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Studies on myelin-basic-protein methylation during mouse brain development

Latika P. CHANDERKAR, Woon Ki PAIK and Sangduk KIM*
Fels Research Institute, Temple University School of Medicine, Philadelphia, PA 19140, U.S.A.

The synthesis and methylation *in vivo* of myelin basic protein (MBP) during the mouse brain development has been investigated. When mice ranging in age from 13 to 60 days were injected intracerebrally with L-[*methyl*-^{3}H]methionine, the incorporation of radioactivity into MBP isolated from youngest brain was found to be the highest and declined progressively in mature brains. This pattern of radioactivity incorporation was inversely correlated with the total amount of MBP in the brains, suggesting a higher ratio of MBP methylation to synthesis in younger brain. To differentiate the relative rate of protein synthesis and methylation, animals were given intracerebral injections of a L-[*methyl*-^{3}H]methionine and L-[^{35}S]methionine mixture and the ratio of ^{3}H/^{35}S (methylation index) was determined. The ratios in the isolated MBP fractions were higher than those of 'acid extracts' and 'breakthrough' fractions, with a maximal ratio in the youngest brain. This high ratio was well correlated with the higher protein methylase I (PMI) activity in younger brains. The MBP fractions were further separated on SDS/polyacrylamide-gel electrophoresis into several species with apparent M_r ranging from 32400 to 14500. The results indicated that each protein species accumulated at a characteristic rate as a function of age. The high-M_r (32400) species was predominant in younger brain, whereas the smaller MBP was the major species in older brain tissue. The importance of this developmental pattern of MBP synthesis and methylation is discussed in relation to PMI activity.

INTRODUCTION

In the central nervous system, myelin is produced by the oligodendrocytes. The major structural proteins of myelin are proteolipid and MBPs, with the latter comprising about 30–40% of the total myelin protein. In mouse brain, myelin first appears at about 10 days after birth and accumulates rapidly until 30 days, and declines thereafter (Morell *et al.*, 1972; Barbarese *et al.*, 1978; Campagnoni *et al.*, 1978). The myelination of brain is an important postnatal development in brain. During the myelination period, MBP accumulates at a maximum rate in the early stages of life and slower at the later stages. Mouse myelin contains four structurally related forms of MBP with apparent M_r values of 21500, 18500, 17000 and 14000 (Barbarese *et al.*, 1977). The amino acid sequence of all these species were found to have a greater deal of homology. The ratio of these four MBPs in myelin changes drastically during active myelination, going from 1:5:2:10 to 1:10:3.5:35 in young and adult myelin respectively (Barbarese *et al.*, 1978).

In 1971 the presence of methylated arginine in MBP from bovine brain was reported (Baldwin & Carnegie, 1971; Brostoff & Eylar, 1971), and this occurrence of methylarginine at position 107 of MBP was shown to be catalysed by a transmethylation reaction to the preformed MBP (Baldwin & Carnegie, 1971; Paik & Kim, 1980). It had been postulated that the introduction of a methyl group to the side chain of an amino acid induces the hydrophobicity of the protein (Brostoff & Eylar, 1971), thus influencing the interaction of MBP and phospholipid molecules in the formation of the myelin sheath. Using inhibitors of transmethylation, Dinn *et al.* (1980) and Jacobson *et al.* (1973) have demonstrated that perturbation of methylation *in vivo* induces malformation of myelin.

The methylation of arginine residues in proteins is catalysed by the enzyme protein methylase I (PMI; *S*-adenosyl-L-methionine:protein-L-arginine *N*-methyltransferase, EC. 2.1.1.23) (Paik & Kim, 1968; Lee *et al.*, 1977). It has been demonstrated that the enzyme responsible for methylating MBP is distinctly different from the histone-methylating enzyme (Lee *et al.*, 1977; Miyake, 1975; Kim *et al.*, 1984). It was therefore felt important to study the methylation pattern along with MBP-specific PMI activity during mouse brain development to understand a possible involvement of MBP methylation during myelination. The present paper deals with the pattern of methylation *in vivo* of mouse brain MBP and MBP-specific PMI activity during development. High-M_r MBPs in addition to those already known were also detected during the course of these studies, and the possible biochemical significance of these MBP species is discussed in relation to the development of brain.

MATERIALS AND METHODS

Materials

L-[*methyl*-^{3}H]Methionine (70–85 Ci/mmol), L-[^{35}S]-methionine (1450 Ci/mmol), *S*-adenosyl-L-[*methyl*-^{14}C]-methionine (60 mCi/mmol) and L-[4,5-^{3}H]-leucine (51.6 Ci/mmol) were obtained from Amersham, Arlington Heights, IL, U.S.A. CM-52 cation-exchanger was purchased from Whatman. Rabbit anti-mouse IgG was purchased from Sternberger–Meyer and 3,3′-diamino-

Abbreviations used: MBP, myelin basic protein; PMI, protein methylase I; SAM, *S*-adenosyl-L-methionine.

* To whom correspondence and reprint requests should be sent.

benzidine tetrahydrochloride dihydrate was purchased from Aldrich Chemical Co. All other chemicals used were obtained from various commercial sources and were of the highest purity grade available. Male CD-1 Swiss mice were obtained from Charles River Laboratories and maintained in our animal-care facility.

