Turnover of Myelin and Other Structural Proteins in the Developing Rat Brain

By M. I. SABRI, A. H. BONE* and A. N. DAVISON
*Miriam Marks Department of Neurochemistry, Institute of Neurology,
The National Hospital, Queen Square, London WC1N 3BG, U.K.*

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1. Protein metabolism of myelin and other subcellular components from developing rat brain was studied for periods from 5 h to 210 days after intraperitoneal injection of [^3H]-lysine and [^{14}C]glucose. 2. Half-lives for total brain proteins ($t_{0.5}$) were 27 days after [^3H]lysine and 4 days after [^{14}C]glucose injection. 3. Factors accounting for the difference in the turnover rates obtained with different precursors, and the problem of reutilization of the label were investigated. 4. The catabolism of purified myelin proteins was studied and the half-lives of individual myelin proteins were calculated. 5. Myelin basic proteins turned over at two different rates. Half-life of the fast component of myelin basic proteins was 19–22 days and the slow component exhibited a high degree of metabolic stability. 6. Proteolipid protein underwent slow turnover. High-molecular-weight Wolfram (1966) proteins underwent (relatively) fast metabolism ($t_{0.5}$ of 17–22 days).

Although early workers (Furst *et al.*, 1958; Davison, 1961) concluded that white-matter and myelin-sheath proteins were metabolically stable, this view has been widely challenged (Smith, 1972; D'Monte *et al.*, 1971; Wood & King, 1971). For example, the turnover of myelin basic protein was shown to occur at a comparable rate with that of other brain proteins. On the other hand, Fischer & Morell (1973) demonstrated relative metabolic stability of labelled myelin basic and proteolipid proteins in the mouse brain (see Table 8). They also showed fast turnover of a labelled myelin-like membranous protein fraction suggesting that it may be a precursor of compact myelin formation. Shapira *et al.* (1972) attribute stability of myelin basic protein to reutilization of radioactive amino acid.

There is therefore a need to re-investigate the whole problem of the metabolism of myelin proteins. In the present paper we describe metabolic experiments with two different radioactive precursors for examining the various factors involved in determining the turnover rate of myelin and other brain subcellular fraction proteins.

Experimental

Sterilized aqueous solution of L-[4,5- ^3H]lysine monohydrochloride (sp. radioactivity 5.5 Ci/mmol) and D-[U- ^{14}C]glucose (sp. radioactivity 285 mCi/mmol) were purchased from The Radiochemical

Centre, Amersham, Bucks., U.K. The experiments were carried out on 22–23-day-old male albino rats. The litter size was restricted to 8–10 rats weighing 35–40 g. Each rat received an intraperitoneal injection of either [^3H]lysine or [^{14}C]glucose (1 $\mu\text{Ci/g}$ body wt.). Development of the rats as judged by increasing body and brain weight was quite normal. Groups of four animals were killed by decapitation at intervals of 5 h to 210 days after the injection of labelled precursors. Blood, withdrawn by cardiac puncture with heparinized syringes before decapitation, was pooled and centrifuged at 4°C to obtain plasma. Whole brains were rapidly excised, placed on ice, superficial blood was removed and then they were weighed. Each brain was homogenized in a glass homogenizer fitted with a Teflon pestle (clearance 0.228 mm) at 500 rev./min for 30 s in 10 ml of ice-cold 0.32 M-sucrose solution and the volume of the homogenate adjusted to give 5% (w/v) wet wt. of brain. Uniform uptake of radioisotope in each brain (differences not exceeding 10%) was checked by determining radioactivity in samples of whole homogenate (0.1 ml). Homogenates from four brains were combined and 5 ml of the homogenate was used for determining total acid-soluble radioactivity.

Subcellular fractions and the preparation of purified myelin

The isolation of subcellular fractions of the brain was carried out essentially by the method of Gray & Whittaker (1962). Myelin was isolated by the method of Norton (1971) from 75 ml of pooled brain

* Present address: Department of Biochemistry, Charing Cross Hospital Medical School, Fulham, London S.W.6, U.K.

homogenate. Purified myelin was suspended in water to give 2–4 mg of myelin protein/ml.

