

Synthesis and Incorporation of Myelin Polypeptides into CNS Myelin

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ABSTRACT The distribution of newly synthesized proteolipid protein (PLP, 23 kdaltons) and myelin basic proteins (MBPs, 14–21.5 kdaltons) was determined in microsomal and myelin fractions prepared from the brainstems of 10–30 d-old rats sacrificed at different times after an intracranial injection of ^{35}S -methionine. Labeled MBPs were found in the myelin fraction 2 min after the injection, whereas PLP appeared first in the rough microsomal fraction and only after a lag of 30 min in the myelin fraction. Cell-free translation experiments using purified mRNAs demonstrated that PLP and MBPs are synthesized in bound and free polysomes, respectively. A mechanism involving the cotranslational insertion into the ER membrane and subsequent passage of the polypeptides through the Golgi apparatus is consistent with the lag observed in the appearance of the *in vivo*-labeled PLP in the myelin membrane. Newly synthesized PLP and MBPs are not proteolytically processed, because the primary translation products synthesized *in vitro* had the same electrophoretic mobility and N-terminal amino acid sequence as the mature PLP and MBP polypeptides. It was found that crude myelin fractions are highly enriched in mRNAs coding for the MBPs but not in mRNA coding for PLP. This suggests that whereas the bound polysomes synthesizing PLP are largely confined to the cell body, free polysomes synthesizing MBPs are concentrated in oligodendrocyte processes involved in myelination, which explains the immediate incorporation of MBPs into the developing myelin sheath.

The myelin sheath has long been recognized as a rich source of plasma membranes with a relatively simple biochemical composition. In the central nervous system the sheath is derived from the plasma membrane of the oligodendrocyte (c.f. reference 44), a cell that during myelinogenesis greatly expands its surface while developing cytoplasmic processes that wrap around neighboring axons. Compact myelin is formed when the oligodendrocyte cytoplasm is extruded from the myelinating process, thus allowing for a close apposition of adjacent plasma membrane surfaces. In the resulting periodic multilamellar structure, apposed cytoplasmic aspects of the oligodendrocyte plasma membrane form what, in thin-section electron microscopy, is recognized as a *major dense line*, whereas the extracellular membrane faces form a thinner *intraparallel line*.

The major integral membrane protein of CNS myelin is the myelin proteolipid protein (PLP, or lipophilin, *M_r* 23 kdaltons) (21, 23) a proteolipid apoprotein that comprises ~50% of the total protein (19) and can only be released from the membrane bilayer by treatment with strong detergents or certain organic

solvents (23). Myelin also contains a comparable amount of one or a set of peripheral membrane proteins that are easily extracted by acids or high salt treatment, and are known as myelin basic proteins (MBPs), (c.f. reference 11). In the mouse, four different MBP polypeptides have been identified (4). The two major ones (18.5 kdaltons and 14 kdaltons), which were previously identified in certain rodent species (39) have been designated L and S (for large and small) and have the same amino acid sequence, except for the deletion of 40 amino acids near the carboxy terminal of S (17, 40). The two other species (21.5 kdaltons and 17 kdaltons) differ from the L and S proteins only by the presence of an additional polypeptide segment common to both (4). Although the disposition of PLP in the bilayer is not known, and the nature of the association of MBP with the myelin membrane is not well understood, it seems likely that specific interactions between the major myelin proteins play a role in myelinogenesis and that the regular arrangement of these polypeptides within the mature myelin sheath has important functional implications.

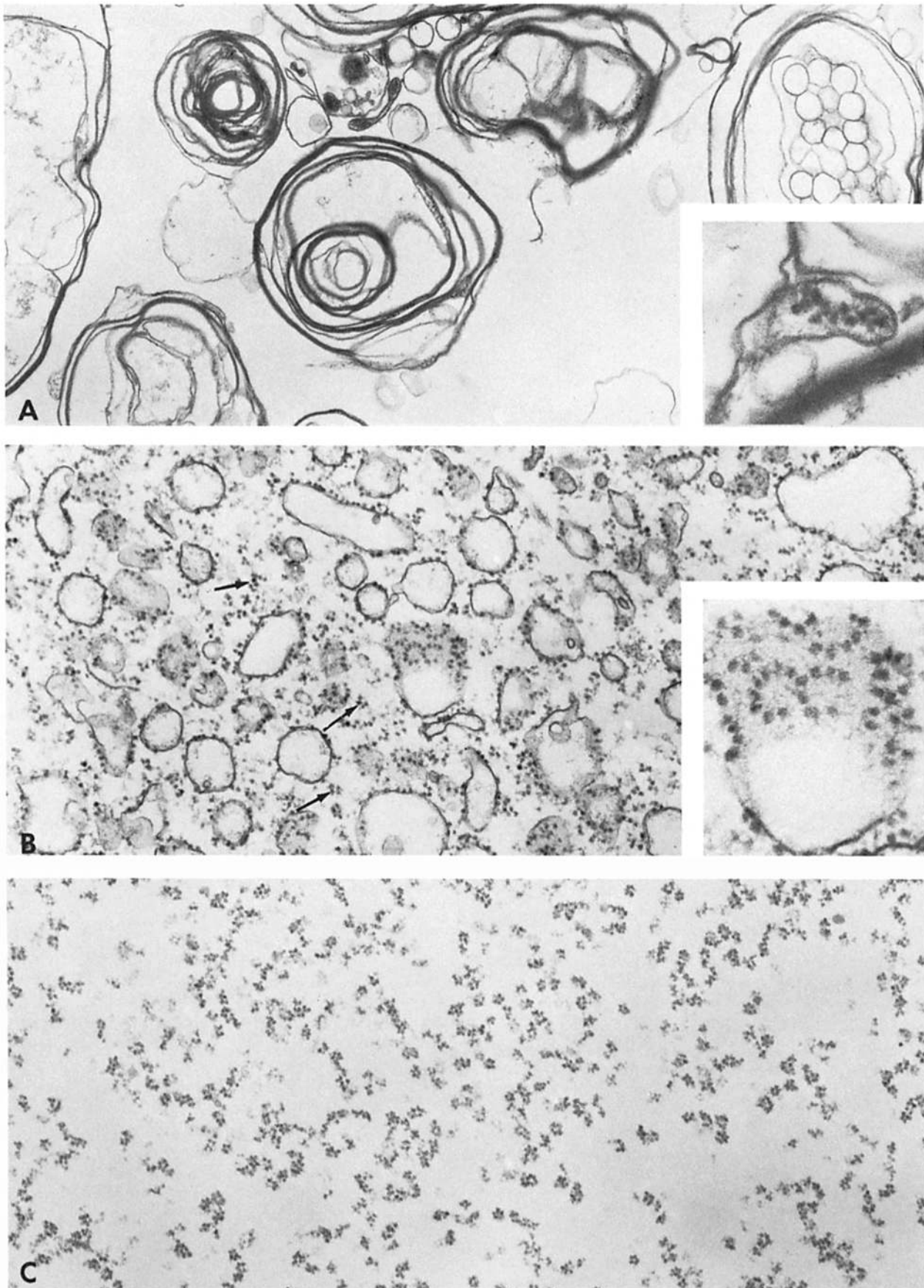


FIGURE 1 Subcellular fractions from myelinating brainstems. Subcellular fractions were prepared from the brainstems of 15-d-old Sprague-Dawley rats by the procedure described in Materials and Methods. (A) Crude myelin fraction containing fragments of axons surrounded by swollen and partially vesiculated myelin. This fraction is a rich source of oligodendrocyte plasma membranes and of free polysomes synthesizing the MBPs. The inset shows a polysome within the developing myelin sheath, recovered with the myelin fraction. $\times 17,000$; inset, $\times 90,000$. (B) Rough microsomal fraction containing ribosome-studded membrane vesicles derived from the RER. Free polysomes that contaminate this fraction are indicated by arrows. The inset shows a grazing section of a rough microsome showing typical configurations of membrane-bound polysomes. $\times 30,000$; inset, $\times 90,000$. (C) Free polysomes not contaminated by membranes, recovered in the pellet. $\times 20,000$.