Radioisotope labelling

Animals were slightly anaesthetized with diethyl ether and then injected intracerebrally with 10 μl (10 μCi) of L-[*methyl*-^{3}H]methionine per 10 g body weight. At 18 h after the injection, the animals were killed by asphyxiation with solid CO_2. Their brains were rapidly excised and frozen immediately in a solid-CO_2/acetone bath. For double-labelling experiments, L-[*methyl*-^{3}H]methionine and L-[^{35}S]methionine were mixed in the ratio of 200 μCi/50 μCi (the actual d.p.m. ratio of the mixture, by a counter programmed for both isotopes, was found to be 3.6). Each animal was then given about 10 μl of the solution containing 12.3 μCi/10 g body weight. Injections of the mice younger than 20 days old were given without anaesthesia.

Determination of total incorporation in brain

Frozen brains were weighed and homogenized individually (1 g of brain in 6.33 ml of methanol). To ensure complete introduction of the radioisotope, proteins from 0.2 ml of each homogenate were precipitated with 0.2 ml of 10% (w/v) trichloroacetic acid solution containing 1 mg of methionine/ml. After the precipitates had been left for 15 min they were collected by centrifugation, washed twice with 5% trichloroacetic acid, once with ethanol/diethyl ether (1:1, v/v), once with diethyl ether and then finally counted in ACS scintillator (Amersham). Those brain samples having extremely low radioactivity were discarded, since the low counts may have been due to radioisotope leakage during injection.

Isolation of MBP by CM-52 chromatography

Brain homogenate was delipidated overnight in a chloroform/methanol (2:1, v/v) mixture as described by Campagnoni *et al.* (1978). The tissue residue was then collected on a sintered-glass funnel and washed, twice with the chloroform/methanol mixture and once with acetone. The residue was then suspended in cold deionized water (2.2 ml/g of brain) and the pH of the suspension was adjusted to 3.0 with 0.1 M-HCl. The mixture was then centrifuged at 27000 *g* for 20 min to obtain the 'acid extract'.

Different forms of MBP were isolated from this acid extract by the method of Chou *et al.* (1977, 1983). An acid extract prepared from about 10 mice brains was applied on a CM-52 column (1 cm × 6 cm), which was equilibrated with 0.08 M-glycine/NaOH buffer, pH 10.4, containing 6 M-urea. The column was first washed with approx. 40 ml of the equilibrating buffer ('breakthrough') and the MBPs were eluted with a linear gradient consisting of 0–0.3 M-NaCl in the equilibration buffer containing 2 M-urea. As shown in Fig. 1(*c*) (below), three classes of MBPs were separated; they were eluted at 0.085 M-(component 3), 0.112 M-(component 2) and 0.132 M-(component 1)salt respectively. Radioactivity incorporated into each fraction was determined, and the specific radioactivity of each component was calculated by using the absorption coefficient of MBP to 5.89 (1% solution) at 276 nm (Eylar & Thompson, 1969).

Separation of MBPs by SDS/polyacrylamide-gel electrophoresis

An acid extract prepared from frozen brains was first chromatographed on a CM-52 column as described above. After removing the 'breakthrough' fraction, all forms of MBP were eluted together with 0.2 M-NaCl instead of a linear gradient (see Fig. 1). The eluate was then desalted on a Sephadex G-25 column in 0.01 M-HCl and freeze-dried. This fraction was designated as '0.2 M-NaCl eluate' and was used for electrophoresis. SDS/polyacrylamide-gel electrophoresis was carried out on 15% (w/v) acrylamide/0.8% bisacrylamide gel as described by Laemmli (1970) with 3% acrylamide in the stacking gel. Electrophoresis was performed at constant voltage of 150 V (direct current) for the sample to pass through the stacking gel (about 90 min) and then at 200 V until the dye front reached to the lower edge of the slab (about 2.5 h). Gels were fixed in 10% trichloroacetic acid for 1 h, stained with Coomassie Blue R-250 overnight and then destained by diffusion (Chou *et al.*, 1983). Gels were scanned on a Bio-Rad Model-1650 Transmittance Reflectance Scanner. The relative mobility of each MBP species was determined and the M_r was estimated from the standard protein scan. The scans were then cut into individual protein peaks and each peak was weighed to quantify the distribution of different MBP species. The relative percentage of each band was calculated on the basis of total area taken as 100.

Preparation of antisera

Antisera against MBP were produced in New Zealand White rabbits as described by Reidl *et al.* (1981). Each rabbit was immunized intradermally over the back with 1.5 mg of bovine MBP emulsified with an equal volume of complete Freund's adjuvant (Difco), once a week for 3 weeks. The rabbits were boosted 2 weeks after the last injection with 5.0 mg of MBP in complete Freund's adjuvant. The formation of anti-MBP antiserum was tested by means of Ouchterlony double immunodiffusion. A single immunoprecipitin line was observed between MBP or brain acid extract against MBP antiserum. Generally 2–3 weeks after the booster the animals were bled from ear artery. Anti-MBP IgG was partially purified from the serum by precipitation with 40%-satd. $(NH_4)_2SO_4$ (Hall *et al.*, 1982). The precipitate was dissolved in 0.15 M-NaCl/10 mM-phosphate buffer, pH 7.2, and dialysed against the buffered saline. After dialysis it was centrifuged at 1000 *g* for 15 min to remove any insoluble material and used for immunoblotting.