Amino acid pool

For blood free amino acids, 1 ml of plasma was deproteinized with 5 ml of ice-cold 6% (w/v) HClO_4 and the acid-insoluble residue washed in a further 2 ml of HClO_4 . For determination of the brain amino acid pool 5 ml of homogenate was pipetted into 10 ml of ice-cold HClO_4 and centrifuged at 10000g for 15 min at 2°C and the pellet washed with 5 ml of HClO_4 . The combined perchloric acid extract (5 ml for plasma, 15 ml for brain) was passed through a column (1.3 cm $\times$ 11 cm) of Zeo-Karb 325 (H^+ form). The column was washed with 30 ml of water and the adsorbed amino acids were eluted with 50 ml of 1 M- NH_3 . NH_3 was removed by vacuum evaporation in a bath maintained at 40°C. The samples were freeze-dried, dissolved in 2 ml of water and transferred into a scintillation counting vial. Water was removed in a vacuum desiccator containing silica desiccant and the residue was dissolved in 0.10 ml of water and the radioactivity determined. In the [^{14}C]glucose experiments freeze-dried samples were dissolved in 0.2–0.4 ml of water and 0.1–0.2 ml was removed for determination of total radioactivity. Labelled amino acids in samples of 0.1 ml were separated by high-voltage electrophoresis as described below.

Other methods

High-voltage electrophoresis. High-voltage electrophoresis was in pyridine-acetic acid buffer, pH 5.2 (prepared by mixing 20 g of pyridine and 9.5 g of glacial acetic acid and making up to 1 litre), on Whatman 3MM paper run at 75 V/cm for 1 h. As controls aspartic acid, glutamic acid and alanine were run and detected by using ninhydrin; corresponding strips of amino acids were eluted with 0.1 M-HCl, dried under vacuum and the radioactivity was determined.

Polyacrylamide-gel electrophoresis. Disc-gel electrophoresis in sodium dodecyl sulphate was carried out essentially as described by Agrawal *et al.* (1972). Protein (20–40 μg ; in 0.05–0.10 ml) was usually applied on the gel and separated proteins were made visible by staining with 0.25% (w/v) Coomassie Blue (E. Merck, 61 Darmstadt, Germany) in methanol-acetic acid-water (5:5:1, by vol.).

Separation of basic proteins from myelin. Basic proteins from aqueous suspensions of myelin were separated by the method of Martenson *et al.* (1969). The myelin suspension containing 10–20 mg of protein was delipidated twice with acetone mixed with 5 vol. of 0.1 M-HCl at 2°C and then stirred at 2°C for 30 min. The suspension was centrifuged at 35000g for 20 min. The clear supernatant was decanted and the acid-insoluble residue again extracted with 5 vol. of 0.1 M-HCl at 2°C. The residue was

washed three times with water by centrifugation and all the supernatants were combined, dialysed at 2°C against 0.1 M-acetic acid for 2 days and freeze-dried.

Proteolipid protein and high-molecular-weight (Wolfgram, 1966) protein. The proteolipid and the high-molecular-weight proteins contained in the acid-insoluble residue were extracted with 20 ml of chloroform-methanol (2:1, v/v). The chloroform-methanol ratio was made 1:1 (v/v) and then the suspension was centrifuged at 35000g for 30 min. The pellet, containing the high-molecular-weight (Wolfgram, 1966) protein, was given three rinses with 5 ml of chloroform-methanol (2:1, v/v) and the supernatants were combined. The residue was finally dried with diethyl ether and weighed. The proteolipid protein was precipitated from the chloroform-methanol solution after remaking the chloroform-methanol ratio 2:1 (v/v) by the addition of the synthetic citrate upper phase (Banik & Davison, 1973). The protein precipitate at the interface was collected, washed with 5 ml of water and then with 10 ml of chloroform-methanol (2:1, v/v) and finally dried with ether. The purity of separated proteins was checked by gel electrophoresis.

Protein and radioactivity determination. Proteins in brain homogenate or myelin suspension were precipitated with 5% (w/v) trichloroacetic acid (final concn.) containing 0.5% sodium phosphotungstate at 2°C for 30 min, heated for 15 min at 90°C and centrifuged. The protein pellet was washed three times with 5 ml of 5% (w/v) trichloroacetic acid, three times with 5 ml of ethanol-ether (3:1, v/v) at 50°C, centrifuged and dried in air. The pellet was then dissolved in 1–2 ml of tissue-solubilizing fluid (NCS solubilizer; Amersham/Searle, High Wycombe, Bucks., U.K.) at 50°C or in 1–2 ml of 1 M-NaOH. Portions were taken for protein determination by the method of Schmukler & Yienst (1968) with bovine serum albumin as standard prepared in tissue-solubilizing fluid (NCS solubilizer), or by the method of Lowry *et al.* (1951) with bovine serum albumin standard.

