroform:methanol (1:1, v/v) for the separation of fatty acids by argentation or reverse-phase TLC. Precast Silica Gel G plates were impregnated with a 5% (w/v) aqueous solution of silver nitrate (freshly prepared) by allowing the solution to migrate to the top of the plate in the dark at room temperature. The plates were immediately activated at 110 °C for 1 h and used the same day (23). The released methylated fatty acids as well as methyl esters of known fatty acids were spotted using a Hamilton syringe and developed in the dark with a freshly prepared solution of chloroforcomepropanol (98:5:1.5, v/v/v). The TLC plates were sprayed with 2,7'-dichlorofluorescin (0.4 g/100 ml of ethyl alcohol) to visualize the spots under long wavelength ultraviolet light. The entire lane was divided into 1-cm sections, each section was scraped from the plate, and the radioactivity was determined after adding 10 ml of scintillation fluid (4 g of Omnifluor/liter of toluene). The counting efficiency was 30%. The reverse-phase TLC plates were heated at 120 °C for 15 min for the separation of the methyl esters of fatty acids. The developing solvent, acetone: methanol:water (70:30:3.5, v/v/v), was allowed to migrate 12 cm above the origin; the plates were air-dried for 30 min. This step was repeated 3 more times. The plates were sprayed with phosphomolybdic acid (20% in 50% ethanol, w/v), and heated at 150 °C for 15 to 30 min to visualize the spots (24). To remove background staining, the plates were exposed to ammonia vapors for 30 min. The entire lane of PLP hydrolysate was divided into 1-cm sections, each section was scraped, and radioactivity was determined after adding 2 ml of water and 10 ml of Insta-Gel. The counting efficiency was 20%. Hydrolyzed material released from the PLP and control eluate were examined both by isothermal (18 °C) and programmed temperature (protocol described by Iversion and Sheppard (25)) gas-liquid chromatography using a Varian gas chromatograph, model 3700, equipped with a flame ionization detector, differential flow controller, and temperature programmer.

**RESULTS AND DISCUSSION**

The electrophoretically eluted PLP was re-examined by SDS-slab gel electrophoresis in the Tris/glycine discontinuous gel system (16) and it migrated as a single but diffuse band (Fig. 1, Lane B). The molecular weight of the purified preparation of MDPF-labeled PLP was found to be 24,000 as described previously (9). This value is in excellent agreement with the previously reported molecular weight of PLP by this laboratory (9) and by other investigators (5, 26-28). The electrophoretic mobility of PLP was not altered by coupling with MDPF. The amino acid composition of PLP (Table I) was similar to that previously reported (8) and the NH2-terminal sequence of the first 4 amino acid residues (Gly-Leu-
of residues acids. Cystine was determined after treatment with performic acid as described.

4590 Acylation were made for hydrolytic losses of serine, threonine, and other amino acids. Cystine was determined after treatment with performic acid as described. (21). Results are nearest integer numbers of residues $M_r = 24,000$.

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>Numbers</th>
</tr>
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<tr>
<td>Half-cystine</td>
<td>7</td>
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<tr>
<td>Lysine</td>
<td>9</td>
</tr>
<tr>
<td>Histidine</td>
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</tr>
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<td>Arginine</td>
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</tr>
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<td>Aspartic acid</td>
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<td>Threonine</td>
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</tr>
<tr>
<td>Serine</td>
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<tr>
<td>Glutamic acid</td>
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<td>Proline</td>
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</tr>
<tr>
<td>Glycine</td>
<td>21</td>
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<td>Alanine</td>
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<td>Tyrosine</td>
<td>9</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>17</td>
</tr>
</tbody>
</table>

Results are the means of 3 separate determinations. No corrections were made for hydrolytic losses of serine, threonine, and other amino acids. Cystine was determined after treatment of the protein by performic acid as described (21). Results are nearest integer numbers of residues $M_r = 24,000$.

It is of interest to mention that only 6% (4100 dpm) and 3% (2990 dpm) of the total radioactivity in delipidated myelin (65,400 dpm) and PLP-enriched fraction (99,700 dpm), respectively, was associated with PLP when the gel sections were excised and the radioactivity was determined by liquid scintillation spectrometry. Reduced radioactivity of PLP in the PLP-enriched fraction could be due to the aggregation of this protein which might have occurred during the preparation of this fraction, solubilization in SDS, gel electrophoresis, or a combination of these factors (Fig. 2, lane 5). It has been shown that PLP aggregates after heating in high concentrations of 2-mercaptoethanol (32) and during delipidation of the Folch-Lees apoprotein (27). A large portion of the radioactivity also migrates with the fast moving lipid front (Fig. 2, lanes 4 and 5).

Since basic proteins of myelin were not labeled (Fig. 2, lane 6).

Fig. 2. SDS-slab gel electrophoresis of proteins of whole myelin, PLP-enriched fraction, and purified PLP. Electrophoresis followed by fluorography clearly shows that both PLP and DM-20 are acylated 24 h after the intracerebral injection of [3H]palmitic acid. The gel was stained with Coomassie blue, lane 1, whole myelin proteins (100 µg); lane 2, proteins of PLP-enriched fraction (100 µg); lane 3, purified PLP (73 µg); lanes 4, 5, and 6, fluorographs of lanes 1, 2 and 3, respectively; lane 7, whole myelin protein (92 µg); lane 8, purified PLP (59 µg); lanes 9 and 10 are fluorographs of lanes 7 and 8, respectively. WP, wolkgram protein; LBP, large basic protein; SBP, small basic protein.

Leu-Glu) is identical to that reported for myelin PLP from brains of rat (5) and other species (29-31). These results firmly established that the purified protein is, in fact, PLP and not another protein migrating to the position of PLP.