Immunoblot analysis of MBP polypeptides

The '0.2 M-NaCl eluate' containing all species of mouse MBPs were first separated by SDS/polyacrylamide-gel electrophoresis in duplicate. While one gel was stained with Coomassie Blue, the separated proteins on the other gel were transferred electrophoretically to nitrocellulose paper and detected by double-antibody immunoperoxidase labelling as described by Towbin *et al.* (1979). The immunoblotting was developed using anti-MBP IgG as the primary antibody and non-fat dry milk to block non-specific binding in an incubation medium (Johnson *et al.*, 1984). The secondary antibody used was rabbit anti-mouse IgG. The blot was then incubated with clonal peroxidase–anti-peroxidase com-

plex for 1–2 h (at 4 °C) and the bands were revealed by incubating the blot in diaminobenzidine solution as described by Glass *et al.* (1981).

Amino acid analysis

The '0.2 M-NaCl eluate' isolated from 59-day-old mouse brain was hydrolysed in 20% formic acid/2 M-HCl at 110 °C for 4 h by the methods of Gurnani *et al.* (1955) and Ro *et al.* (1984). Amino acid analysis of the hydrolysate was performed on an h.p.l.c. instrument equipped with a model 721 programmable system controller, two model 510 pumps, model 730 data module and an automatic sampler (WISP model 710B), using Model 2 analysis, which utilizes *o*-phthaldialdehyde derivatization (Waters Associates amino acid analysis system, operator's mannual no. 07124, June 1984, revision C). The analysis was performed on a Resolve C_{18} column (3.9 mm internal diameter × 15 cm) from Waters Associates. Solvent A was methanol/tetrahydrofuran/aq. 50 mM-sodium acetate/50 mM-Na_2HPO_4, pH 7.5 (2:2:96, by vol.) and solvent B was methanol/water (65:35, v/v). Fractions were collected at a rate of 1 ml/min and counted for radioactivity using ACS scintillator.

PMI assay

Mouse brain was homogenized in 4 vol. of 0.25 M-sucrose containing 5 mM-$CaCl_2$ and 5 mM-sodium phosphate, pH 7.4, in a Teflon/glass homogenizer by ten up-and-down strokes. The homogenate was centrifuged at 39000 *g* in a Sorvall RC-2B centrifuge for 30 min (Paik & Kim, 1968), and the supernatant was used for the PMI assay.

PMI assays were performed as described by Kim *et al.* (1984). The total reaction mixture, 0.25 ml, containing 0.05 ml of 0.5 M-sodium phosphate buffer, pH 7.2, 0.05 ml of protein substrate (purified bovine MBP, 30 mg/ml), 0.05 ml of enzyme preparation and 0.05 ml of *S*-adenosyl-L-[*methyl*-^{14}C]methionine (6 nmol) was incubated at 37 °C for 10 min. The reaction was terminated by the addition of 3.0 ml of 15% trichloroacetic acid. Nucleic acid and phospholipids were removed by successive treatment with hot trichloroacetic acid (at 90 °C) and then ethanol. Protein carboxymethyl esters, a product of protein methylase II (Kim *et al.*, 1975), were removed by hydrolysing the mixtures in 1.0 ml of 0.5 M-sodium phosphate buffer pH 8.0, at 60 °C for 5 min. The mixtures were re-precipitated with trichloroacetic acid, washed with ethanol and transferred quantitatively into scintillation vials for monitoring of the radioactivity.

Preparation of bovine MBP used for the PMI substrate

Substrate MBP for the PMI assay and for antibody production was prepared from frozen bovine brain (Pel-Freez) as described by Deibler *et al.* (1984); this method yields MBP free of associated proteinases. Briefly, the acid extract prepared as described above was made 2 M in urea with an 8 M-urea solution. It was then titrated to pH 9.0 the addition of several small portions of DE-52. The suspension was vacuum-filtered and the filtrate was then further fractionated on a CM-52 column as described previously. After the breakthrough elution, the MBP fraction was eluted with the equilibrating buffer containing 0.2 M-NaCl. It was then desalted by gel filtration on Sephadex G-25, freeze-dried and used as the substrate for the PMI assay. This MBP preparation was homogeneous on SDS/polyacrylamide-gel electrophoresis, with a single broad band at M_r approx. 18000, which corresponded to the M_r (18500) of mouse MBP. The bovine brain did not contain MBP polypeptides of differing M_r values, as observed in case of mouse brain (Deibler *et al.*, 1984). Protein determinations were performed by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

Turnover studies of MBP

About 60 mice (12 days old) were injected intracerebrally with L-[^{3}H]leucine at a dose of 15 μCi/15 μl per animal without anaesthesia and were kept on a normal diet. A group of animals (10–15, depending on their ages) were then killed at 1, 5, 14, 37 and 47 days after the injection, and the brains were quickly removed. In each experiment each brain was homogenized individually and total radioactivity introduced into the brain was determined as described above before mixing the homogenates for MBP isolation. The pooled homogenate was first delipidated and 'acid extract' was prepared (Campagnoni *et al.*, 1978). It was then chromatographed on a CM-52 column (see the previous subsection for further details) by the method of Chou *et al.* (1977, 1983). After removing 'breakthrough', the MBP fraction ('0.2 M-NaCl eluate') was obtained. A portion of the fraction was counted for radioactivity and the amount of MBP was estimated by its A_{276} (Eylar & Thompson, 1969). Finally, the specific radioactivity of MBP fraction at each experimental point was expressed as d.p.m./mg of MBP, after correcting the total amount of MBP accumulated at that age group.

