

Phosphate Groups Modifying Myelin Basic Proteins Are Metabolically Labile; Methyl Groups Are Stable

K. C. DESJARDINS and P. MORELL

Department of Biochemistry and Nutrition and Biological Sciences Research Center, University of North Carolina, Chapel Hill, North Carolina 27514

ABSTRACT Young and adult rats received intracranial injections of [^{33}P]orthophosphoric acid. The time course of the appearance and decay of the radioactive label on basic proteins in isolated myelin was followed for 1 mo. Incorporation was maximal by 1 h, followed by a decay phase with a half-life of approximately 2 wk. However, radioactivity in the acid-soluble precursor pool (which always constituted at least half of the total radioactivity) decayed with a similar half-life, suggesting that the true turnover time of basic protein phosphates might be masked by continued exchange with a long-lived radioactive precursor pool. Calculations based on the rate of incorporation were made to more closely determine the true turnover time; it was found that most of the phosphate groups of basic protein turned over in a matter of minutes. Incorporation was independent of the rate of myelin synthesis but was proportional to the amount of myelin present. Experiments in which myelin was subfractionated to yield fractions differing in degree of compaction suggested that even the basic protein phosphate groups of primarily compacted myelin participated in this rapid exchange. Similar studies were carried out on the metabolism of radioactive amino acids incorporated into the peptide backbone of myelin basic proteins. The metabolism of the methyl groups of methylarginines also was monitored using [*methyl*- ^3H]methionine as a precursor. In contrast to the basic protein phosphate groups, both the peptide backbone and the modifying methyl groups had a metabolic half-life of months, which cannot be accounted for by reutilization from a pool of soluble precursor. The demonstration that the phosphate groups of myelin basic protein turn over rapidly suggests that, in contrast to the static morphological picture, basic proteins may be readily accessible to cytoplasm *in vivo*.

Myelin is a compact, multilamellar extension of the membrane of specialized cells that surrounds nerve axons and functions to increase conduction velocity (for reviews, see references 15 and 43). The oligodendroglial cells in rat brain start producing myelin in substantial quantities between post-natal days 10 and 12. Myelin then accumulates at an increasingly rapid rate until 20 d of age, when the rates of synthesis and accumulation decrease progressively (47). Because of its high concentration of lipid (70%) and resulting low buoyant density, myelin of the central nervous system (CNS) is readily isolated by sucrose density gradient centrifugation (46). During the period of rapid myelin accumulation, it is possible to isolate subfractions of myelin that appear to represent different degrees of compaction and possibly different stages in the development of this specialized membrane system (3, 9, 34).

In mammals, one-third of the total myelin protein of the

CNS is present as a group of proteins that are closely related in sequence, the myelin basic proteins (12, 31). These proteins are located at the apposition of the very closely apposed cytoplasmic faces of the myelin membrane (48). The peptide backbones of the basic proteins have been shown to be metabolically very stable, with half-lives of many months (22, 53). This metabolic stability has been attributed in large part to the highly compacted, multilamellar structure of myelin, in which protein components may not be easily accessible to cytoplasm (13). If this is true, it is to be expected that any secondary modifying groups of these proteins may also be metabolically very stable.

Posttranslational modifications of the myelin basic proteins include methylation and phosphorylation. A cytosolic *S*-adenosylmethionine-myelin basic protein (arginine) methyltransferase catalyzes the conversion of a specific arginine

residue in a fraction of the basic protein molecules to either N^G -monomethylarginine or N^G, N'^G -dimethylarginine (5, 14, 16, 18, 26, 37, 38). It is not known whether basic protein methyl groups turn over independently of the peptide backbone, although it has been shown that methylarginine in chicken myelin basic protein is quite stable, turning over with an apparent half-life of ~ 40 d (54).

Phosphorylated basic protein from several mammalian species has been shown to contain 0.2 mol of phosphorus per mol of protein, of which at least 0.07 and 0.09 mol are associated with phosphoserine and phosphothreonine, respectively (32, 40). Both an endogenous kinase (32, 39, 40, 49, 55–57) and a phosphatase (36, 41) have been identified in purified myelin. Although the turnover rate of basic protein phosphate groups labeled by intracranial injection of [32 P]-phosphate into rodents has been studied *in vivo*, the interpretations of previously reported results have been contradictory, ranging from hours (40) to weeks (33) to possible stability (2). This report demonstrates that data from the previously published studies were not sufficient to draw quantitative conclusions of phosphate group turnover on myelin basic protein because, after intracranial injection, much of the radioactive phosphate may remain in a precursor pool for several weeks. We used a more appropriate experimental design that demonstrates that the turnover of myelin basic protein phosphate groups takes place in a matter of minutes, or even more rapidly. In contrast, the methyl groups of basic proteins were found to be as stable as the peptide backbone. Our findings suggest a need for the reassessment of the metabolic dynamics of myelin; it is possible that the tightly packed, multilamellar membrane stack is a more accessible structure than indicated by static morphological studies.

MATERIALS AND METHODS

Materials: [32 P]Orthophosphoric acid (carrier free), [$2\text{-}^3\text{H}$]glycine (30–60 Ci/mmol), [$\text{methyl-}^3\text{H}$]methionine (5–15 Ci/mmol), and Protosol were obtained from New England Nuclear (Boston, MA). [32 P]Orthophosphoric acid (carrier free) and [$\gamma\text{-}^{32}$ P]ATP were obtained from ICN (Irvine, CA). Radioactive compounds were prepared for injection by evaporation to dryness under a stream of nitrogen and resuspension in 0.9% (wt/vol) sodium chloride.

Thin-layer chromatography was carried out on precoated (250 μm thick) Analtech Silica Gel G plates (20 \times 20 cm) (Fisher Scientific Co., Raleigh, NC). Amino acid standards were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were reagent grade or as specified in the appropriate reference.

Long-Evans rats, either obtained from Charles River Breeding Laboratories (Wilmington, MA) or raised from these animals in our animal care facility, were used for all studies. Litters were reduced to 8 to 10 animals at birth and were weaned at 21 d of age.

Radioisotope Labeling: Rats were lightly anesthetized with ether and received intracranial injections of 15 μl of one of the isotope solutions described below. The injection was into the right cerebrum by means of a 50- μl syringe (Glenco Scientific, Inc., Houston, TX) equipped with a needle guard to stop penetration at 3 mm brain surface depth.

For study of the metabolism of myelin basic protein phosphate groups relative to the peptide backbone, the isotope solution contained both [32 P]-orthophosphoric acid as the precursor for protein phosphorylation and [$2\text{-}^3\text{H}$]glycine as the precursor for protein synthesis. (For studies longer than 1 d, ^{33}P was used in place of ^{32}P because of the severe radiotoxicity to the developing brain of ^{32}P at the required levels of radioactivity.) In one set of short-term experiments, the time-staggered injection of [32 P]orthophosphoric acid and [32 P]orthophosphoric acid was used. For study of the metabolism of basic protein methyl groups, the isotope solution contained [$\text{methyl-}^3\text{H}$]methionine as the precursor for protein methylation (via [$\text{methyl-}^3\text{H}$]S-adenosylmethionine) and for peptide backbone synthesis.

Preparation of Myelin: Rats were killed by decapitation. The forebrain was dissected and homogenized in 0.32 M sucrose. Aliquots were removed for determination of protein and of the distribution of radioactivity between

the 10% (wt/vol) trichloroacetic acid-soluble and -insoluble fractions. Myelin was isolated from the remainder of the homogenate by sucrose density gradient centrifugation (8, 46), lyophilized, and stored at -70°C .

Subfractionation of Myelin: Subfractions of myelin were prepared from crude myelin as described by Benjamins *et al.* (10). The procedure included centrifugation of a partially purified myelin preparation on a discontinuous sucrose gradient. Myelin membrane fractions were collected from the 0.32 M–0.52 M sucrose interface (subfraction A), the 0.52 M–0.63 M sucrose interface (subfraction B), the 0.63 M–0.72 M sucrose interface (subfraction C), and the pellet (subfraction D). The myelin subfractions were then lyophilized and stored at -70°C . The characterization of these subfractions has been described (1, 9, 34).