Although the subject of considerable attention (5, 6, 10, 28, 35), the mechanisms by which the major myelin proteins are synthesized and incorporated into the developing sheath have not as yet been elucidated. In this paper we report the results of cell fractionation studies that allowed us to infer the distribution of the newly synthesized polypeptides in the oligodendrocyte and to compare the kinetics of their incorporation into the forming myelin membranes. We have also determined the sites of synthesis of PLP and MBP from the distribution of the mRNAs in purified fractions of free and membrane-bound ribosomes, and in myelin fragments derived from actively myelinating rat brainstems. A preliminary report of this work has been presented in abstract form (14).

MATERIALS AND METHODS

Preparation of Subcellular Fractions from Brainstems

Sprague-Dawley rats of 12–30 d of age, a time when brainstem myelin deposition is maximal (2), were used for all experiments. Following decapitation, the brain was removed and transferred to ice-cold 0.25 M sucrose where the medulla, pons, and ventral midbrain, which are rich in white matter, were dissected out.

FRACTIONS USED FOR RNA EXTRACTION: A 15% (wt/vol) homogenate containing 500 mM KCl, 25 mM HEPES (pH 7.4); 5 mM MgCl₂, 50 U/ml Trasylol, and 3 mM dithiothreitol (DTT) was prepared and a nuclear fraction was removed by centrifugation (2,000 RPM, 2 min in a Sorvall HB-4 rotor, DuPont Instruments-Sorvall Biomedical Div., Newtown, CT). After adjusting the postnuclear supernatant to 1.40 M sucrose and 250 mM KCl, without changing the concentration of other constituents, an aliquot (9 ml) was layered above a cushion (2 ml) of 1.9 M sucrose and then overlaid with 0.85 M sucrose (1 ml) and 0.25 M sucrose (.25 ml). All these sucrose layers had the same salt concentration as the diluted homogenate. After centrifugation (40,000 RPM for 20 h in a Beckman SW41 rotor, Beckman Instruments, Inc., Fullerton, CA), fractions were recovered from the 0.25–0.85 M and 1.4–1.9 M sucrose interfaces. Electron microscopy (Fig. 1A and B) showed that these consisted of myelin fragments and rough microsomes, respectively. Free polysomes (Fig. 1C) were recovered in the pellet.

CELL FRACTIONATION PROCEDURE USED TO DETERMINE THE DISTRIBUTION OF IN VIVO LABELED POLYPEPTIDES: This was similar to that described above, but in this case for each brainstem the homogenization medium (6 ml) contained 0.25 M sucrose, 10 mM HEPES, 3 mM DTT, and 50 U/ml Trasylol, and no KCl or MgCl₂ was added either to the homogenization medium, to the postnuclear supernatant, or to the sucrose layers in the discontinuous gradient. High salt was not used in these experiments, because under high salt conditions MBPs associated with in vivo labeled myelin or rough microsomes would have been released from the membranes (c.f. reference 11).

Myelin fragments (from the 0.25–0.85 M interface) were subjected to an osmotic shock by homogenization in 20 vol of 10 mM HEPES (pH 7.4) and 100 U/ml Trasylol. The suspension was then adjusted to 0.85 M sucrose, keeping other constituents constant, and 12 ml of this suspension was overlaid with 0.5 ml of 0.25 M sucrose in 10 mM HEPES. After centrifugation (40,000 RPM for 4 h in a Beckman Instruments, Inc. SW41 rotor), a pellicle at the 0.25–0.85 M sucrose interface was collected and subjected two more times to the osmotic shock and centrifugation procedure. The final pellicle, consisting of purified myelin membranes, was used immediately for immunoprecipitation or frozen in liquid nitrogen and stored at –80°C for purification of myelin proteins. Analysis of this fraction by SDS gel electrophoresis followed by Coomassie Blue or silver staining (Fig. 5, lanes b, c and h) demonstrated that these fractions contain the characteristic major myelin proteins (c.f. reference 19; 27).

PURIFICATION OF MBPS AND PLP: Osmotically shocked myelin fragments were partially delipidated in acetone, washed in H₂O, and suspended in 15 vol of 0.5 N acetic acid or 0.5 N HCl (35). The suspension was kept on ice for 1 h and then centrifuged (10,000 RPM for 20 min in a Sorvall HB4 rotor) to separate a supernatant, from which the MBPs were extracted, and a sediment, used to prepare PLP. The supernatant received 10 vol of cold acetone and was kept overnight at –20°C to precipitate a crude basic protein fraction. For extraction of PLP the sediment was resuspended in chloroform:methanol (2:1) and maintained at 37°C for 1 h. After centrifugation (as above) a clear yellowish supernatant was obtained to which 4 vol of ether were added. This material was incubated overnight at –20°C to precipitate a proteolipid protein fraction from which PLP was prepared. After heating (100°C) in sample buffer containing 10%

SDS, the proteolipid fraction and the crude basic protein fraction were cooled to room temperature and treated with 500 mM DTT (final concentration) before loading onto gels. Final purification of the MBPs and PLP was accomplished by SDS electrophoresis in 7–17% polyacrylamide gradient gels (Maizel, J. V., reference 38, as modified by Kreibich, G., and D. D. Sabatini, reference 33).