RESULTS

Incorporation of L-[*methyl*-^{3}H]methionine into MBP

To assess initially the synthesis and methylation pattern of MBP during development, mice at different ages were injected with 10 μCi of L-[*methyl*-^{3}H]methionine and killed 18 h later. Subsequently, MBP was isolated from the 'acid extract' of mouse brain on a CM-52 column with a linear salt gradient. A typical elution profile is shown in Fig. 1. MBPs were eluted in three protein peaks, between 0.08 and 0.132 M-NaCl, after the major breakthrough protein (Fig. 1*a*). Peak 1 is mainly a small MBP (M_r 14000), whereas 2 and 3 are mixtures of small and large MBPs (Chou *et al.*, 1983). The specific radioactivity in each MBP species was calculated by using the absorption coefficient of 5.89 at 276 nm for MBP and is expressed as d.p.m./mg. The experiments were carried out three times. The variation in the specific incorporation was about 7%. The typical incorporation is shown in Table 1. The incorporation of methionine was found to be greater at a younger age when the myelination process and protein synthesis are active, whereas it was diminished in adult animals. It is evident that the incorporation in each form was 15–25 times higher at a younger age (13 days) compared with 60-day-old animals. At the same time, the relative incorporation in the young brain was highest in the small MBP (peak 1), the major form in mouse brain.

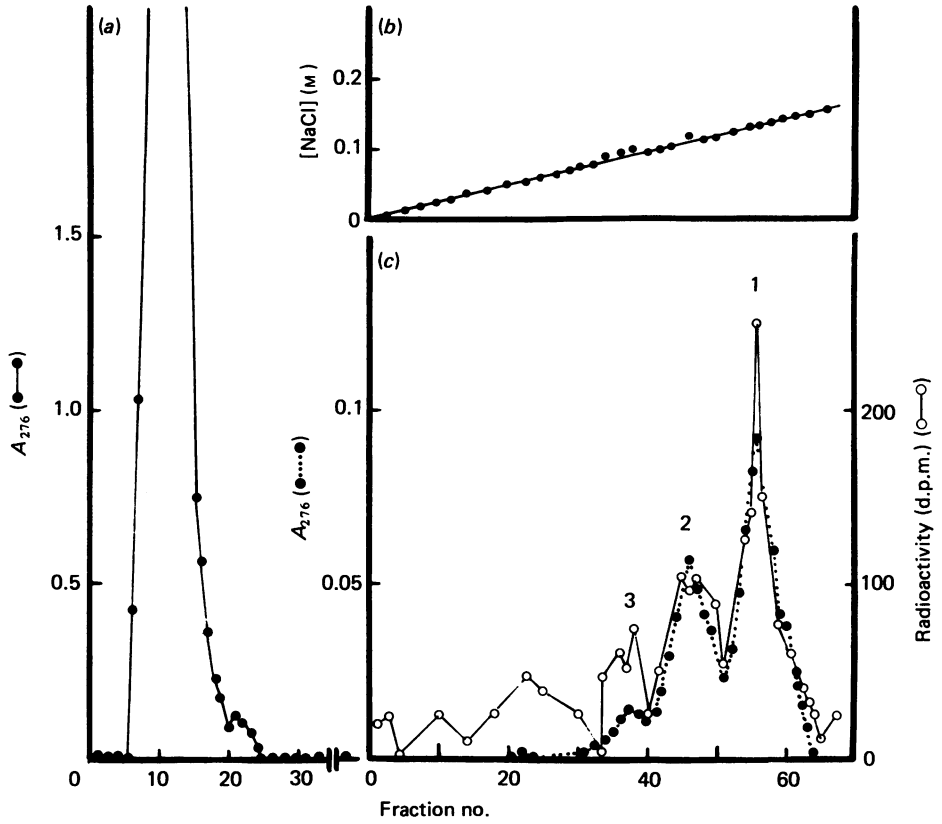

Fig. 1. Elution profile of the 'acid extract' on CM-52 chromatography

The 'acid extract' from ten mouse brains (33 days old) was applied to a CM-52 column (1 cm × 6 cm) which had been equilibrated with 0.08 M-glycine/NaOH, pH 10.4, containing 6 M-urea. MBP was eluted with a linear gradient of 0–0.3 M-NaCl in 0.08 M-glycine/NaOH, pH 10.4, containing 2 M-urea. The incorporation of radioactivity into each fraction was determined (*c*). (*b*) shows the NaCl gradient. 1, 2 and 3 indicate MBP components described by Chou *et al.* (1983).

Differential radioactivity incorporation in the backbone and methylation of MBP during development

In order to discern the methyl-group incorporation and protein-backbone labelling by methionine, two labels of methionine, L-[^{35}S]methionine for backbone synthesis and L-[*methyl*-^{3}H]methionine for methyl group via *S*-adenosyl-L-[*methyl*-^{3}H]methionine, were used. A higher ratio of ^{3}H to ^{35}S incorporated would indicate a greater amount of methylation (Paik & Kim, 1980). In these experiments, total MBP was eluted with 0.2 M-NaCl buffer instead of separating MBPs into three components on CM-52 (Fig. 1), because radioactivity incorporated was not sufficient for individual separation. It is clear from Table 2 that the ratio of ^{3}H/^{35}S in the 'acid extract' and 'breakthrough' fractions remained about the same as the originally injected ratio of 3.6, whereas those ratios in the MBP fractions were always higher. The ratio was greater at 17 days of age when myelination and protein synthesis were at a maximum and decreased during growth, remaining at about 6–8 in mature animals. This high ratio at a younger age indicates a higher methylation rate compared with the protein-backbone synthesis.