Polyacrylamide Gel Electrophoresis: Lyophilized myelin or myelin subfractions were delipidated with diethyl ether/absolute ethanol (3:2) and dissolved in 1% SDS (25). Protein concentration was determined. For determination of the distribution of radioactivity in myelin proteins, 800 μg of protein was subjected to discontinuous PAGE in buffers containing SDS (30) as described previously (25, 45). The procedure was modified by increasing the bisacrylamide concentration in the 15% acrylamide running gel solution so that the ratio of acrylamide/bisacrylamide was 20:1; this modification increased resolution in the low molecular weight region of the gel and provided better separation of radioactively labeled phospholipids from the basic proteins (the last slices of each gel containing the radioactive phospholipid were ignored for purposes of calculating radioactivity distribution of proteins). Cylindrical gels were cast in tubes with an 11-mm inner diameter to allow electrophoresis of a relatively large amount of protein. The gels were fixed with acetic acid/methanol/water (25) and sliced into 1-mm sections with an electric gel slicer (Hoefer Scientific Instruments, San Francisco, CA). The distribution of radioactivity across the gel was determined by liquid scintillation spectrometry after digestion of each slice with 0.6 ml Protosol and addition of 10 ml xylene containing 3 g of 2,5-diphenyloxazole per liter. When ^3H and ^{33}P were determined simultaneously, spillover of ^{33}P radioactivity into the ^3H channel was $\sim 15\%$; counting efficiencies were $\sim 40\%$ for ^3H and 70% for ^{33}P . When ^{33}P and ^{32}P were determined simultaneously, spillover of ^{32}P radioactivity into the ^{33}P channel was approximately 20%; counting efficiencies were $\sim 50\%$ for ^{32}P and 70% for ^{33}P . All samples were corrected for spillover and radioactive decay. Radioactivity in each basic protein was calculated by summing the radioactivities of all slices in each peak.

When specific radioactivities of the myelin proteins were to be determined, 100 μg of the protein sample used for radioactivity determination (as above) was subjected to electrophoresis in the gel system described above but with cylindrical gels with an inner diameter of 6 mm. The gels were fixed, stained with 1% acid Fast Green, and dye-binding distribution was determined by scanning at 635 nm with a spectrophotometer equipped with a linear transport system (Gilford Instrument Laboratories, Inc., Oberlin, OH). The scans were cut into individual protein peaks and each peak was weighed to quantitate the myelin protein distribution (44).

Myelin Basic Protein Extraction and Hydrolysis: For analysis of methyl group turnover, basic proteins were extracted from myelin and myelin subfractions with dilute HCl as described by Golds and Braun (24). In brief, the lyophilized myelin was partially delipidated with acetone (-20°C). The basic proteins were extracted from the acetone pellet with 0.1 N HCl (0°C) and recovered from the acid-soluble fraction by precipitation with acetone (-20°C). The acid-soluble, acetone-insoluble material (containing the basic proteins) was resuspended in water. Aliquots were taken for determination of protein and for PAGE. The remainder was lyophilized, oxidized with performic acid to convert methionine completely to methionine sulfone, and hydrolyzed in 6 N HCl at 110°C for 18 h (42). Methionine, N^G -monomethylarginine, and N^G, N'^G -dimethylarginine standards were subjected to the same treatment. The hydrolyzates were evaporated to dryness under nitrogen and resuspended in 0.1 N HCl.

Separation of Amino Acids: Amino acids were separated from the acid hydrolyzates by thin-layer chromatography using chloroform/methanol/17% ammonium hydroxide (2:2:1) as solvent, as described by Randerath (51). The regions of the plate containing methionine sulfone, N^G -monomethylarginine, and N^G, N'^G -dimethylarginine were located by comparison with standards and were scraped into scintillation vials. Radioactivity was determined by liquid scintillation counting after the addition of 0.5 ml of water and 10 ml of a toluene-Triton X-100-based scintillation mixture (58).

Other Analytical Methods: Aliquots of forebrain homogenates were precipitated with 10% trichloroacetic acid. Aliquots of the supernatant were taken for total phosphorus analysis (52) and for determination of radioactivity with ScintiVerse (Fisher Scientific Co., Fairlawn, NJ) as the scintillation solvent. The acid precipitable material was washed once with 5% (wt/vol) trichloroacetic acid, once with absolute ethanol, and once with diethyl ether. The pellet was dried, dissolved in 0.3 ml Protosol, and counted in 5 ml of

xylene containing 3 g of 2,5-diphenyloxazole per liter.

Protein determinations were carried out by the method of Lowry et al. (29) with bovine serum albumin as a standard.

RESULTS

Metabolism of Phosphate Groups

DECAY OF BRAIN POOLS OF RADIOACTIVE PRECURSORS: Rats either 18 or 60 d old were injected intracranially with [^{33}P]orthophosphoric acid and [$2\text{-}^3\text{H}$]glycine and were killed at various times over a 4-wk period. Acid soluble and acid insoluble radioactivity were determined at each time point (Fig. 1). The data are shown for only one age group, rats that had been injected at 18 d old; the curves are similar for rats that had been injected at 60 d of age. The amino acid label, [^3H]glycine, was rapidly incorporated into acid insoluble material and rapidly removed from the acid soluble pool (Fig. 1A); by 1 d after injection, acid-insoluble radioactivity in brain was much greater than acid-soluble radioactivity (Fig. 1A, inset). [^{33}P]Phosphate also was rapidly incorporated into acid-insoluble material; however, the percent of the total label incorporated into macromolecules was always much less than in the case of the amino acid label (Fig. 1B). Even at 4 wk after injection, radioactivity in the acid-soluble pool was greater than radioactivity in the insoluble pool (Fig. 1B, inset). The apparent half-life for removal of radioactive phosphate from the acid-soluble pool was calculated from the slope of a plot of the logarithm of the soluble radioactivity versus time after injection and was found to be ~ 16 d in rats injected at 18 d of age and 9 d in rats injected at 60 d of age. In contrast to the amino acid label, the fact that a significant amount of radioactive phosphate was present in the acid-soluble pool throughout the time-course of the study must be considered in any analysis of protein phosphate group turnover (see below).

APPARENT TURNOVER OF PHOSPHATE GROUPS AND PEPTIDE BACKBONE OF MYELIN BASIC PROTEINS: Groups of rats either 18 or 60 d old were injected intracranially with [^{33}P]orthophosphoric acid and [$2\text{-}^3\text{H}$]glycine. At times ranging from 1 d to 4 wk after injection, the animals were killed, and myelin was isolated from forebrain. Myelin proteins were separated by PAGE, and the distribution of radioactivity was quantitated. As expected, both ^3H and ^{33}P were incorporated into the four known molecular weight forms (6) of the myelin basic proteins (Fig. 2). It is well established that both radioactive phosphate (2) and radioactive amino acids (22, 53) that are biosynthetically incorporated into low molecular weight proteins ($<22,000$) in a

purified myelin fraction are in the basic proteins. It is of interest that the peak of incorporated ^{33}P showed a slight, but consistent, higher apparent molecular weight than the peak of incorporated ^3H in each of the four molecular weight forms. This is apparent only on gels with a high bisacrylamide concentration (see Materials and Methods); the resolution is lost on the 15% acrylamide, 0.4% bisacrylamide gels ordinarily used (as described in references 30 and 45). This apparent molecular weight shift may be a result of a slight anomaly of SDS binding by the phosphorylated form of the protein. One side effect of using the high bisacrylamide gels is that the resolution of the 21,500-mol wt protein from another (proteolipid protein related) peptide that migrates slightly ahead of proteolipid protein (1, 44) is lost. The overlap of this non-phosphorylated peptide with the 21,500-mol wt basic protein