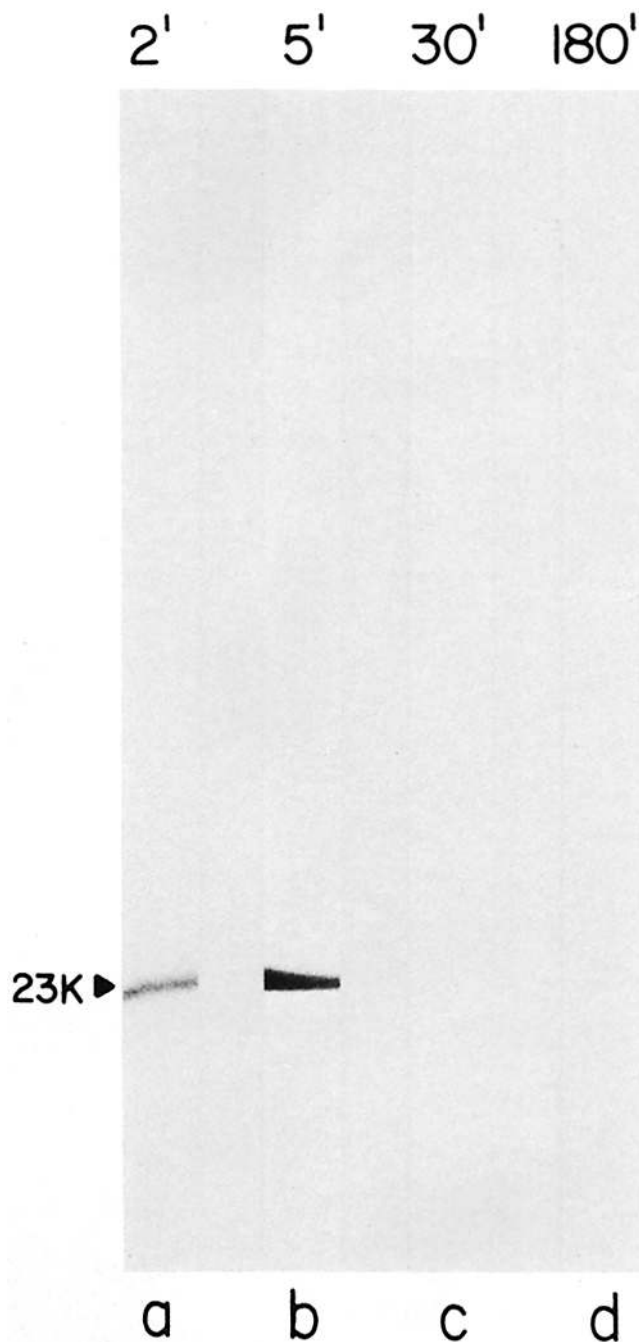


FIGURE 2 Immunoprecipitation of newly synthesized PLP from rough microsomes. Rough microsome fractions were prepared (see Materials and Methods) from four 12-d-old Sprague Dawley rat littermates that received an intracranial injection into the metencephalon of 0.5m Ci of ³⁵S-methionine (sp act = 1,000 Ci/mmol) in 10 μl of phosphate buffer (pH 7.4). Animals were killed by decapitation 2, 5, 30, and 180 min later. PLP was immunoprecipitated with 25 μl of antiserum from rough microsome samples containing 40 μg of protein in 200 μl. Immunoprecipitates were analyzed by SDS acrylamide (7–17%) gel electrophoresis followed by fluorography (8). The numbers above each gel track indicate the time in minutes after injection of label at which each animal was killed. K, kdaltons.

Four polypeptides, which corresponded in electrophoretic mobility to the different MBP proteins identified in the mouse (4), were found in rat myelin. The nomenclature introduced for mouse MBPs was, therefore, adopted for the rat myelin MBPs although we have not directly determined the relationships between the various polypeptides.

After light staining (5 min) of gels with Coomassie Blue (0.04% dye in 50% methanol), the major protein bands corresponding to the small myelin basic protein (SMBP, M_r 14 kdaltons) and PLP (M_r 23 kdaltons) were excised. Gel slices were placed in a Corex tube (Corning Glass Works, Corning, NY), pulverized by grinding with a glass rod, and then treated with 0.5% SDS at 95°C for 2 h. A supernatant was prepared by centrifugation, and 10 vol of acidified acetone were added to precipitate the purified proteins. Determination of the amino acid composition (SMBP, reference 41) and amino terminal sequence (PLP, see below) of these proteins confirmed their identity (11, 42).

ANTIBODIES: The same procedure was used for purified PLP and SMBP.

Each of these proteins (50 μ g) was dissolved in 250 μ l of 0.1% SDS with 150 mM NaCl, and the solutions were mixed with equivalent volumes of complete Freund's adjuvant. Aliquots (50 μ l) of each suspension were injected into the popliteal lymph nodes of New Zealand rabbits under Na pentobarbital anesthesia (15). After 2 wk, a total of 100 μ g of antigen suspended in 0.5 ml of incomplete Freund's adjuvant was distributed intradermally in several injections across the back. Animals were bled at weekly intervals thereafter and booster injections of 10 μ g of purified antigen in incomplete Freund's adjuvant were given monthly. The titer of the antibodies was determined by indirect immunoprecipitation (see below) using 125 I-labeled antigen (7). All antisera bound \sim 50 μ g of purified antigen per ml. As is the case for the mouse (4), antibodies against SMBP recognized all four MBP polypeptides. A single polypeptide of an apparent molecular weight of 23 kdaltons was immunoprecipitated with PLP antisera.

IMMUNOPRECIPITATION: Samples (100 μ l for translation mixtures, and 200 μ l containing 100 μ g of protein for in vivo-labeled myelin and 40 μ g for in