Amino acid analysis of MBP

MBPs isolated on the CM-52 column from the brain of a 59-day-old mouse that had been injected with [*methyl*-^{3}H]methionine were hydrolysed and the amino acids were analysed by h.p.l.c. in order to verify the presence of methylarginine. It should, however, be noted that this MBP preparation is a '0.2 M-NaCl eluate' from the CM-52 column and thus contains all the MBP polypeptides as shown in SDS/polyacrylamide-gel electrophoresis (Fig. 3, lane D), the major species being the small MBP and various amounts of the other species. Studies from several laboratories indicate that these species are derived from a common gene as a result of different splicing mechanisms (Takahashi *et al.*, 1985) and, therefore, are not due to intracellar proteinases. Although our MBP preparation is a mixture of several species, its amino acid composition may be compared with the various reported values (Table 3). Of course the main purpose of this analysis was to identify [^{3}H]methyl-group incorporation into arginine residues. It should be noted that, with the present h.p.l.c. method, N^{G}-monomethylarginine is almost co-eluted with alanine at 14.2 min and N^{G}-dimethylarginine is eluted at 17.8 min. When fractions (1 ml/min) were collected from the h.p.l.c. instrument and counted for radioactivity, 42% of the total applied activity was recovered as methylated arginine: 27% with N^{G}-dimethylarginine and 15% with N^{G}-monomethylarginine respectively. In a similar study carried out on chick MBP, Small & Carnegie (1982) also reported about 46% of the radioactivity in methylated arginine.

Table 1. Incorporation of L-[*methyl*-^{3}H]methionine into mice brain MBP

Age of animal (days)	Component*	Amount of MBP (mg/ten mouse brains)	^{3}H incorporation (d.p.m./mg)
13	1	0.162	23682
	2	0.167	12768
	3	0.173	20590
20	1	0.486	13900
	2	0.381	11600
	3	0.091	9400
33	1	1.13	901
	2	0.743	916
	3	0.161	1510
45	1	2.010	401
	2	1.230	341
	3	0.144	1070
60	1	1.640	923
	2	1.070	786
	3	0.243	813

* The numbers, 1, 2 and 3 indicate the MBP components described by Chou *et al.* (1983) and shown in Fig. 1.

Table 2. Incorporation of L-[*methyl*-^{3}H]methionine and L-[^{35}S]methionine into protein fractions during mouse brain development

The ratio of ^{3}H/^{35}S was calculated by counting the fractions in a Packard Prias 240 Tri-Carb liquid-scintillation counter programmed for dual-label counting. Results are expressed as averages for three independent experiments. The MBP fraction was eluted from the CM-52 column with 0.2 M-NaCl buffer. Details of the experimental conditions are as described in the text.

Age of animal (days)	^{3}H/^{35}S		
	Acid extract	Breakthrough	MBP fraction
13	3.8	3.4	9.1
17	3.7	3.7	9.4
21	3.8	3.5	5.7
49	3.8	3.3	7.5
59	3.5	3.3	6.9

PMI activity during brain development

With the ^{3}H/^{35}S ratio derived from the methionine-injection experiment described above being higher in younger animals, we studied PMI activity in brains during development (Fig. 2). It is clear from the Figure that PMI activity gradually increased as a function of age and was parallel with the myelination process. Activity peaked at 17 days of age, and then declined to the baseline level. This is in good agreement with previous reports from this laboratory (Kim *et al.*, 1984) as well as from Crang & Jacobson (1982).

Molecular species of MBPs during aging

The '0.2 M-NaCl eluate' from the CM-52 column was separated by SDS/polyacrylamide-gel electrophoresis

Table 3. Amino acid composition of mouse brain MBP

Results are averages for two independent analyses.

Amino acid	Composition (residues/molecule)			
		Reported		
	Found	Small MBP*	Small MBP†	Large MBP†
Aspartic acid	16	9	13	13
Threonine	8	8	7	13
Serine	13	16	9	20
Glutamic acid	18	8	20	11–12
Glycine	24	14	14	20
Alanine	12‡	7	12	9
Valine	4	3	5	3
Methionine	1	2	3	2
Isoleucine	4	3	3	3–4
Leucine	10	6	4	9
Tyrosine	1	2	1	3
Phenylalanine	8	6	3	8
Histidine	8	8	2	7
Lysine	17	7	9	9
Arginine	19	18	3–4	18
Proline§	N.D.‖	9	6	13
Tryptophan	N.D.	1	N.D.	N.D.

* Values reported by Chou *et al.* (1983).
† Values reported by Rauch *et al.* (1981).
‡ The value represents both alanine and methylarginine, because both amino acids are co-eluted on h.p.l.c. However, error will be less than 8%, since MBP is fractionally methylated at position 107 (Baldwin & Carnegie, 1971).
§ Proline is not detectable by the *o*-phthaldialdehyde derivatization method.
‖ N.D., not determined.