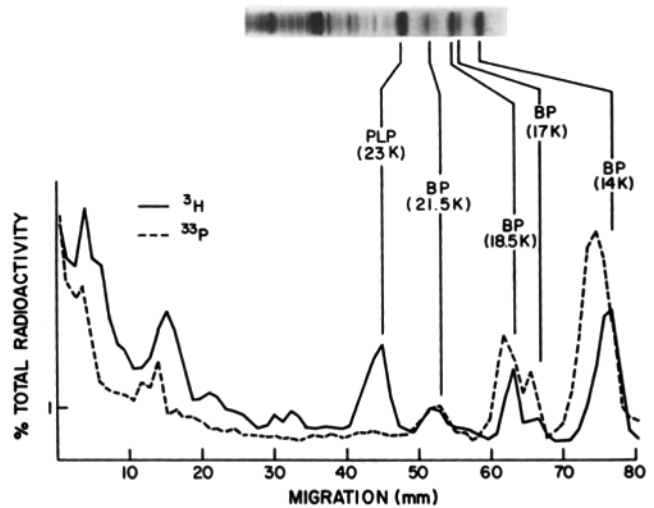


FIGURE 2 Electrophoretic separation of CNS myelin proteins. Rats were injected intracranially with both 200 μCi [$2\text{-}^3\text{H}$]glycine and 400 μCi [^{33}P]orthophosphoric acid, and forebrain myelin was isolated. Myelin proteins were separated by PAGE. A photograph of a Fast Green-stained gel is shown above a plot of the distribution of ^3H and ^{33}P radioactivity along the matched gel (see Materials and Methods). The last slices of the gel containing ^{32}P -labeled phospholipid were deleted from the plot, so the label on the ordinate refers only to protein bound radioactivity. These particular gels were from a 30-d-old rat killed 1 h after injection. The gel used to determine the distribution of radioactivity contained 37,142 ^3H cpm and 12,016 ^{33}P cpm. Major myelin proteins and their apparent molecular weights are indicated: proteolipid protein (PLP), 21,500-mol wt basic protein (21.5K BP), large basic protein (18.5K BP), 17,000-mol wt basic protein (17K BP), and small basic protein (14K BP).

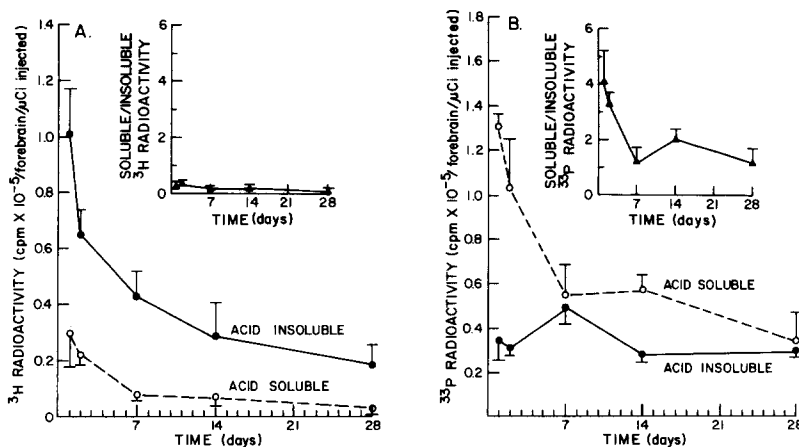


FIGURE 1 Distribution of radioactivity between acid-soluble and acid-insoluble material in forebrain at various times after the intracranial injection into 18-d-old rats of (A) 100 μCi [$2\text{-}^3\text{H}$]glycine and (B) 225 μCi [^{33}P]orthophosphoric acid. The change in ratio of soluble to insoluble radioactivity with time is shown in the insets. The points represent the mean \pm SD of three determinations.

probably accounts for the slightly lower $^{33}\text{P}/^3\text{H}$ ratio in this peak relative to that in the other proteins. The total radioactivity in the two major molecular weight forms of the basic proteins, 18,500 (large) and 14,000 (small), was determined at each time point. Since the 18,500- and 17,000-mol wt forms were not consistently well resolved, the two forms were considered together (see Discussion).

Apparent half-lives for turnover were calculated from the slope of a semi-log plot of total radioactivity versus time after injection (Fig. 3). In both 18- and 60-d old rats, ^3H glycine in the peptide backbone of both basic proteins remained stable during the study, with apparent half-lives much greater than the 4-wk course of the experiment. ^{33}P Phosphate groups on basic proteins in animals receiving injections when they were young (18 d) also appeared to be very stable, with half-lives greater than 4 wk. In contrast, ^{33}P phosphate groups on the basic proteins in animals injected when they were adults (60 d) appeared to turn over with a half-life on the order of 10–12 d. As discussed below, the calculated apparent turnover rates of basic protein phosphate groups may not accurately represent actual turnover rates.

CORRECTED TURNOVER OF PHOSPHATE GROUPS OF MYELIN BASIC PROTEINS: The interpretation of the turnover rate of basic protein phosphates must take into account the fact that radioactive phosphate was present in acid-soluble form throughout the study. It has long been known that much of the acid soluble phosphate pool of brain is in ATP and that intracranially injected ^{32}P phosphate equilibrates rapidly with the γ -phosphate of ATP (28). In younger, rapidly myelinating animals, continued incorporation of label from this precursor pool due to continuing myelin synthesis would be

an especially serious problem and might account for the apparent stability of basic protein phosphates in young animals. As shown in Fig. 4A, rats continue to synthesize a considerable amount of myelin for several weeks after injection at 18 d old. A correction was made for the contribution of label due to myelin basic protein synthesized during the experiment. All calculations were relative to the 1 d after injection time point and were based on the amount of myelin basic protein synthesized during the interval between time points (data from Fig. 4) and the specific radioactivity of the soluble pool of ^{33}P (data from Fig. 1). When the data for the apparent turnover of ^{33}P in basic proteins in young animals were corrected for the estimates of continued labeling due to synthesis, the apparent half-life of ^{33}P in basic proteins was on the order of 2 wk (Fig. 5). Note that this is close to the turnover of soluble ^{33}P (half-life of 16 d). A similar correction for myelin synthesis in adult animals was calculated and, as expected, was found to be negligible, since myelin is accumulating at only a very slow rate (Fig. 4A). Without the correction, the turnover of basic protein phosphates in adult animals was on the same order as that of the soluble pool of radioactive phosphate (half-life of 9–12 d).

A potentially even more serious complication due to the long-lasting presence of a significant precursor pool of radioactive phosphate is that the basic proteins may continue to be labeled through turnover of the phosphate groups. If the basic protein phosphate groups turned over with an actual half-life of much less than 2 wk, the apparent half-life would be detected as the same as the half-life of disappearance of radioactivity from the soluble pool of ^{33}P . In fact, the data indicate that this is the case; after correction for any accumulation of myelin, the basic protein phosphate groups in both rapidly and slowly myelinating rats had an apparent turnover rate on the same order as that of the radioactive precursor pool. This may differ from the real turnover rate by orders of magnitude. Thus, a different experimental approach, analysis of the incorporation of radioactive phosphorus into myelin, was used.

INCORPORATION OF ^{33}P PHOSPHATE INTO MYELIN BASIC PROTEINS DURING DEVELOPMENT: The incorporation of ^{33}P phosphate and ^3H glycine into basic proteins by 1 h after injection was studied in rats 18, 30, and 60 d old. Incorporation of ^3H glycine was assumed to reflect the developmental pattern of the rate of myelin basic protein accumulation because the label is relatively stable once in the peptide backbone (Fig. 3). As expected, either absolute incorporation (not shown) or incorporation per milligram of myelin protein (Fig. 6) was highest at 18 d, lower at 30 d, and lower still at 60 d old. In marked contrast, ^{33}P incorporation was not proportional to the rate of myelin synthesis but was proportional to the amount of myelin present in forebrain. This is clear because the ^{33}P phosphate in basic proteins per milligram of myelin protein is the same at the three ages (Fig. 6). These results indicate that injected ^{33}P phosphate rapidly exchanges with phosphate groups on basic proteins in pre-existing, presumably mature, myelin.