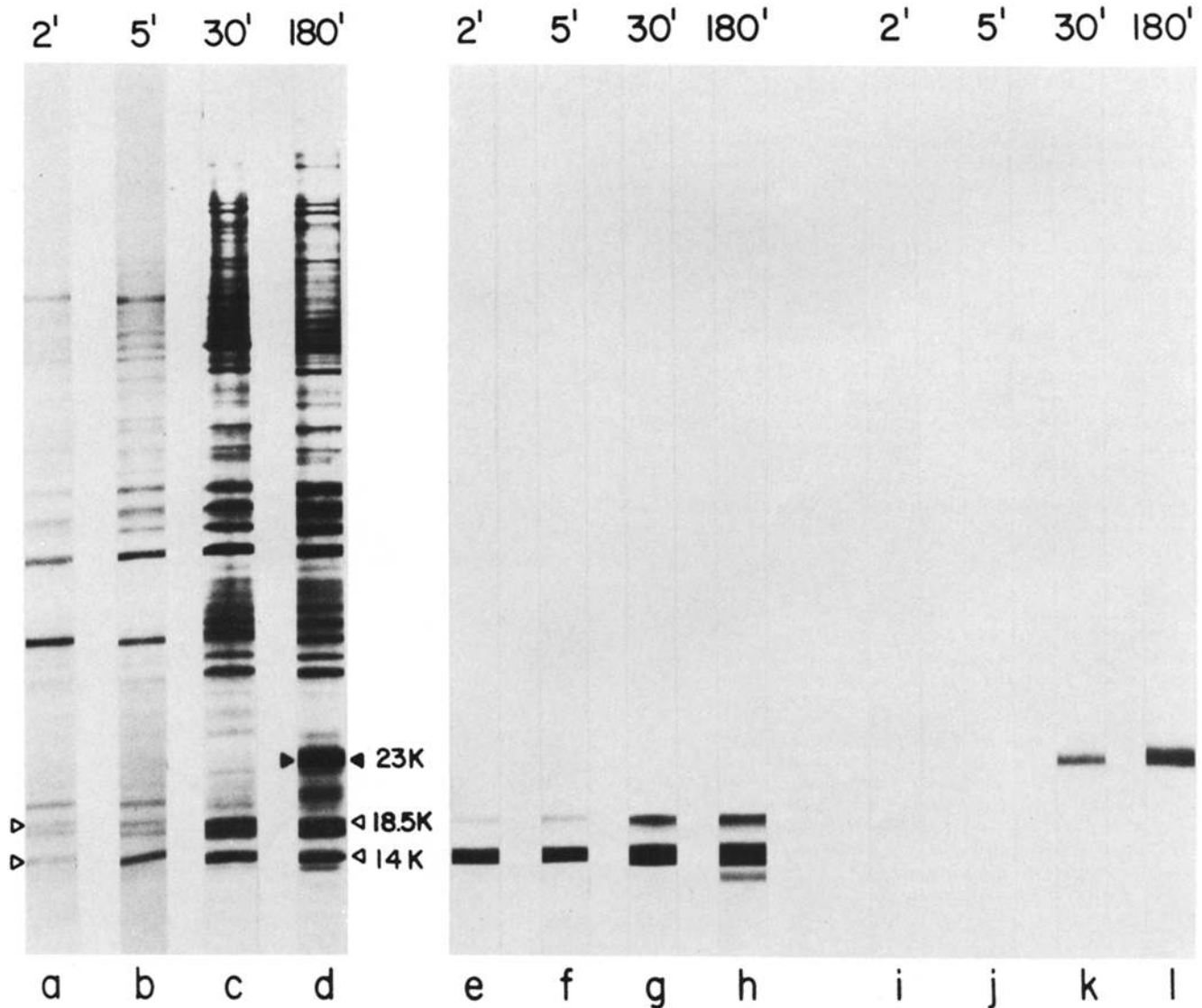


FIGURE 3 Kinetics of incorporation of newly synthesized membrane proteins into myelin. Myelin fractions were prepared (see Materials and Methods) from animals labeled as described in the legend to Fig. 2, and samples (100 μ g protein in 200 μ l) were used for immunoprecipitation with 25 μ l of SMBP or PLP antiserum. Lanes a–d: Resuspended purified myelin fractions (25 μ g of protein each) analyzed by SDS electrophoresis in 7–17% acrylamide gels followed by fluorography. Lanes e–h: Analysis of immunoprecipitates obtained with SMBP antibodies from aliquots (200 μ l containing 100 μ g of protein) of a suspension of purified myelin. The short exposure (1 d) of the fluorogram necessary to resolve SMBP and LMBP as separate bands does not allow the demonstration of the 17-kdalton and 21.5-kdalton MBPs, in the same figure. These proteins contain <5% of the total radioactivity and, in this figure, correspond to very weak bands that only become apparent after much longer exposures. Lanes i–l: Immunoprecipitates obtained with anti-PLP antibodies. The time after the injection at which each animal was killed is indicated above each lane. The rapid appearance of labeled MBPs in the myelin fraction (lanes a–d) is in marked contrast to the slow accumulation of labeled PLP, which becomes clearly visible in the total pattern after >30 min (lanes c and d). K, kdaltons.

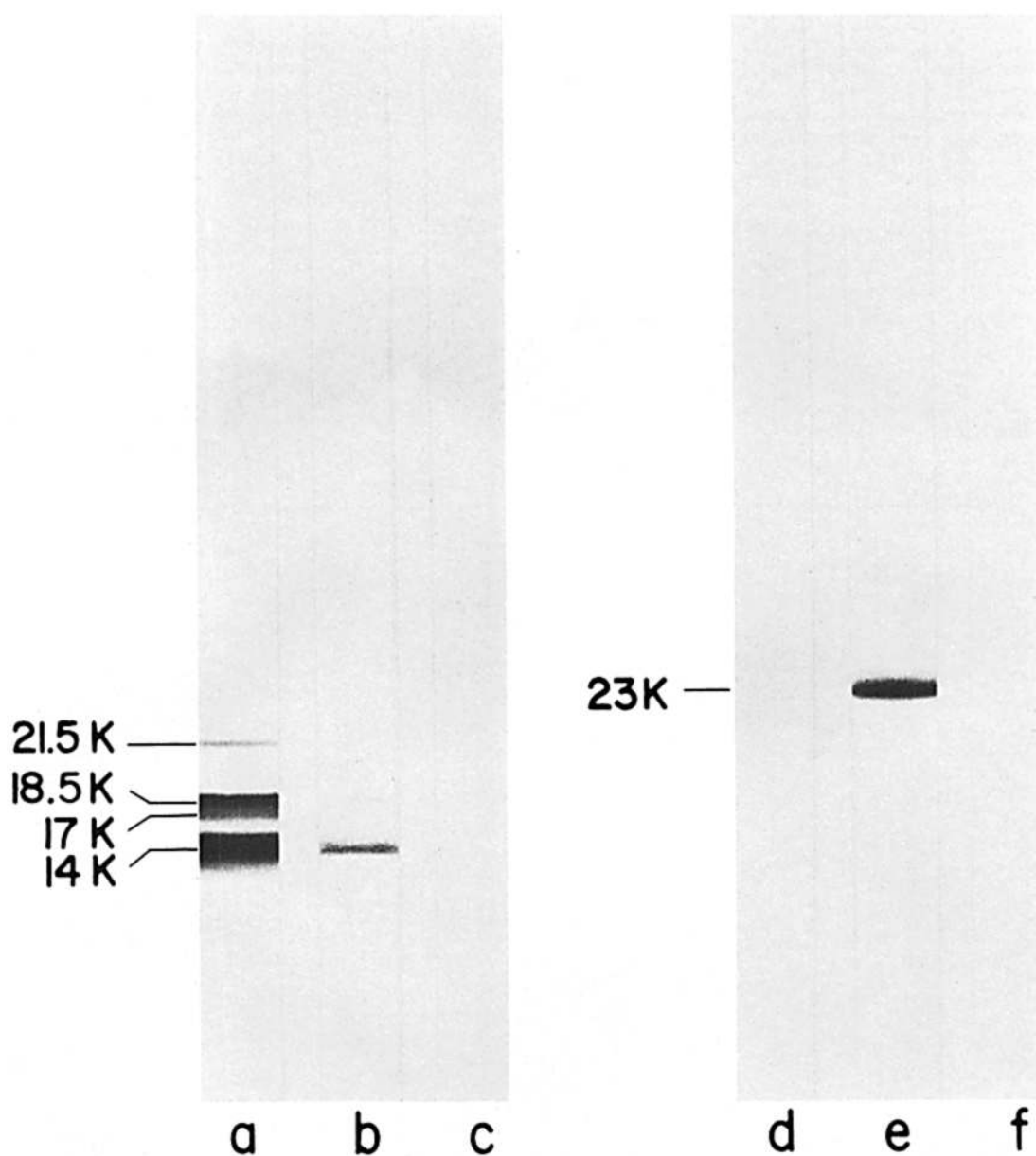


FIGURE 4 Site of synthesis of myelin proteins. Poly (A)⁺ mRNA (1.5 OD U/ml of translation mixture) extracted from free polysomes (*a*, *c*, and *d*) and rough microsomes (*b*, *e*, and *f*) isolated from rat brainstems of 8-d-old rats (see Materials and Methods) was used to program a wheat germ translation system (Roman et al., 1976) containing ³⁵S-methionine (0.5 mCi/ml) (sp act = 1000 Ci/mmol). RNA was extracted using 6 M guanidine-HCl (37) and poly(A)⁺ mRNA was prepared as described by Aviv and Leder (1). After translation for 2 h at 25°C, samples (100 μl each) were treated for immunoprecipitation with 10 μl of SMBP (*a*, *b*, and *c*) or PLP antiserum (*d*, *e*, and *f*) in the presence (*c* and *f*) or absence (*a*, *b*, *d*, and *e*) of 5 μg of the corresponding competing antigen. Immunoprecipitates were analyzed by electrophoresis on a 7–17% polyacrylamide gradient gel followed by fluorography. After exposures (1 d), the fluorogram was placed over the dried gel to locate the relevant bands, which were excised and counted (New England Nuclear, LSC Applications Notes #39) to determine relative ratios of radioactivity in each myelin basic protein. In this case, 10–12% of the radioactivity incorporated into MBPs was present in the 21.5 kdalton- and 17 kdalton-proteins, and all four polypeptides are therefore detected in lane *a* after 1 d of exposure. When mRNA from older animals (20 d) was used (as in Figs. 5 and 9), <5% of the MBP radioactivity was in the two minor proteins. *K*, kilodaltons.

vivo-labeled rough microsomes) were heated (100°C, 2 min) in 2% SDS (final) and diluted by addition of 4 vol of a buffer containing Triton X-100 (Solution A, reference 25). Antisera (10 μl for in vitro translations, 25 μl for in vivo-labeled samples) were added and samples were incubated overnight (4°C). Protein A Sepharose CL-4B (Pharmacia Inc., Piscataway, NJ) was used to retrieve immune complexes. After incubation (4°C, 3 h) on a rotary shaker, beads were recovered by sedimentation, washed extensively in Solution A, and prepared for electrophoresis by heating (100°C, 2 min) in a SDS gel sample buffer.