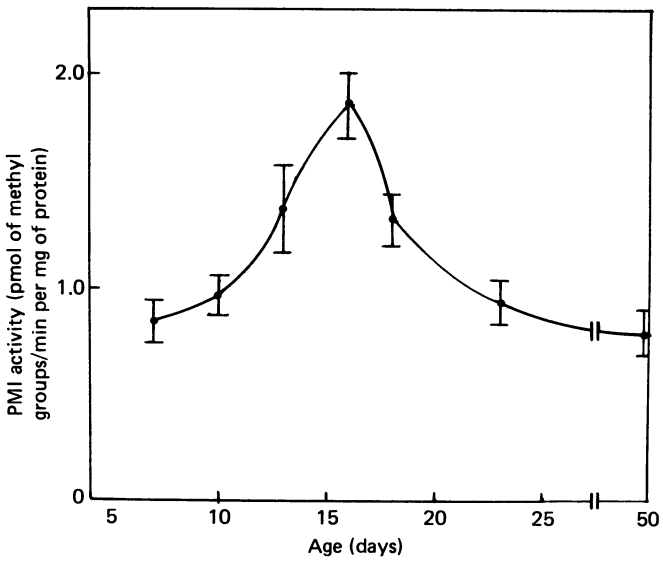

Fig. 2. PMI activity in mouse brain during development

Enzyme activity was determined as described in the text by using 1.5 mg of purified bovine MBP as the methyl acceptor substrate. Values are averages from three independent experiments.

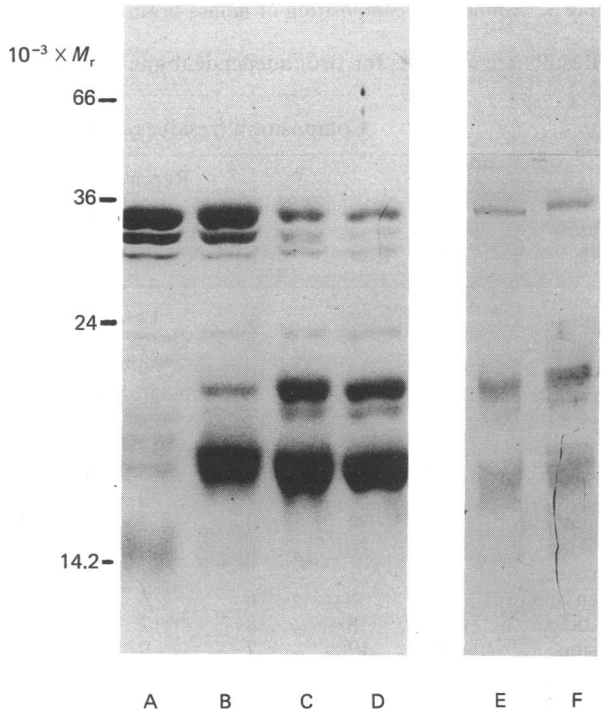

Fig. 3. Electrophoretic evaluation of different species of MBP during mouse brain development

MBP (10 μg) purified on a CM-52 column (0.2 M-NaCl eluate) was analysed by SDS/polyacrylamide-gel electrophoresis by the method of Laemmli (1970) as described in the Materials and methods section. Lanes A, B, C and D represent MBP isolated from 13-, 17-, 49- and 59-day-old mouse brains respectively. Molecular markers applied were bovine serum albumin (M_r 66000), glyceraldehyde-3-phosphate dehydrogenase (36000), trypsinogen (24000) and α-lactalbumin (14200). The lanes E and F represent the immunoblot of lanes C and D respectively.

Table 4. Relative amounts of individual MBP species isolated by SDS/polyacrylamide-gel electrophoresis during mouse brain development

$10^{-3} \times M_r$	Age of animal (days)...	Amount (% of total)*			
		13	17	49	59
32.4		32.4	22.9	8.25	7.7
31.0		16.6	9.9	3.4	2.65
29.1		5.78	2.6	2.6	2.52
23.6		2.2	Traces	Traces	Traces
21.0		3.3	2.3	2.1	2.68
18.5		4.5	8.4	17.8	19.8
17.0		6.40	2.5	3.9	4.45
16.0†		8.1	45.8	58.5	55.8
14.5		20.6	5.56	3.6	3.49

* Results are expressed as a percentage of total recovered MBP species on SDS/polyacrylamide-gel electrophoresis (see Fig. 3).

† '16.0' represents the small MBP having an M_r of 14000 by amino acid analysis (Campagnoni & Magno, 1974).

after desalting and freeze-drying. This fraction was resolved into eight different M_r species ranging from 32400 to 14500 (Fig. 3). The proportion of each species was quite different depending on the age of animal. The high-M_r species (32400) was predominant at a young age, whereas in older animals the larger MBP was decreased in amount and the small MBP accumulated with age. It should be noted in the Figure that the small MBP (M_r 14000 on the basis of amino acid analysis) always moved as a 16000-M_r species on SDS/polyacrylamide-gel electrophoresis as has also been observed by others (Campagnoni & Magno, 1974; De Rosbo *et al.*, 1984). The presence of another, smaller, MBP (M_r 14500), other than the usual small MBP, was also observed in the young animal (Fig. 3, lane A).