At all ages, by 1 h after injection of 400 μCi of ^{32}P -phosphate, the basic protein phosphates had reached nearly maximum radioactivity, $4,768 \pm 1,000$ cpm/mg of myelin protein ($n = 9$) as compared with $4,973 \pm 403$ cpm/mg myelin protein ($n = 5$) at 1 d after injection. Assuming that the precursor ATP was largely in equilibrium with the forebrain acid-soluble phosphate pool (determined to be $\sim 1.31 \times 10^6$

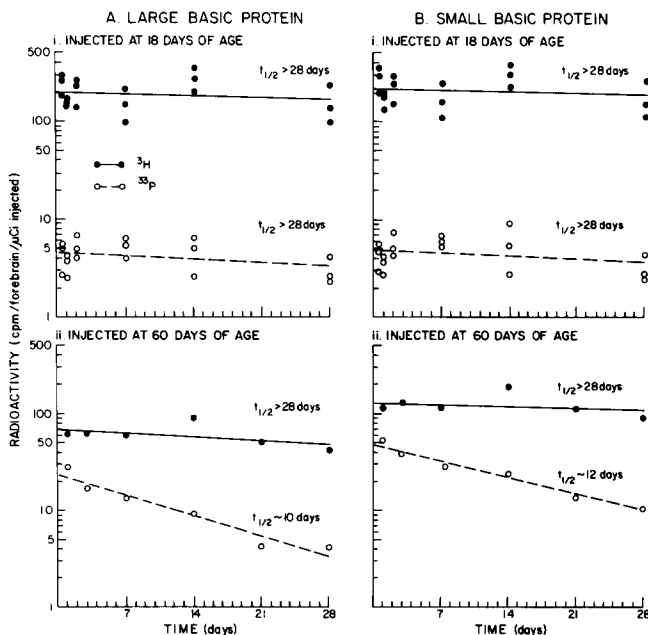


FIGURE 3 Decay of ^3H and ^{33}P radioactivity in (A) large and (B) small basic proteins with time after the intracranial injection of either 100 μCi [^3H]glycine and 225 μCi [^{33}P]orthophosphoric acid into 18-d-old rats (i) or 200 μCi [^3H]glycine and 400 μCi [^{33}P]orthophosphoric into 60-d-old rats (ii). Myelin proteins (800 μg) were separated by PAGE; radioactivity was determined for each band on the gel, and the results were used to calculate the total radioactivity for each basic protein in total isolated myelin. Each point represents a single determination. The lines were fit by linear regression.

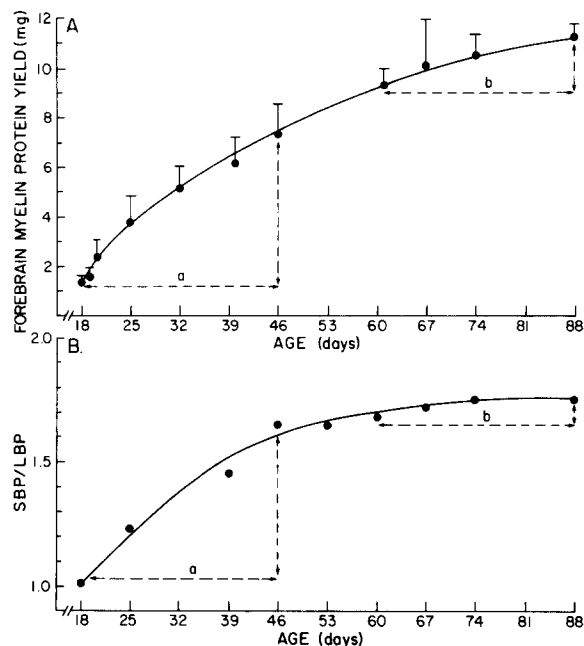


FIGURE 4 (A) The accumulation of myelin protein isolated from forebrain as a function of age. Each point represents the mean \pm SD of three determinations. (B) The change in ratio of the small basic protein (SBP) to the large basic protein (LBP) with age. The percent of each protein relative to total myelin protein was determined after electrophoresis of 100 μ g of myelin protein, Fast Green staining, and densitometry. The percent concentration of LBP relative to total myelin protein did not change significantly with age (not shown); therefore, the change in the SBP/LBP ratio with age represents primarily relative accumulation of SBP. Each point represents a single determination. The 4 wk after injection at 18 and 60 d of age are indicated by a and b, respectively.

cpm/ μ mol of phosphate at each age), we calculated that the rapid incorporation of radioactive phosphate into basic proteins corresponded to ~ 0.25 mol of phosphate per mole of basic protein. This value is in agreement with previous reports of the level of endogenous phosphorylation of the basic protein from several species (19, 40).

A more sensitive way of measuring the rate at which myelin basic protein is phosphorylated to its maximum specific activity is the double-label technique, which allows comparison of specific activities directly by measurement of isotope ratios. Animals 60 d old received intracranial injections of 300 μ Ci of [33 P]phosphate and were killed 1 wk later. At 5, 30, or 60 min or 24 h before death, the animals were injected with 300 μ Ci of [32 P]phosphate. The 32 P/ 33 P ratio was obtained for myelin basic proteins by isolation of myelin and, following PAGE, quantitation of radioactivity in basic protein-containing gel slices. The 32 P/ 33 P ratio so obtained was divided by the 32 P/ 33 P ratio of the acid-soluble supernatant. We expected that if the basic protein phosphate turnover were as rapid as indicated by the previous experiments and if equilibration were occurring with a radioactive precursor pool, the 1-wk period would allow complete equilibration of 33 P with myelin basic protein. After the injection of the second radiolabel, [32 P]phosphate, the isotope ratio for basic protein would approach that of the acid-soluble pool, with a time-course proportional to the rate of equilibration of basic protein phosphate groups with the whole-brain acid-soluble pool of phosphate. The values for the 32 P/ 33 P ratio in basic proteins relative to that in the acid-soluble supernatant were 0.54 at 5

min, 0.73 at 30 min, 0.95 at 60 min, and 1.01 at 24 h (average of results from two animals at each time, except that results from only one animal were available for the 60-min time point). This indicates that approximately half of the myelin

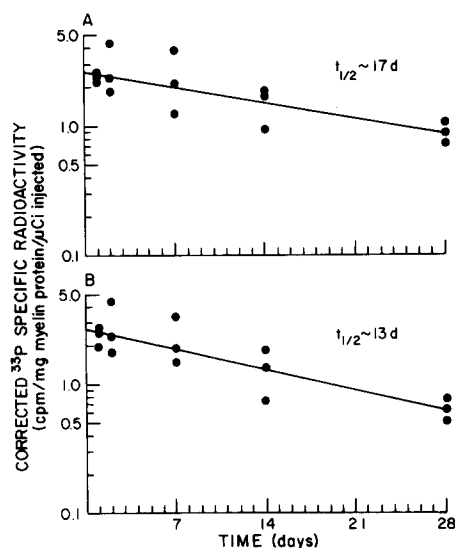


FIGURE 5 The corrected decay of radioactivity in the (A) large and (B) small basic proteins with time after the intracranial injection of 225 μ Ci [33 P]orthophosphoric acid into 18-d-old rats. The data presented in Fig. 3 have been corrected by a factor estimating the amount of radioactivity contributed by continuing myelin basic protein accumulation in the presence of a radioactive precursor pool. The following formula was used: corrected relative specific radioactivity equals $z/[x_0y_0 + \sum_{n=1}^N (x_ny_n)]$, where z = radioactivity in basic protein per total myelin isolated from forebrain, x_0 equals initial myelin protein yield (1 d after injection), y_0 equals 1 = initial fraction of radioactivity remaining in acid soluble pool, and x_n equals amount of myelin accumulated since the previous time point. (Although the accumulation of LBP is proportional to the accumulation of total myelin protein, the accumulation of SBP is not [see Fig. 4B]. Therefore, x must be multiplied by the ratio of SBP/LBP when SBP accumulation is being determined.) y_n equals average fraction of initial acid soluble pool remaining between time point n and time point $n-1$. N equals time point being considered (1st, 2nd, 3rd . . .). Each point represents a single determination. The lines were fit by linear regression.