N-TERMINAL AMINO ACID SEQUENCING OF IN VITRO-SYNTHESIZED

PLP AND MYELIN BASIC PROTEIN: Immunoprecipitates obtained from translation mixtures were resuspended in 500 μl of 1.0% SDS (43). After heating to 100°C for 5 min and centrifugation to remove aggregates, the supernatant was mixed with 0.5 mg sperm whale apomyoglobin and 1.5 mg ovalbumin and loaded in a Beckman Instruments, Inc., model 890C sequenator. Sequencing was carried out employing a 0.1 M Quadrol program (9), and double-coupling was effected on the first cycle by omitting HFBA. When more than one radiolabeled amino acid was used in in vitro translation, the anilinothiazolinones (ANZ) generated by Edman degradation were converted to the phenylthiohydantoin (PTH) deriv-

atives by incubation for 10 min with 1N HCl at 80°C (18), and these were identified by reverse-phase liquid chromatography on a Hewlett-Packard model 1084A apparatus equipped with an Altex Ultrasphere ODS column (30). When

a single radiolabeled amino acid was used, the radioactivity in the anilinothiazolinones generated in each cycle was measured directly by liquid scintillation counting.

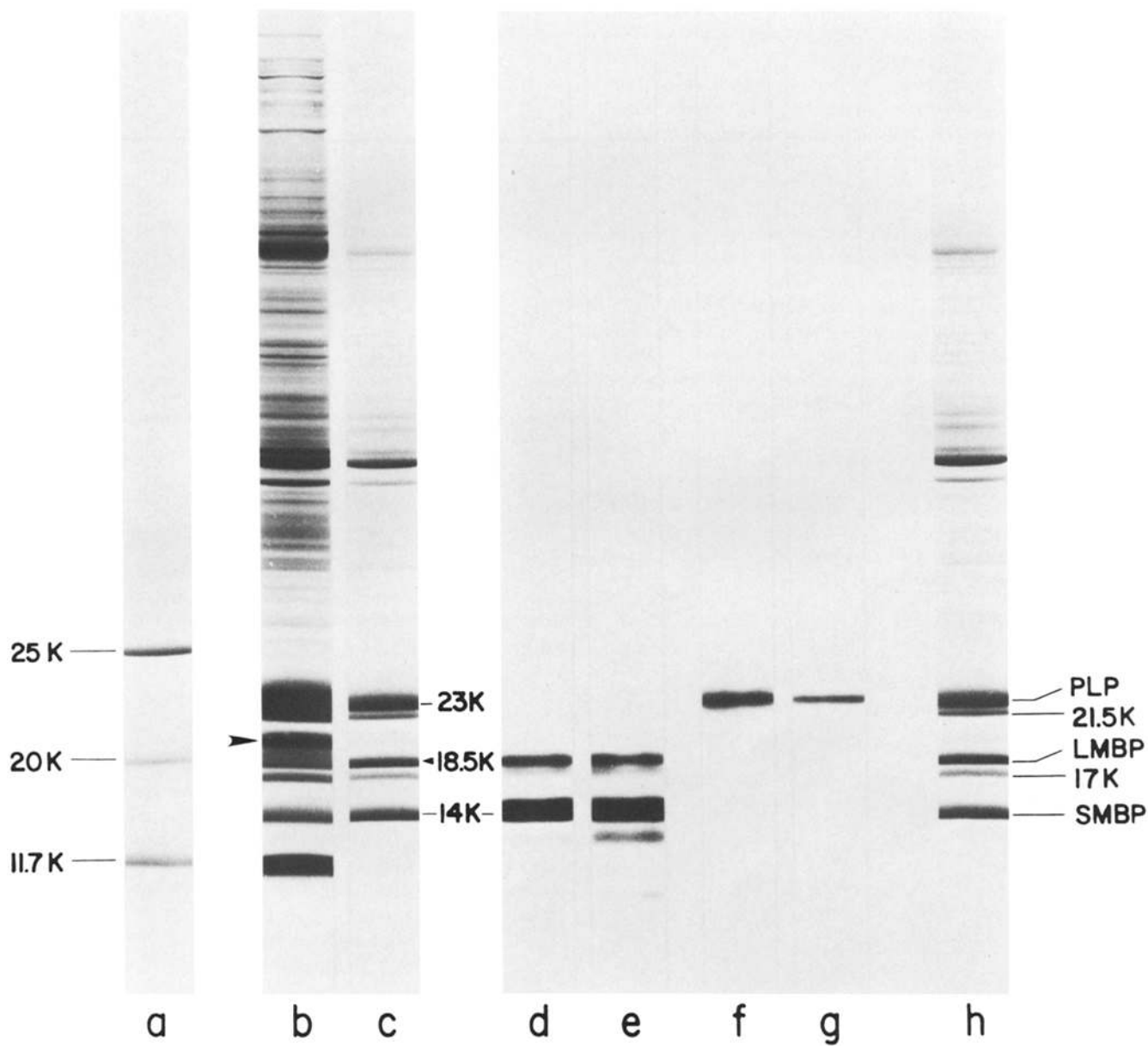


FIGURE 5 Electrophoretic mobilities of myelin proteins labeled in vivo and MBPs and PLP polypeptides synthesized in vitro. Immunoprecipitates containing ^{35}S -methionine-labeled polypeptides (*d-g*) obtained from in vitro translation mixtures (*d* and *f*) (see Fig. 4) or in vivo-labeled myelin (*e* and *g*) treated with SMBP (*d* and *e*) or PLP (*f* and *g*) antiserum (see Fig. 3) were analyzed by electrophoresis in a slab gel together with partially delipidated myelin (75 μg protein; *b*, *c*, and *h*) and molecular weight standards (*a*): cytochrome *c* (11.7 kdaltons); soybean trypsin inhibitor (20 kdaltons); chymotrypsinogen A_4 (25 kdaltons). The myelin sample had been osmotically shocked and partially delipidated by brief (5 min) treatment with acetone followed by washing in water. All samples were solubilized in sample buffer containing 10% SDS, heated to 100°C, and, after cooling, received 500 mM DTT. After electrophoresis (20 mA for 20 h) the gel (2-mm thick) was fixed in acetic acid-methanol and stained with Coomassie Blue. Strips *a*, *c*, and *h*, are photographs of the Coomassie Blue-stained lanes containing the standards (*a*) and myelin samples (*c* and *h*). After photography the slab gel was rinsed in several changes of 50% methanol, stained by a silver staining procedure (57), and rephotographed. Strip *b* shows a photograph of the silver-stained myelin sample. Silver deposits were then removed from the gel by treatment with Kodak Rapid Fixer (1:1 with H_2O). The gel was soaked in Kodak hypo-clearing agent, extensively rinsed in water followed by 50% methanol, and prepared for fluorography (8) to reveal bands of labeled polypeptides in immunoprecipitates (*d-g*). The short exposure (1 d) necessary to resolve SMBP and LMBP bands does not allow for the demonstration in the same figure of the minor 17 kdalton- and the 21.5 kdalton MBPs. The 13 kdalton-polypeptide in lane *e* is not always present and probably results from proteolysis of one of the major MBPs. The protein known as DM-20 stains intensely with the silver method (arrowhead in *b*) but very poorly with Coomassie Blue (*c* and *h*). Note the absence of DM-20 in the PLP immunoprecipitates (*f* and *g*). The Coomassie Blue-stained pattern of total proteins in purified myelin is repeated in *c* and *h* to aid in a comparison of electrophoretic mobilities. *K*, kilodaltons.