SDS-slab gel electrophoresis followed by fluorography of myelin proteins acylated in vivo clearly showed labeling of PLP and DM-20 (Fig. 2, lane 4) 24 h after the intracerebellar injection of [3H]palmitic acid. The radioactivity associated with PLP was retained when proteins in the whole myelin (Fig. 2, lane 4), PLP-enriched fraction (Fig. 2, lane 5), and purified PLP (Fig. 2, lane 6) were subjected to electrophoresis and fluorography. These results provided clear evidence of radioactivity firmly bound to PLP. When the gels were stained and destained before fluorography, there was no loss of radioactivity from the myelin proteins and PLP (Fig. 2, lanes 9 and 10) which is in contrast to the loss of [3H]palmitic acid from the chicken embryo fibroblast protein p20 reported by Schlesinger et al. (17).

Fig. 3. Fluorography of untreated and hydroxylamine-treated SDS slab gels of whole myelin proteins. Lane 1, untreated myelin proteins (75 µg); lane 2, myelin proteins (75 µg) treated for 24 h with 1 M NH$_2$OH at pH 6.6.
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Fig. 4. Thin layer chromatography of fatty acids on silver nitrate plates showing the separation of methyl oleate from methyl palmitate and methyl stearate. A, methyl palmitate (50 μg); B, methyl stearate (50 μg); C, methyl oleate (50 μg); D, methyl linoleate (50 μg); E, PLP hydrolysate (10,556 dpm); F, PLP hydrolysate (10,556 dpm) + 50 μg methyl oleate; spot 1, mixture of methyl palmitate and methyl stearate (8570 dpm); spot 2, methyl oleate (313 dpm); G, mixture of methyl esters of palmitate, stearate, oleate, and linoleate. A number of unidentified spots (?) were consistently seen which were devoid of radioactivity. In addition, the mobility of authentic methyl oleate in lane F is different from lane G.

4), it is reasonable to assume that the [3H]palmitic acid was not cycled in vivo into amino acid and giving us a spurious indication that the labeling of PLP is due to amino acids. In order to establish the nature of the linkage of [3H]palmitic acid with PLP, the gels were treated with 1 M hydroxylamine at pH 6.6 for 24 h (17) and fluorographed using two different procedures, described by Chamberlain (19) and Bonner and Laskey (18). Radioactivity was not removed from PLP in the hydroxylamine-treated gels which were fluorographed after infiltration with 1 M sodium salicylate (19) (data not shown) because the hydroxamates of fatty acids are not water-soluble. Fluorography of the dimethyl sulfoxide:2,5-diphenyloxazole-infiltrated gels (18) revealed that most of the radioactivity associated with PLP was removed after treatment of the gels with hydroxylamine (Fig. 3, lanes 1 and 2). This latter observation indicates that fatty acids were bound to PLP by an ester linkage. Finally, when the purified preparation of PLP (217,950 dpm) was cleaved with methanolic NaOH, most of the radioactivity was recovered in the organic phase (198,800 dpm). Thin layer chromatography of the released fatty acids on silver nitrate plates (Fig. 4) indicated that the bulk of the radioactivity migrated to the position of methyl palmitate and methyl stearate (8570 dpm). A small amount of radioactivity was also associated with methyl oleate (313 dpm). Methyl palmitate and methyl stearate migrate to the same position on silver nitrate plates; however, methyl oleate is distinctly separated from these two fatty acids. While methyl palmitate and methyl oleate migrate to the same position on reverse-phase TLC plates, there is a distinct separation of methyl stearate from these two fatty acids (Fig. 5). On reverse-phase TLC plates, the mixture of methyl esters of palmitate and oleate contained 1511 dpm whereas methyl stearate contained 357 dpm. Therefore, with the use of both silver nitrate and reverse-phase TLC plates, it was possible to determine the radioactivity associated with each fatty acid. In two separate experiments, the methyl esters of palmitate, stearate, and oleate were found to contain approximately 77, 19, and 4%, respectively, of the radioactivity which was recovered from the plates. In addition, gas-liquid chromatography of the fatty acids associated with PLP revealed a distinct peak of methyl palmitate, as well as a small but detectable peak with a retention time similar to methyl stearate (data not shown). A large number of unidentified peaks were observed on gas-liquid chromatography of the PLP hydrolysate. Since identical unidentified peaks were observed in the blank gel hydrolysate, it is more likely that these are contaminants derived from acrylamide and/or SDS rather than short chain fatty acids associated with PLP.

Regardless of the method of isolation of PLP from the brain, a number of investigators have shown the presence of fatty acids covalently linked to PLP (3, 4, 7, 33). This is the first reported evidence of the in vivo acylation of myelin PLP (8) after injection of [3H]palmitic acid. Similarly, Townsend et al. (34) presented preliminary evidence of in vitro acylation of myelin PLP and DM-20 in tissue slices. The ability to label PLP with [3H]palmitic acid will allow determination of the peptides, and subsequently, the amino acids to which fatty acids are linked in the macromolecular sequence of this protein. It will also permit determination of the differential turnover of the fatty acid and amino acid moieties of PLP. It also remains to be established if PLP is acylated in the cytosol of oligodendroglial cells prior to its incorporation into the myelin...
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membrane or whether the enzyme for the acylation of PLP is in fact present within the myelin sheath. These studies are currently in progress.

Acknowledgments—We thank Dr. N. S. Radin for his suggestion to separate methyl stearate from methyl palmitate by reverse phase thin layer chromatography. We also thank Aileen Derbake for typing the manuscript.

REFERENCES