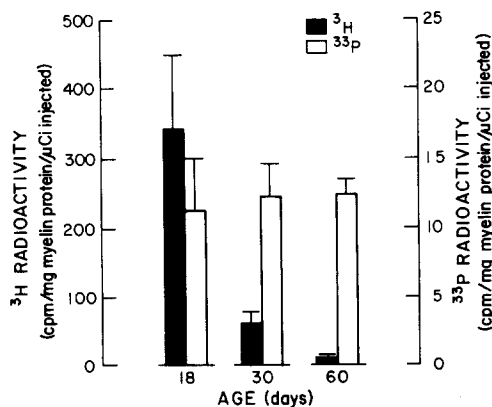


FIGURE 6 Radioactivity incorporated into the basic proteins per milligram of myelin protein at 1 h after the intracranial injection of 200 μ Ci [3 H]glycine and 400 μ Ci [33 P]orthophosphoric acid. Rats 18, 30, and 60 d old were used. Radioactivity in the basic proteins (LBP + SBP) was determined after electrophoresis. Bar, mean \pm SD of three determinations.

basic protein phosphate was available for exchange with the total acid-soluble pool of phosphate within 5 min, with complete equilibration occurring between 60 min and 1 d.

INCORPORATION OF [^{33}P]PHOSPHATE INTO BASIC PROTEINS OF MYELIN SUBFRACTIONS: As noted above and in reference 40, only a portion of the basic protein is phosphorylated, suggesting that the observed rapid incorporation of radioactive phosphate into basic proteins corresponded to preferential incorporation into less compacted regions of myelin, such as the lateral loop region or myelin recently deposited. There is evidence that myelin can be subfractionated by virtue of its heterogeneity in buoyant densities into fractions representing differing degrees of compaction and, possibly, different developmental stages in myelin synthesis (7, 9, 50). We designed an experiment to test whether myelin subfractions presumably enriched in uncompact and/or immature myelin (denser subfractions, subfractions C and D) had higher specific radioactivity than myelin subfractions enriched in more compacted, mature myelin (more buoyant subfractions, subfractions A and B) at very short times after the intracranial injection of radioactive phosphate. A double-isotope (^{32}P and ^{33}P) design was used because it allowed for the examination of two time points in the same animal.

Rat brains were labeled *in vivo* for 45 min with [^{33}P]orthophosphoric acid and for 5 or 15 min with [^{32}P]orthophosphoric acid. Subfractions of myelin were isolated, and radioactivity in the myelin basic proteins was determined after gel electrophoresis. By 5 min after injection, the specific radioactivity of phosphate in both large and small basic proteins was similar in all fractions (Fig. 7), indicating rapid and, within the 5-min time scale of the study, simultaneous incorporation of radioactive phosphate into the basic proteins isolated in all subfractions of myelin. The ratio of $^{32}\text{P}/^{33}\text{P}$ in basic proteins was the same in each subfraction (Fig. 8), confirming that the phosphorylation of basic proteins isolated in subfractions representing both compacted and uncompact myelin was occurring at similar rates.

Because equal amounts of ^{32}P and ^{33}P radioactivity were injected, the isotope ratio (following correction for differences in counting efficiency) should be an accurate indication of the relative incorporation of the two isotopes. As shown in Fig. 8, the incorporation of radioactive phosphate by 5 min after injection had already reached 60–70% of the incorporation at 45 min; by 15 min after injection, the incorporation had reached 90% of the incorporation at 45 min. This indicates very rapid incorporation of radioactive phosphate into basic proteins (half-life < 5 min).

CONTROLS FOR ARTIFACTS DURING THE PREPARATIVE PROCEDURES: To determine whether any incorporation of radioactive phosphate into basic proteins was occurring during the isolation procedure, an unlabeled rat forebrain was homogenized in sucrose containing 35 μCi [$\gamma\text{-}^{32}\text{P}$]ATP. Myelin was isolated, and the amount of radioactivity in the basic proteins was determined. We found that the incorporation of radioactive phosphate into the basic proteins during the isolation was <3% of the *in vivo* labeling and concluded that it was insignificant.

To control for preferential dephosphorylation (loss of basic protein phosphate greater than could be accounted for by losses in recovery of total basic protein), a rat was injected intracranially with [^3H]glycine and [^{33}P]phosphate. The rat was killed 1 d later, and myelin was isolated. A portion of the

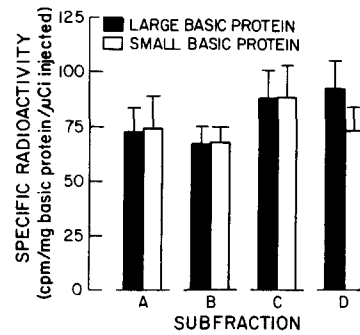


FIGURE 7 Specific radioactivity of the large and small basic proteins in subfractions of myelin 5 min after injection of 200 μCi [^{32}P]orthophosphoric acid. The radioactivity in each protein was determined after the electrophoretic separation of myelin subfraction protein. The basic protein concentration in each subfraction was determined after electrophoresis of myelin proteins, Fast Green staining, and densitometry with correction for differential dye-binding capacity (see Materials and Methods). Bar, mean \pm SD of three determinations.

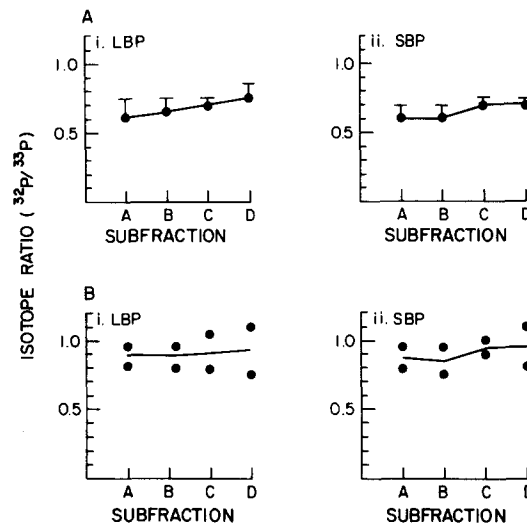


FIGURE 8 Isotope ratio in basic protein of myelin subfractions either 5 (A) or 15 min (B) after the intracranial injection of 200 μCi [^{32}P]orthophosphoric acid and 45 min after the intracranial injection of 200 μCi [^{33}P]orthophosphoric acid. For each experiment, four 18-d old rats were injected intracranially with 200 μCi [^{33}P]orthophosphoric acid, then either 40 (A) or 30 min (B) later with 200 μCi [^{32}P]orthophosphoric acid. After 5 (A) or 15 min (B), the rats were killed and the brains were pooled. Myelin subfractions were isolated as described in Materials and Methods. Radioactivity in the large (i) and small (ii) basic proteins was determined after the electrophoretic separation of the proteins from each subfraction. Differences in counting efficiency between ^{32}P and ^{33}P were accounted for by calculating the data on the basis of dpm. The points in panels *i* and *ii* represent the mean \pm SD of three such experiments. The points in panels *Bi* and *Bii* represent single determinations; the lines connect the means. Pair differences between fractions were calculated for each experiment by Student's *t* test; no significant differences were found.

myelin was lyophilized, and myelin proteins were separated by electrophoresis. The isotope ratio in the basic proteins was determined. The remainder of the myelin was resuspended in 0.32 M sucrose and homogenized with an unlabeled rat forebrain. Myelin was isolated from this homogenate, and myelin proteins were again separated by electrophoresis. The

isotope ratio in the basic proteins from this reisolated myelin was determined and found not to differ significantly from the isotope ratio in the basic proteins from the first isolation, indicating that basic proteins were not dephosphorylated during the isolation procedure.