RESULTS

Intracellular Pathway Followed by Myelin Proteins

The distribution of newly synthesized myelin proteins was determined in myelin and microsomal fractions obtained from myelinating brainstems of young rats killed at different times after an intracranial injection of ^{35}S -methionine. Newly synthesized PLP was found in purified rough microsomes immediately (2 min) after the injection (Fig. 2) but the labeled polypeptide did not reach the myelin fraction until >5 min later, when it had already disappeared from the microsomal fraction (compare Figs. 2 and 3). No labeled MBPs were detected in the rough microsomal fraction throughout the labeling period, but, as early as 2 min after the injection, newly synthesized MBPs were present in the purified myelin fraction, where they accumulated continuously thereafter (Fig. 3). The early appearance of PLP in rough microsomes and the substantial delay with which it reached the myelin sheath are consistent with a mechanism for the transfer of this protein to the plasma membrane that involves its synthesis in membrane-bound ribosomes and a relatively slow passage through an intracellular pathway which, it may be presumed, includes the Golgi apparatus. On the other hand, the rapid incorporation of MBPs into developing myelin and their absence from *in vivo* labeled rough microsome fractions suggest that these proteins are synthesized in free polysomes and are therefore incorporated directly into regions of the plasma membrane forming myelin.

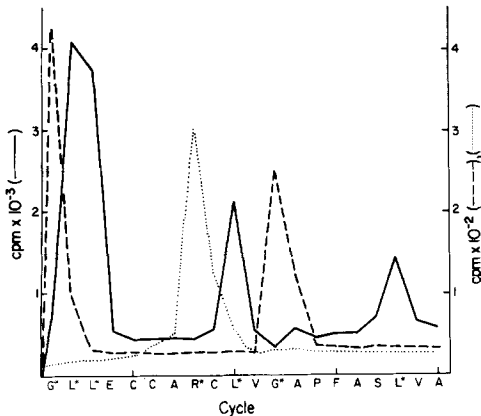


FIGURE 6 N-terminal sequence analysis of *in vitro*-synthesized PLP. Immunoprecipitates were obtained with PLP antiserum from two wheat-germ translation mixtures (1 ml each), which were programmed with total brainstem poly (A)⁺ mRNA and contained either [^3H]Leu (2 mCi; 100 Ci/mmol) or a mixture of [^3H]Gly (3 mCi; 15 Ci/mmol) and [^3H]Arg (3 mCi; 17 Ci/mmol). The sequence of labeled amino acids in the N terminal regions of the products was determined by automated Edman degradation as described in Materials and Methods. The radioactivity released in each step from the sample labeled with [^3H]leucine (total 60,000 cpm) was measured directly by scintillation counting of (ANZ) derivatives. In the case of the sample labeled with a mixture of [^3H]Gly and [^3H]Arg (a total of 18,000 cpm), the ANZ derivatives were converted to the PTHs, and these were separated and identified by HPLC. The repetitive yield was 93.5% as calculated from steps 2 and 18. Capital letters in the abscissa indicate the N terminal sequence of amino acids in PLP (32) using the one letter code. Asterisks in the sequence of PLP indicate radioactive residues identified in the *in vitro*-synthesized product. (—) [^3H]Leu, (---) [^3H]Gly, (.....) [^3H]Arg.

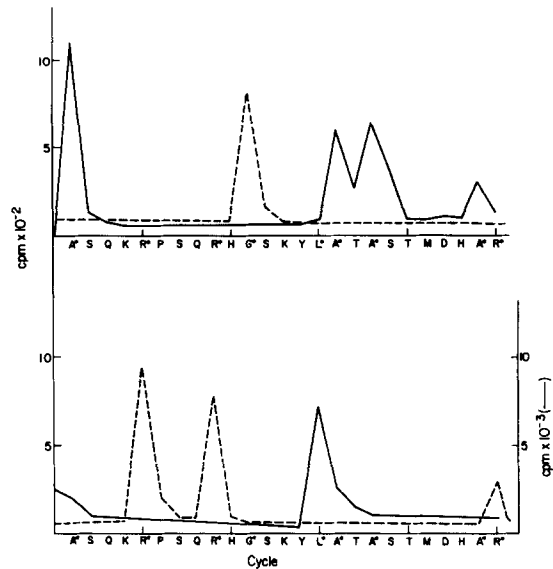


FIGURE 7 N-terminal sequence analysis of *in vitro*-synthesized MBP. Immunoprecipitates were obtained with anti-SMBP from two translation systems similar to those described in the legend to Fig. 6 except that [^3H]Ala (2 mCi; 60 Ci/mmol) was also present in the mixture containing [^3H]Gly and [^3H]Arg. The immunoprecipitates contained 290,000 cpm [^3H]Leu and 110,000 cpm [^3H]Ala, [^3H]Gly, [^3H]Arg. Analysis of the immunoprecipitates (see legend to Fig. 4) showed that the large (18.5 kdaltons) and small (14 kdaltons) MBP polypeptides together contained 95% of the radioactivity. The repetitive yield of the Edman degradation calculated from steps 1 and 16 was 96%. For clarity, the results are depicted in two different panels. Top: (—) [^3H]Ala, (---) [^3H]Gly. Bottom: (—) [^3H]Leu, (---) [^3H]Arg.

In Vitro Synthesis of Myelin Membrane Proteins

The synthesis of MBPs and PLP in free and membrane-bound polysomes, respectively, was demonstrated by cell-free translation of mRNA derived from each polysome population (Fig. 4). From translation systems programmed with free polysomal mRNA, four labeled polypeptides were immunoprecipitated with antiserum raised against SMBP. These corresponded in electrophoretic mobility to the 21.5 kdalton, 18.5 kdalton (LMBP)-, 17 kdalton- and 14 kdalton (SMBP)-polypeptides described for mouse myelin (4). A single polypeptide of apparent molecular weight 23 kdaltons was immunoprecipitated with PLP antiserum from translation mixtures programmed with mRNA from bound polysomes. As expected from the occasional contamination of rough microsome fractions with free polysomes (Fig. 1B), smaller amounts of MBPs were usually immunoprecipitated from translation systems programmed with mRNA prepared from rough microsomes (Fig. 4, lane *b*).

It should be noted that the cell-free translation products of mRNAs coding for PLP and the MBPs were indistinguishable in electrophoretic mobility from the mature polypeptides (Fig. 5). Because the molecular weights of the major myelin proteins are relatively low, the removal of even short peptide segments (10 amino acids in length) during *in vivo* synthesis would have been easily detected. This suggests that, aside from the loss of the initiator methionine, neither PLP nor the MBPs undergo co- or posttranslational proteolytic cleavages. Indeed, partial sequencing of the products of *in vitro* translation, which were labeled with selected amino acids (Figs. 6, 7), demonstrated

that they contain the same amino terminal segments that are present in the mature proteins (17, 20, 31, 42).

The interaction of in vitro-synthesized myelin proteins with microsomal membranes was examined in translation mixtures programmed with total brainstem poly (A)⁺ mRNA and supplemented with dog pancreas microsomes and a high salt extract of microsomes, which has been shown to facilitate the cotranslational insertion of nascent polypeptides into membranes (56). Under these conditions, >50% of the in vitro synthesized PLP was recovered with the sedimentable microsomes (Fig. 8 *b*). This association, which was not accompanied by a detectable change in the size of PLP, appeared to result from insertion of the polypeptide into the ER membranes, because all the PLP remained in the supernatant when the

microsomes were added after translation was completed (Fig. 8 *c*), or when the high salt extract was omitted (Fig. 8 *a*). In the same experiments a large fraction (50%) of the labeled MBPs synthesized in vitro was also consistently recovered with the sedimentable microsomes (Fig. 8 *d-f*). In contrast to the situation with PLP, however, the association of MBPs with the microsomal membranes did not require the addition of a high salt extract and occurred even when the microsomes were added after translation was completed.