To verify that these different species of polypeptides isolated by SDS/polyacrylamide-gel electrophoresis were indeed MBPs, a separate gel was run in parallel and developed by immunoblotting with anti-MBP immunoglobulin (Fig. 3, lanes E and F). As shown in Figure, all the Coomassie Blue-stained species were immunoreactive, indicating that these various molecular species are MBPs and not other contaminating proteins.

In order to quantify SDS/polyacrylamide-gel-electrophoretic data, each lane was scanned densitometrically and the scans were cut into individual protein peaks with a common baseline and weighed individually. The percentage attributable to individual MBP peaks were then calculated by taking the total weight of all peaks as 100 (Table 4). In the younger animals (13 days old), 32400-, 31000- and the lower-M_r (14500) species are predominant. On the other hand, in adult animals, the high-M_r proteins are diminished and the small MBPs, particularly the 18500-M_r and 16000-M_r species, are increased. However, it is not clear at present whether the high-M_r species are the precursors of the lower-M_r ones and what is their significance in the myelination process.

Turnover studies of MBP

Animals were injected intracerebrally with L-[^{3}H]leucine at 12 days of age and were killed 1, 5, 14, 22, 37 and 47 days later. The MBP fraction was isolated on a CM-52 column and the radioactivity incorporation (d.p.m./mg of MBP) was calculated. The values were corrected for the dilution of radioactivity caused by continuous synthesis of new MBP during the respective experimental period (Small & Carnegie, 1982). This corrected specific radioactivity was plotted against time and is shown in Fig. 4. The rate of decay of radioactivity was plotted as a semilogarithmic plot (inset to Fig. 4) to calculate the half-life of MBP. The half-life of MBP was found to be around 36 days.

DISCUSSION

The different molecular species of MBP isolated from mice brains that had been injected with L-[*methyl*-^{3}H]methionine showed differences in their specific radioactivities (d.p.m./mg of MBP), being highest in the small MBP at the youngest age. The incorporation was inversely correlated with the total amount of MBP in the brain and thus was greater in MBP isolated from the younger animals (Table 1) at ages where MBP synthesis and myelination processes are known to be the most active (Barbarese *et al.*, 1978). Since the ^{3}H label can be incorporated into MBP both in the backbone and via side-chain transmethylation, the results in Table 1 reflect

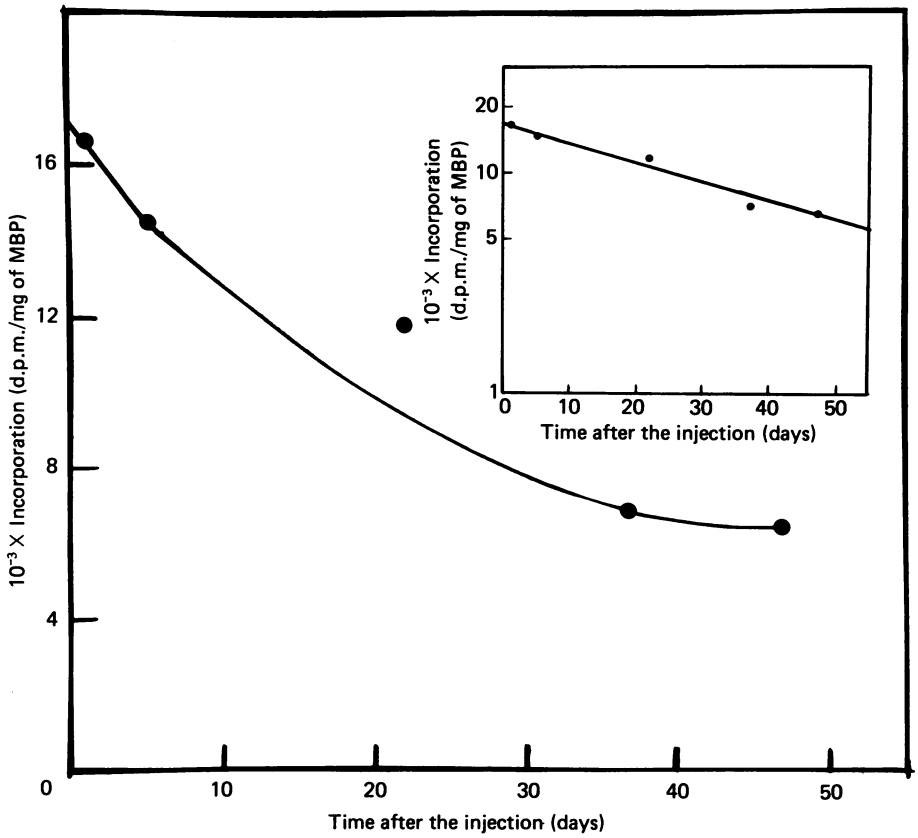

Fig. 4. Time course of incorporation of [^{3}H]leucine into purified MBP

Each point represents a determination of MBP pooled from five to seven brains. Details of the experiment are given in the Materials and methods section. The data were corrected for the dilution of label caused by accumulation of MBP during the experimental period. The inset (a semilogarithmic plot) was used to calculate the apparent half-life of MBP, which was about 36 days.

to enhanced MBP synthesis and accompanying methylation in these young animals. In order to distinguish their respective contributions, a dual-labelling technique utilizing L-[*methyl*-^{3}H]- and L-[^{35}S]methionine was employed. In these experiments the latter isotope cannot contribute as a methyl donor, but only a backbone methionine, thus the ratio of ^{3}H/^{35}S can serve as a methylation index. As can be seen in Table 2, the high ratio of ^{3}H/^{35}S in MBP from young brain clearly indicates that methylation is most active in MBP during the period of MBP synthesis and myelination (Benjamins & Smith, 1984), but is not related to the amount of myelin present in the brain. Similar observations have also been made by Desjardins & Morell (1983) in rats utilizing [*methyl*-^{3}H]methionine and analysing the incorporation of radioactivity into MBP. It is not clear, however, at what stage of MBP synthesis (during or after backbone synthesis of MBP) the methyl group is introduced to the arginine residue.