Metabolism of Methyl Groups

DECAY OF BRAIN POOLS OF THE RADIOACTIVE PRECURSOR: Rats were injected intracranially with [*methyl-³H*]methionine and were killed at various times over a 12-wk period. Acid-soluble and acid-precipitable radioactivity in the forebrain homogenate were determined at each time point. Radioactivity was rapidly incorporated into acid-insoluble material and was even more rapidly removed from the acid-soluble pool than was [³H]glycine (data not shown). By 1 d after injection, >95% of the radioactivity in brain was found in macromolecular material.

TURNOVER OF METHYL GROUPS ON MYELIN BASIC PROTEINS: Groups of rapidly myelinating (22 d of age) and slowly myelinating (60 d old) rats were injected intracranially with [*methyl-³H*]methionine. The animals were killed and forebrain myelin was isolated at times ranging from 1 d to 12 wk after injection. Basic proteins were purified by acid extraction; gel electrophoresis of this material indicated that >95% of the radioactivity coelectrophoresed with the basic protein bands. The basic protein extract was hydrolyzed and then oxidized, and the amino acids were separated by thin-layer chromatography. Methionine sulfone (the oxidized form of methionine), *N*^G-monomethylarginine, and *N*^G,*N*^G-dimethylarginine were resolved from each other with relative migrations of 0.51, 0.17, and 0.25, respectively. The rate of decay of radioactivity in methionine was determined as a measure of basic protein peptide backbone turnover. The rate of decay of radioactivity in the methylarginines was determined as a measure of basic protein methyl group turnover. When rats were given injections at 22 d old, the apparent half-lives for ³H in both basic protein methionine and methylarginines were found to be much longer than the 12-wk study period (Fig. 9A). When rats received injections at 60 d of age, the apparent half-life for ³H in methionine was found to be 11 wk, whereas that for ³H in methylarginines appeared to be longer than the 12-wk study period (Fig. 9B). These results indicate that myelin basic protein methyl groups in both young and adult rats are as stable as the peptide backbone to which they are bound. In fact, in adult rats the label in methyl groups has an apparent half-life greater than the label in backbone methionine (see Discussion).

INCORPORATION OF [³H]METHYL GROUPS INTO MYELIN BASIC PROTEINS DURING DEVELOPMENT: Because the results indicate that, unlike phosphate groups, the methyl groups of myelin basic protein are as stable as the peptide backbone, we expected that the rate of incorporation of methyl groups into arginines of myelin basic protein would be proportional to the rate of myelin synthesis. To test this we designed an experiment with internal controls by taking advantage of the fact that [*methyl-³H*]methionine is incorporated into both the backbone (where it serves as a measure of the rate of myelin protein synthesis) and into the methyl groups of methylarginine. Rats 22, 30, and 60 d old were injected with [*methyl-³H*]methionine. 20 h later, the rats were killed, and myelin was isolated. Basic proteins were extracted and hydrolyzed. Radioactivity in methionine and methylarginines was determined. It was expected that if methylation

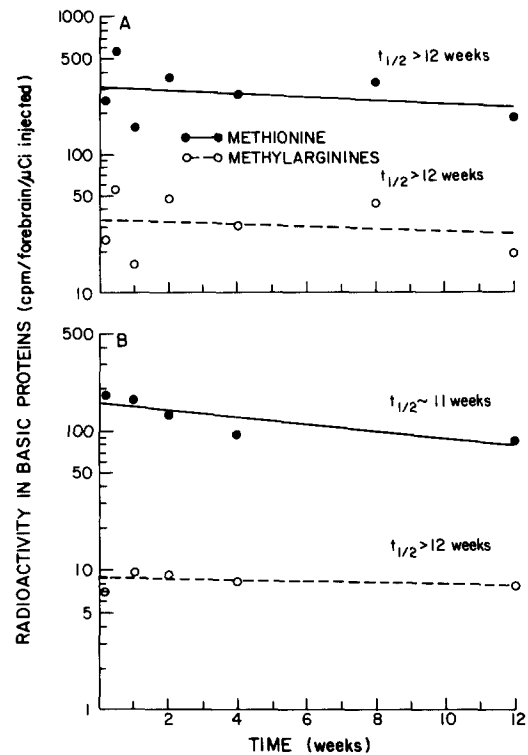


FIGURE 9 Decay of radioactivity in methionine and methylarginines of basic proteins with time after the intracranial injection of (A) 500 μ Ci [*methyl-³H*]methionine into 22-d-old rats or (B) 700 μ Ci [*methyl-³H*]methionine into 60-d-old rats. Basic proteins were isolated by acid extraction from forebrain myelin and were hydrolyzed to constituent amino acids. Radioactivity in methionine and mono- and dimethylarginine was determined and used to calculate the total radioactivity in each amino acid in total basic protein isolated. Each point represents a single determination. The lines were fit by linear regression.

paralleled the rate of accumulation of myelin, the incorporation of ³H into methionine of the peptide backbone relative to incorporation into methylarginine would be similar at different ages, even though the rate of myelin accumulation was changing. This was indeed the case (Fig. 10). The counterhypothesis, that methylation is proportional to the amount of myelin present in forebrain, is clearly not correct, because if it were the incorporation of ³H into methylarginine relative to incorporation of ³H into methionine should increase more than 20-fold during development (as it does in the case of phosphate incorporation; see Fig. 10). A minor flaw in the experimental design is the possibility that the pools of metabolic intermediates between [*methyl-³H*]methionine and the methylarginines (for example, *S*-adenosylmethionine) vary during development in such a way as to convert data from an unexpected mechanism to give the anticipated results; but this seems unlikely.

DISCUSSION

Peptide Backbone of Myelin Basic Protein Is Metabolically Very Stable

We have shown that the basic protein peptide backbone, labeled by [³H]glycine or [³H]methionine, is metabolically very stable, confirming several previous reports (17, 22, 53). The basic proteins appeared to be slightly more stable in rats injected at 18–22 d of age than in rats injected at 60 d old.

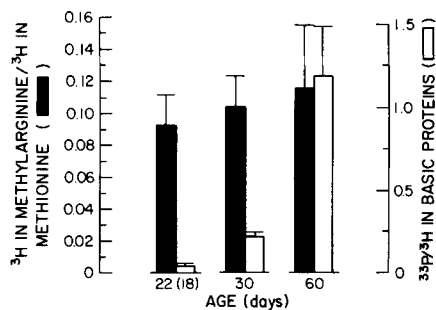


FIGURE 10 A comparison of developmental changes in the incorporation of radioactive methyl and phosphate groups into basic proteins in myelin relative to the incorporation of radioactive amino acids. 22-, 30-, and 60-d old rats were injected intracranially with 700 μCi [*methyl*- ^3H]methionine. Myelin was isolated 20 h later, and basic proteins were extracted. The ratio of radioactivity in methylarginines to the radioactivity in methionine of basic proteins was determined. For comparison, data for phosphate groups (see Fig. 6) are presented as the ratio of $^{33}\text{P}/^3\text{H}$ radioactivity incorporated into basic proteins (LBP + SBP) at 18, 30, and 60 d of age. Bar, mean + SD of three determinations.

Myelin Basic Protein Methyl Groups Are Metabolically Very Stable

The methyl groups of basic protein were found to be as stable as the peptide backbone to which they are bound. In adults, the apparent half-life of label in methyl groups is greater than that of the label in the peptide backbone (Fig. 9). It seems unlikely that this is a result of continued reutilization of labeled methyl groups, inasmuch as myelin is synthesized at only a very slow rate during this time period (Fig. 4). One possible explanation for this is that the fraction of the basic protein that is methylated (as reflected by labeled methylarginine) may be slightly more stable than the bulk of the basic protein (as reflected by labeled methionine). Aspillaga and McDermott (4) showed that the methylarginines of basic protein accumulate in myelin to a greater extent than basic proteins, providing indirect evidence that methylated basic proteins are more stable than nonmethylated basic proteins.