Recovery of MBP mRNA's in Myelin Fractions

The preparation by flotation of a myelin fraction from myelinating brainstems allows for the separation of cytoplasmic

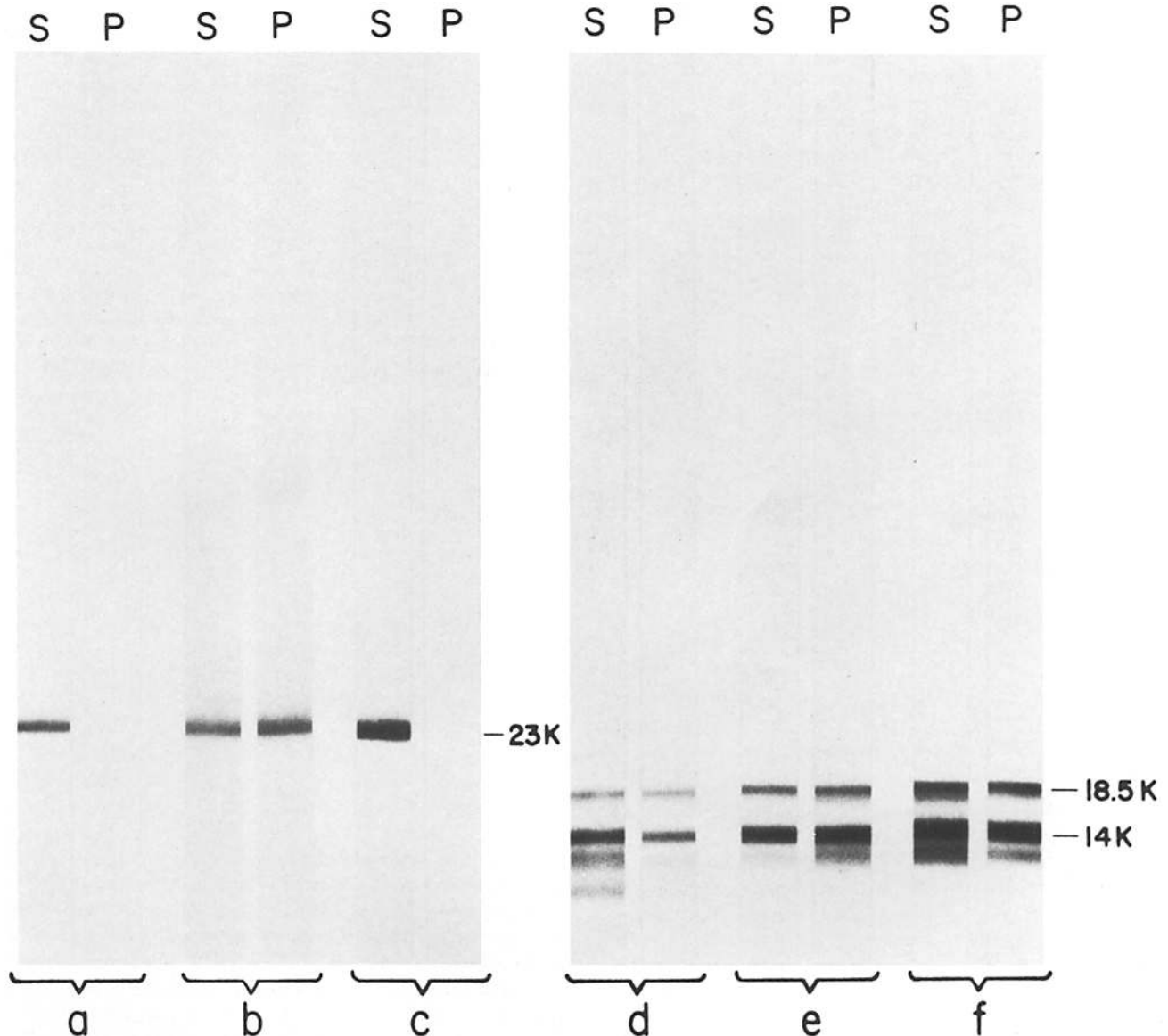


FIGURE 8 Cotranslational insertion of PLP into microsomal membranes. In vitro translation was carried out in wheat germ systems programmed with poly (A)⁺ mRNA from unfractionated brainstem. A set of samples (*a*, *b*, *d*, and *e*) received dog pancreas microsomes at the beginning of translation (52) with (*b* and *e*) or without (*a* and *d*) a high salt microsomal extract (1 μ l/25 μ l translation mixture) that facilitates insertion of nascent polypeptides into membranes (56). Another set (*c* and *f*) received the microsomal membranes and high salt extract after 2 h of incubation when translation had ceased, and incubation was continued for an additional hour. Supernatants (S) and sediments containing membranes (P) were collected from all samples after centrifugation for 5 min at 13,000 *g*. Sediments were washed once by resuspension in 250 mM sucrose with 25 mM HEPES (pH = 7.4) and centrifugation. Supernatants and pellets were solubilized in SDS and prepared (as described in Materials and Methods) for immunoprecipitation, first with PLP (*a*, *b*, and *c*) and then with SMBP antisera (*d*, *e*, and *f*). *K*, kdaltons.