Possible variations in the SAM pool for the transmethylation reaction during mouse development does not seem to have any significant effect on the observed methylation index. If it had any, it would have been to a minimum extent in younger brain, since Gharib *et al.* (1985) have recently reported that the SAM level is high in the newborn and diminishes during rat brain maturation. Furthermore, they also observed that the concentration of *S*-adenosyl-L-homocysteine, a potent product inhibitor for the reaction, is lower in the newborn animal.

The fact that the ratios of ^{3}H/^{35}S in all MBP fractions are 2–3-fold higher than those in the 'acid extract' and 'breakthrough' fractions suggests that this methylation reaction occurs with MBP rather than with other soluble brain proteins. This is in accordance with the results of Aspillaya & McDermott (1977), who have shown that the methylarginine of MBP in myelin accumulated to the greatest extent among all the basic proteins of brain. It should be mentioned that the presently employed MBP isolation method is highly unlikely to extract nuclear proteins which contain methylated arginine such as heterogeneous-RNA proteins and protein C23 (Karn *et al.*, 1977; Lischwe *et al.*, 1985). All these proteins are acidic in nature and, therefore, will be removed during the acid precipitation and DE-52 treatment during MBP purification (see the Materials and methods section).

The increased ratio of ^{3}H/^{35}S in the young brain is correlated temporally with increased MBP-specific PMI activity (Fig. 2); PMI activity was found to be higher at about 17 days of life when the myelination process is at its maximum level. Similar observations have been made previously by our laboratory (Kim *et al.*, 1984) and also by others on mouse spinal-cord enzyme activity (Crang & Jacobson, 1982). It is worthwhile to mention here that the developmental pattern of MBP (arginine) methyltransferase levels in embryonic mouse brain culture has been reported to be regulated by thyroid hormone, which has a profound effect on the differentiation of the oligodendroglia (Amur *et al.*, 1984).

Another important observation described in the present paper is the presence of high-M_r MBPs (32 2000, 31 000 and 29 100) in mouse brain. Only four different MBPs in mouse have been generally reported. Recently Agarwal *et al.* (1985) have also reported six different forms of MBP from developing rat brain (M_r ranging from 23 600 to 14 000). However, we have detected as many as eight species of MBP (M_r ranging from 32 400 to 14 000). It was noteworthy that the concentration of these different forms of MBP changed during aging. A gradual decrease in high-M_r species and an increase in small MBP in older brain is clearly seen in Fig. 3 and Table 4. In addition to these changes, one more species, M_r 14 500, was also detectable in 13-day-old animals, but not in adult animals. Similar developmental patterns for MBPs were reported by Carson *et al.* (1983) in mouse brain homogenate by an immunoblotting procedure. However, it is not clear at present whether there exists any precursor–product relationship between these high- and low-M_r MBPs during development.

It seems quite likely that synthesis of different MBPs takes place independently, with specified rates at different ages. Carson *et al.* (1983) have shown that MBP gene expression proceeds concurrently with myelin morphogenesis and that translatable mRNA specific for MBP encodes predominantly that particular MBP at each developmental stage. Yu & Campagnoni (1982) have shown that four MBPs are translated from four independent brain mRNAs and are not metabolically related. Interestingly, Takahashi *et al.* (1985) have recently shown that a single gene apparently gives rise to mRNAs for multiple MBPs by alternate RNA splicing pathways. Although this is a highly speculative theory, it is possible that different forms of MBP may have particular functions during the myelination process. For example, high-M_r MBPs may have significant roles during initial phases of myelin deposition, whereas small MBPs may be essential in the maturation process or possible repair of the myelin membrane in case of damage.

Finally, from the studies of radioactive leucine incorporation into the brain during development, the half-life of MBP was found to be 36 days (Fig. 4). This is very close to the value of 40 days for methylarginine in chicken MBP (Small & Carnegie, 1982); methyl groups in MBP have been shown to be as stable as the polypeptide backbone (Desjardins & Morell, 1983). However, the reported half-life for MBP varies from 21 to 95 days (Smith, 1972; Sabri *et al.*, 1974; Fisher & Morell, 1974) and the half-life of basic proteins isolated from myelin-like material is 16 days (Fisher & Morell, 1974). One of the reasons for these variations, as suggested by Fisher & Morell (1974), is the variation in the experimental conditions and the age of the animals selected. The origin of the myelin-like material is still uncertain. It is therefore possible that, when myelin and myelin-like material are not well separated before MBP extraction, one is likely to get variable half-lives depending on the ratio of the two basic-protein fractions. Another possibility is the difference in turnover rate of each MBP species; each species may have different half-lives, as evidenced by their changing ratios during growth.

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