Myelin Basic Protein Phosphate Groups Turn Over Very Rapidly

Radioactive phosphate was incorporated into myelin proteins with approximate molecular weights of 21,500, 18,500, 17,000, and 14,000. Agrawal et al. (2) showed that radioactive phosphate in that molecular weight range, as determined by PAGE in the presence of SDS, was covalently bound to serine and threonine residues of the four molecular weight forms of basic protein. To simplify study of the metabolism of these phosphate groups, quantitative data were collected for only the two major forms of basic protein, the 18,500- (large) and 14,000-mol wt (small) basic proteins. It is the large basic protein that is almost identical in sequence to human myelin basic protein. The small basic protein differs from the large basic protein by a deletion of 40 amino acids in the interior of the sequence. As noted in Results, the large basic protein (18,500 mol wt) was consistently contaminated with one of the minor forms of basic protein (see Fig. 2), the 17,000-mol wt protein. The 17,000-mol wt protein appeared to be labeled to the same specific radioactivity as the other myelin proteins (our data, not shown; see also reference 2). Since we do not attempt to make an argument for any differences in metabo-

lism of the phosphate groups among the basic proteins, contamination of the 18,500-mol wt protein by the minor 17,000-mol wt protein was not considered a problem.

We have shown that a precursor pool of radioactive phosphate, with which basic protein phosphates could exchange, persists long after intracranial injection. The continued presence of this pool renders previously published turnover studies in brain (2, 33, 40) difficult to interpret. Although the results of the turnover studies indicate that the half-life of basic protein phosphate groups in both rapidly and slowly myelinating rats is on the order of 2 wk, as is the half-life of the brain pool of acid-soluble radioactive phosphate, we conclude that turnover could be much faster and would not be detected because of exchange with the radioactive precursor pool.

Studies of the rate of incorporation of radioactively labeled phosphate groups into the basic proteins were undertaken to test whether the turnover rate was more rapid than detected by monitoring the rate of removal of the radiolabel. We found that the incorporation of radioactive phosphate into basic proteins had peaked before 1 h from the time of injection. The incorporation was independent of the rate of myelin basic protein synthesis but was proportional to the amount of myelin protein, and presumably to the amount of basic protein, present in brain (Fig. 6). These results suggest that phosphate groups on preexisting basic proteins in mature myelin, possibly in cytoplasmic incisures, were involved in the exchange. Studies of the incorporation of radioactive phosphate into basic proteins isolated in different subfractions of myelin provided further evidence that the turnover of phosphate groups on basic proteins in mature, multilamellar myelin and uncompacted myelin is very rapid (half-life <5 min).

The actual turnover time may be even more rapid than 5 min, but detection would be limited by the rate at which intracranially injected (extracellular) radioactive orthophosphoric acid equilibrates with the labile phosphates of intracellular ATP. Lindberg and Ernster (28) showed that intracranially injected [^{32}P]phosphate exchanges rapidly with the labile phosphates of ATP (half-life <5 min), with the two pools reaching virtual equilibrium by 45 min after injection (kinetics similar to those for basic protein phosphorylation). However, the phosphates in the γ position of ATP (a very large pool, since in brain there is almost twice as much phosphate in ATP as there is in free orthophosphate; reference 35) are very metabolically active, with half of these phosphates turning over in about 3 s (23, 27).

In conclusion, radioactively labeled phosphate groups on myelin basic proteins turn over very rapidly in both rapidly and slowly myelinating rats and in both immature and mature myelin sheaths. This suggests a tight coupling in the myelin membrane of the kinase, phosphatase, and phosphorylated basic protein, as well as ready accessibility to cytoplasmic substrates. Incorporation of radioactive phosphate was proportional to the amount of basic protein present and was calculated to be ~ 0.25 mol of phosphate per mol of basic protein. If that represents all of the basic protein phosphate, as it does for several species, it appears that all of the phosphate groups may be involved in rapid exchange. Although the function of the phosphorylation of the basic proteins is not known, it seems unlikely that it is involved directly in myelin synthesis and assembly, because the metabolism of the phosphate groups is as rapid in slowly myelinating animals as it is in rapidly myelinating animals. It also seems unlikely that it

is involved in maintaining any structurally stable contacts in compact myelin. In fact, it is possible that phosphorylation and dephosphorylation of myelin basic protein may be involved in keeping cytoplasmic incisures open in the otherwise compact structure of myelin. The only published reports on an analogous extremely rapid metabolism of a myelin component are those describing the exchange of the monoesterified polyphosphoinositide phosphates (20, 21).

We thank Drs. Jeffery Goodrum, Arrel Toews, John Wilson, and Gerhard Meissner for discussions and assistance.

This research was supported by U. S. Public Health Service grants NS11615 and HDO3110.

Received for publication 29 November 1982, and in revised form 15 March 1983.