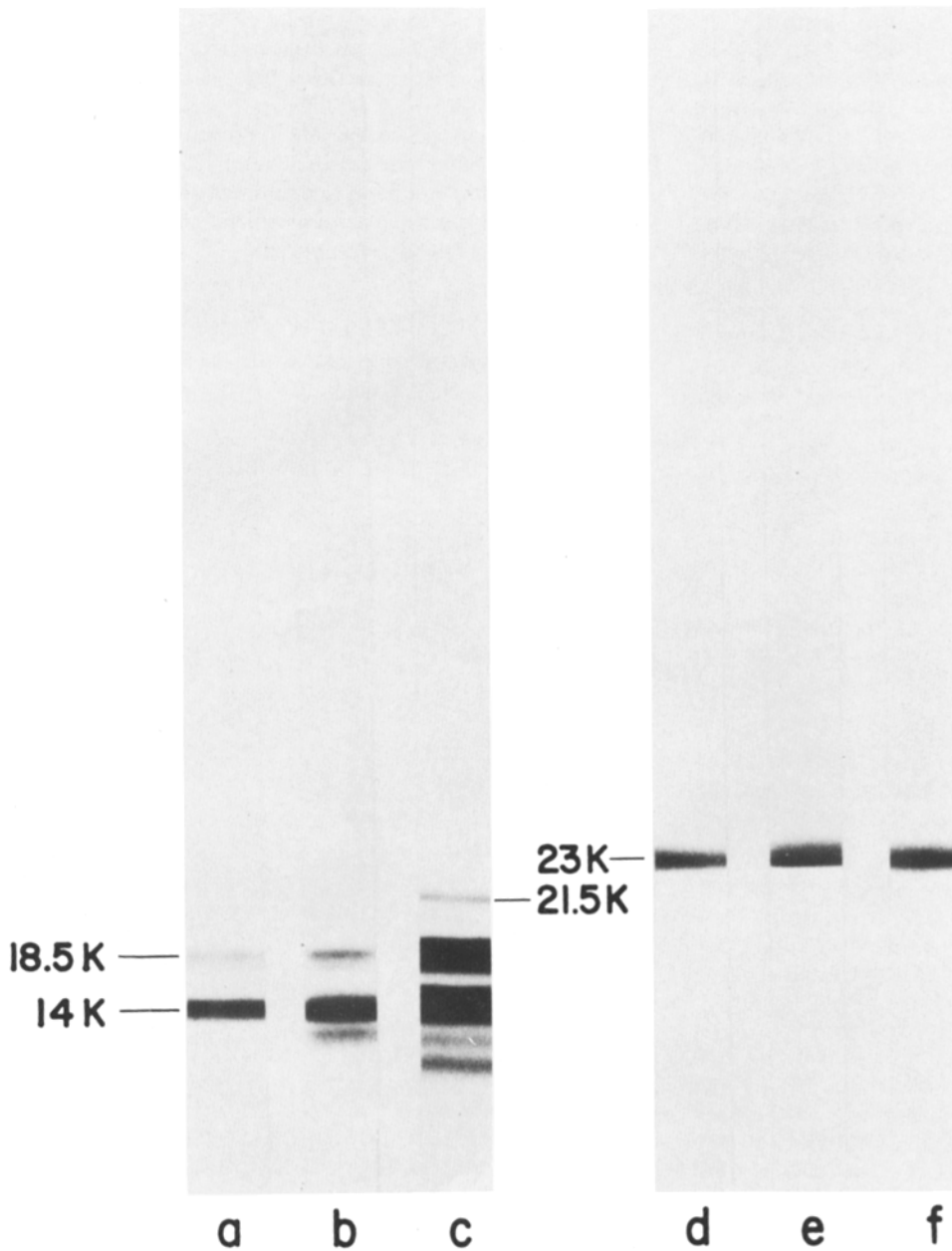


FIGURE 9 Translation products of mRNA extracted from the myelin fraction. Translation systems were programmed with poly (A)⁺ mRNA extracted from the myelin fraction (c and f), total brainstem (a and d), free polysomes (b) or rough microsomes (e). After incubation for 120 min, samples were treated for immunoprecipitation with SMBP (a, b and c) or PLP (d, e, and f) antiserum and immunoprecipitates were analyzed by SDS PAGE followed by fluorography. The bands of higher mobility than 14 kdaltons in lanes b and c probably represent products of mild proteolytic degradation during immunoprecipitation. K, kdaltons.

processes of actively myelinating oligodendrocytes from the rest of the cytoplasm of these cells, as well as from other glial and neuronal elements that are present in white matter. Translation of mRNA extracted from the myelin fraction revealed that this is highly enriched in messengers coding for the myelin basic proteins (Fig. 9). Whereas MBPs represented <0.3% of the cell-free products synthesized *in vitro* with unfractionated brainstem mRNA or with mRNA prepared from free polysomes, they amounted to 2–3% of the total polypeptides synthesized under the direction of mRNA purified from the myelin fraction. This finding and the fact that only a much smaller increase in mRNA coding for PLP was occasionally obtained in the myelin fraction (0.2% in brainstem, and 0.3%–0.4% in rough microsomes, versus 0.3%–0.4% in the myelin fraction) suggest that free polysomes synthesizing MBPs are preferentially segregated in the myelinating cell processes, whereas the RER where PLP synthesis takes place is restricted almost exclusively to the cell body.

DISCUSSION

The preceding *in vivo* labeling and *in vitro* translation experiments demonstrate that nascent PLP chains are inserted into ER membranes during their synthesis in membrane-bound ribosomes. By contrast with other membrane proteins, such as the G protein of VSV (36) and the heavy chain of histocompatibility antigens (16), the myelin PLP does not undergo a proteolytic cleavage during this process and therefore must retain the signal sequence that triggers the cotranslational insertion. In this respect PLP resembles several other proteins of the plasma membrane, such as erythrocyte band 3 (48), the glycoprotein subunit of the Na⁺,K⁺-ATPase (51) and envelope glycoproteins of toga viruses (7a, 24), as well as proteins characteristic of ER membranes, such as cytochrome P-450 (3) epoxide hydratase, NADPH cytochrome P-450 reductase (43), and the Ca⁺⁺-transporting ATPase of the sarcoplasmic reticulum of muscle cells (13). An understanding of the relationship

between the process of incorporation of the myelin PLP into the ER membrane and its final disposition with respect to the phospholipid bilayer in the myelin sheath will greatly benefit from the determination of the complete amino acid sequence of this protein. Partial sequence data (31, 32, 34, 42) indicated that the myelin PLP contains several hydrophobic segments, which probably traverse the myelin membrane. It seems likely that, as is apparently the case with other membrane proteins (c.f., 47), at least some of these segments serve as components of insertion or halt transfer signals that during synthesis establish a disposition in the ER that is maintained in the myelin sheath.

In agreement with the results of others (5, 6) we observed that newly synthesized PLP and MBPs are incorporated into developing myelin with different kinetics. We found that newly synthesized PLP reaches the myelinating processes of the oligodendrocyte only after a significant lag period. This interval is probably required for the transfer of the polypeptide from the ER to the specialized plasma membrane domains which surround the myelinating axons. The presence of covalently attached fatty acid molecules in PLP (23, 55) suggests that the polypeptide traverses the Golgi apparatus—the site where viral envelope glycoproteins appear to be similarly modified (49, 50) and therefore that it reaches the plasma membrane via Golgi-derived vesicles. Such vesicles could mediate incorporation of PLP into the plasma membrane at the cell body, which would require subsequent lateral diffusion of the polypeptide to regions surrounding an axon or, alternatively, could be transferred directly to the myelinating processes and deliver PLP only to those regions of the plasma membrane engaged in myelin formation.

In contrast to PLP the MBP's were found to be synthesized in free polysomes. A comparison of the ratios of translatable mRNAs for PLP and MBPs in brainstem white matter and in the crude myelin fraction revealed that polysomes containing MBP messenger RNA's are highly concentrated in the myelinating oligodendrocyte processes. Furthermore, when labeled in vivo, the MBPs did not become associated with the ER membranes but rather reached the myelin sheath without any detectable lag. These observations are consistent with the notion, derived from labeling studies with nonpenetrating probes (26, 45), that these peripheral proteins are associated with the cytoplasmic face of the plasma membrane. In mature myelin this disposition would place the MBPs at the major dense line, where they have been localized by immunocytochemical procedures (29).

MBPs are highly basic polypeptides that in vitro associate with acidic lipids (54) or with phospholipid vesicles (53). It would seem likely that the segregation of free polysomes synthesizing MBPs within the myelinating processes serves to prevent the nonspecific association of the newly synthesized polypeptides with intracellular membranes found in the cell body. Several mechanisms could be envisaged to establish and maintain a segregation of free polysomes synthesizing MBP's to the ensheathing regions of the oligodendrocyte. MBP mRNAs could specifically associate with cytoskeletal elements in the myelinating processes, and, once the segregation is established, nascent MBP polypeptides could directly interact with the sites of their deposition in the myelin sheath. An association of total cytoplasmic polysomal mRNA with cytoskeletal elements has been reported (12, 22) and it has been postulated that the protein-synthesizing machinery of all cells may be organized by such interactions. In this regard it should

be noted that the distribution of polysomes translating MBP mRNA's may reflect the distribution of all free polysomes in the oligodendrocyte.

The observation that all four MBPs represent primary translation products is of considerable interest. The known striking arrangement of identical peptide segments within the MBPs (4) suggests that independent messenger RNA's for these polypeptides may be derived from a single genomic arrangement of exons and introns, the primary transcripts of which can be processed along four different splicing pathways.

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