REFERENCES

- Agrawal, H. C., R. M. Burton, M. A. Fishman, R. F. Mitchell, and A. L. Prensky. 1972. Partial characterization of a new myelin protein component. *J. Neurochem.* 19:2083-2089.
- Agrawal, H. C., K. O'Connell, C. L. Randle, and D. Agrawal. 1982. Phosphorylation in vivo of four basic proteins of rat brain myelin. *Biochem. J.* 201:39-47.
- Agrawal, H. C., J. L. Trotter, R. M. Burton, and R. Mitchell. 1974. Metabolic studies on myelin: evidence for a precursor role of a myelin subfraction. *Biochem. J.* 140:99-109.
- Aspillaga, M. D., and J. R. McDermott. 1977. The N⁰-methylated arginine content of rat myelin during development. *J. Neurochem.* 28:1147-1149.
- Baldwin, G. S., and P. R. Carnegie. 1971. Specific enzymic methylation of an arginine in the experimental allergic encephalomyelitis protein from human myelin. *Science (Wash. DC)*. 171:579-581.
- Barbarese, E., P. E. Braun, and J. H. Carson. 1977. Identification of prelarge and presmall basic proteins in mouse myelin and their structural relationship to large and small basic proteins. *Proc. Natl. Acad. Sci. USA*. 74:3360-3364.
- Benjamins, J. A., M. Gray, and P. Morell. 1976. Metabolic relationships between myelin subfractions: entry of proteins. *J. Neurochem.* 27:571-575.
- Benjamins, J. A., M. Jones, and P. Morell. 1975. Appearance of newly synthesized protein in myelin of young rats. *J. Neurochem.* 24:1117-1122.
- Benjamins, J. A., K. Miller, and G. M. McKhann. 1973. Myelin subfractions in developing rat brain. *J. Neurochem.* 20:1589-1603.
- Benjamins, J. A., S. L. Miller, and P. Morell. 1976. Metabolic relationships between myelin subfractions: entry of galactolipids and phospholipids. *J. Neurochem.* 27:565-570.
- Benjamins, J. A., and P. Morell. 1977. Assembly of myelin. In *Mechanisms, Regulation and Special Functions of Protein Synthesis in the Brain*. S. Roberts, A. Lajtha, and W. H. Gispen, editor. Elsevier/North-Holland Biomedical Press, New York. 183-197.
- Benjamins, J. A., and P. Morell. 1978. Proteins of myelin and their metabolism. *Neurochem. Rev.* 3:137-174.
- Benjamins, J. A., and M. E. Smith. 1977. Metabolism of myelin. In *Myelin*. P. Morell, editor. Plenum Press, New York. 233-270.
- Brostoff, S., and E. H. Eylar. 1971. Localization of methylated arginine in the A1 protein from myelin. *Proc. Natl. Acad. Sci. USA*. 68:765-769.
- Bunge, R. P. 1968. Glial cells and the central myelin sheath. *Physiol. Rev.* 48:197-251.
- Crang, A. J., and W. Jacobson. 1982. The relationship of myelin basic protein (arginine) methyltransferase to myelination in mouse spinal cord. *J. Neurochem.* 39:244-247.
- Davison, A. N. 1961. Metabolically inert proteins of the central and peripheral nervous system, muscle and tendon. *Biochem. J.* 78:272-282.
- Deibler, G. E., and R. E. Martenson. 1973. Determination of methylated basic amino acids with the amino acid analyzer: application to total acid hydrolyzates of myelin basic proteins. *J. Biol. Chem.* 248:2387-2391.
- Deibler, G. E., R. E. Martenson, A. J. Kramer, M. W. Kies, and E. Miyamoto. 1975. The contribution of phosphorylation and loss of COOH-terminal arginine to the microheterogeneity of myelin basic protein. *J. Biol. Chem.* 250:7931-7938.
- Deshmukh, D. S., S. Kuizon, W. D. Bear, and H. Brockerhoff. 1981. Rapid incorporation in vivo of intracerebrally injected ³²P into polyphosphoinositides of three subfractions of rat brain myelin. *J. Neurochem.* 36:594-601.
- Eichberg, J., and R. M. C. Dawson. 1965. Polyphosphoinositides in myelin. *Biochem. J.* 96:644-650.
- Fischer, C. A., and P. Morell. 1974. Turnover of proteins in myelin and myelin-like material of mouse brain. *Brain Res.* 74:51-65.
- Gatfield, P. D., O. H. Lowry, D. W. Schulz, and J. V. Passonneau. 1966. Regional energy reserves in mouse brain and changes with ischaemia and anaesthesia. *J. Neurochem.* 13:185-195.
- Golds, E. E., and P. E. Braun. 1978. Cross-linking studies on the conformation and dimerization of myelin basic protein in solution. *J. Biol. Chem.* 253:8171-8177.
- Greenfield, S., W. T. Norton, and P. Morell. 1971. Quaking mouse: isolation and characterization of myelin protein. *J. Neurochem.* 18:2119-2128.
- Jones, G. M., and P. R. Carnegie. 1974. Methylation of myelin basic protein by enzymes from rat brain. *J. Neurochem.* 23:1231-1237.
- Lajtha, A. L., H. S. Maker, and D. D. Clarke. 1981. Metabolism and transport of carbohydrates and amino acids. In *Basic Neurochemistry*. G. J. Siegel, R. W. Albers, B. W. Agranoff, and R. Katzman, editors. Little, Brown and Co., Boston. 329-353.
- Lindberg, O., and L. Ernster. 1950. The turnover of radioactive phosphate injected into the subarachnoid space of the brain of the rat. *Biochem. J.* 46:43-47.
- Lowry, O. H., N. J. Rosebrough, A. J. Farr, and K. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
- Maizel, J. V. 1971. Polyacrylamide gel electrophoresis of viral proteins. *Methods Virol.* 5:179-246.
- Martenson, R. E. 1980. Myelin basic protein: what does it do? In *Biochemistry of Brain*. S. Kumar, editor. Pergamon Press, Ltd., New York. 49-79.
- Martenson, R. E., M. J. Law, and G. E. Deibler. 1983. Identification of multiple *in vivo* phosphorylation sites in rabbit myelin basic protein. *J. Biol. Chem.* 258:930-937.
- Mathieu, J. M., and A. D. Kuffer. 1978. *In vivo* incorporation of ³²P into myelin basic protein from normal and quaking mice. In *Advances in Experimental Medicine and Biology: Myelination and Demyelination*. J. Palo, editor. Plenum Press, New York. 159-170.
- Mathieu, J. M., R. H. Quarles, R. O. Brady, and H. deF. Webster. 1973. Variation of proteins, enzyme markers, and gangliosides in myelin subfractions. *Biochim. Biophys. Acta.* 329-305-317.
- Mellwain, H., and H. S. Bachelard. 1971. The brain and the body as a whole. In *Biochemistry and the Central Nervous System*. (H. Mellwain and H. S. Bachelard, editors). The Williams & Wilkins Co., Baltimore. 540-563.
- McNamara, J. O., and S. H. Appel. 1977. Myelin basic protein phosphatase activity in rat brain. *J. Neurochem.* 29:27-35.
- Miyake, M. 1975. Methylases of myelin basic protein and histone in rat brain. *J. Neurochem.* 24:909-915.
- Miyake, M., and Y. Kakimoto. 1973. Protein methylation by cerebral tissue. *J. Neurochem.* 20:859-871.
- Miyamoto, E. 1976. Phosphorylation of endogenous proteins in myelin of rat brain. *J. Neurochem.* 26:573-577.
- Miyamoto, E., and S. Kakiuchi. 1974. *In vitro* and *in vivo* phosphorylation of myelin basic protein by exogenous and endogenous adenosine 3':5'-monophosphate-dependent protein kinases in brain. *J. Biol. Chem.* 249:2769-2777.
- Miyamoto, E., and S. Kakiuchi. 1975. Phosphoprotein phosphatases for myelin basic protein in myelin and cytosol fractions of brain. *Biochim. Biophys. Acta.* 384:458-465.
- Moore, S. 1963. On the determination of cystine as cysteic acid. *J. Biol. Chem.* 238:235-237.
- Morell, P. 1977. Myelin. Plenum Press, New York.
- Morell, P., S. Greenfield, C. Constantino-Ceccarini, and H. Wisniewski. 1972. Changes in the protein composition of mouse brain myelin during development. *J. Neurochem.* 19:2545-2554.
- Morell, P., R. C. Wiggins, and M. T. Gray. 1975. Polyacrylamide gel electrophoresis of myelin proteins: a caution. *Anal. Biochem.* 68:148-154.
- Norton, W. T., and S. E. Poduslo. 1973. Myelination in rat brain: method of myelin isolation. *J. Neurochem.* 21:749-757.
- Norton, W. T., and S. E. Poduslo. 1973. Myelination in rat brain: changes in myelin composition during brain maturation. *J. Neurochem.* 21:759-773.
- Omlin, F. X., H. deF. Webster, C. G. Palkovits, and S. R. Cohen. 1982. Immunocytochemistry localization of basic protein in major dense line regions of central and peripheral myelin. *J. Cell Biol.* 95:242-248.
- Petrali, E. H., B. J. Thiessen, and P. V. Sulakhe. 1980. Magnesium ion-dependent, calcium ion-stimulated, endogenous protein kinase-catalyzed phosphorylation of basic proteins in myelin fraction of rat brain white matter. *Int. J. Biochem.* 11:21-36.
- Quarles, R. H. 1980. The biochemical and morphological heterogeneity of myelin and myelin-related membranes. In *Biochemistry of Brain*. S. Kumar, editor. Pergamon Press, Ltd., New York. 81-102.
- Randerath, K. 1968. Amino acids, amino acid derivatives, peptides and proteins. In *Thin-Layer Chromatography*. K. Randerath, editor. Academic Press, New York. 110-128.
- Rouser, G., S. Fleischer, and A. Yanamoto. 1966. Two dimensional thin layer chromatographic separation of polar lipids and determination of phosphorus analysis of spots. *Lipids.* 5:494-496.
- Shapira, R., M. R. Wilhelmi, and R. F. Kibler. 1981. Turnover of myelin proteins of rat brain, determined in fractions separated by sedimentation in a continuous sucrose gradient. *J. Neurochem.* 36:1427-1432.
- Small, D. H., and P. R. Carnegie. 1982. *In vivo* methylation of an arginine in chicken myelin basic protein. *J. Neurochem.* 38:184-190.
- Steck, A. J., and S. H. Appel. 1974. Phosphorylation of myelin basic protein. *J. Biol. Chem.* 249:5416-5420.
- Sulakhe, P. V., E. H. Petrali, E. R. Davis, and B. J. Thiessen. 1980. Calcium ion stimulated endogenous protein kinase catalyzed phosphorylation of basic proteins in myelin subfractions and myelin-like membrane fraction from rat brain. *Biochemistry.* 19:5363-5371.
- Turner, R. S., C. H. Jen Chou, R. F. Kibler, and J. F. Kuo. 1982. Basic protein in brain myelin is phosphorylated by endogenous phospholipid-sensitive Ca²⁺-dependent protein kinase. *J. Neurochem.* 39:1397-1404.
- Wiggins, R. C., S. L. Miller, J. A. Benjamins, M. R. Krigman, and P. Morell. 1976. Myelin synthesis during postnatal nutritional deprivation and subsequent rehabilitation. *Brain Res.* 107:257-273.