Radioactivity in amino acid or protein samples was determined in two solvents systems. 1. When protein samples were solubilized in NCS solubilizer, 0.1–0.2 ml of sample was neutralized with 0.03 vol. of acetic acid and radioactivity was determined in 10 ml of toluene scintillation fluid containing 0.5% (w/v) 2,5-diphenyloxazole and 0.025% (w/v) 1,4-bis(5-phenyloxazol-2-yl)benzene. When a sample was dissolved in water or 1 M-NaOH; 1–2 ml of NCS solubilizer was added to 0.1–0.2 ml of sample. The sample was made neutral with acetic acid and radioactivity was determined in 10 ml of scintillation fluid. 2. When the sample was in 1 M-NaOH or water; NaOH was neutralized with acetic acid and radioactivity was determined in 10 ml of toluene scintil-

EXPLANATION OF PLATE I

Electron micrograph of the purified myelin membrane fraction of rat brain

Purified myelin was prepared by the sucrose-density-gradient procedure described in the text. The sample was fixed in glutaraldehyde, post-fixed in OsO_4 and then stained in 3% (w/v) uranyl acetate (see the text). Magnification $\times 24000$ and that of inset $\times 108000$.

EXPLANATION OF PLATE 2

Electron micrograph of myelin-like membrane fraction of rat brain

'Myelin-like' membrane fraction was separated from the myelin by osmotic shock (see the text). Other details are the same as in Plate 1. Magnification $\times 24000$ and that of inset $\times 108000$.

EXPLANATION OF PLATE 3

Sodium dodecyl sulphate-polyacrylamide-gel electrophoresis of purified myelin and myelin-like fraction of rat brain

Aqueous samples of myelin and myelin-like fraction were delipidated with acetone at 0°C and the samples were dissolved in sample buffer, pH 7.4, containing 1% sodium dodecyl sulphate (1mg of protein/ml). A portion (25μl) of each sample was applied on the 1% gel column with 1% sodium dodecyl sulphate in sodium phosphate buffer, pH 7.4. Gel electrophoresis was carried out at about 20°C at constant current for 1 h at 1 mA/gel, then 3 h at 2 mA/gel and finally 2 h at 4 mA/gel. Gels (a, myelin fraction; b, myelin-like protein fraction) were stained with Coomassie Blue (0.25% in 7%, v/v, acetic acid) overnight and destained by washing the gels with methanol-water-acetic acid (1:9:10, by vol.) several times. The densitometric profile was recorded at 650nm in a Gilford spectrophotometric scanner. ----, Myelin; —, myelin-like fraction.

lation fluid containing 30% (v/v) Bio-Solv solubilizer (BBS₃) (Beckman Instruments, Inc. Fullerton, Calif., U.S.A.). Radioactivity determination was carried out in a Beckman LS-230 liquid-scintillation counter with a counting efficiency of around 40%.

Electron microscopy. The samples were fixed in glutaraldehyde and post-fixed in 1% (w/v) OsO₄. They were dehydrated in 50% (w/v), 75% (w/v) and finally absolute ethanol before embedding in araldite. Sections were stained with 3% (w/v) uranyl acetate in methanol for 5 min followed by Reynold's lead citrate (Reynolds, 1963) for 30 min.

Results

Characterization of myelin and myelin-like fractions

In the present study purified myelin and myelin-like fractions were examined as a routine by electron

microscopy (see Plates 1 and 2). Analysis of both preparations for the marker enzyme 2':3'-cyclic nucleotide 3'-phosphohydrolase showed activities of 955 μ mol/h per mg of protein for purified myelin and 616 μ mol/h per mg of protein for the myelin-like preparation. This pattern is in good comparison with the data of Agrawal *et al.* (1970). In addition, protein samples of each fraction were separated by gel electrophoresis (see Plate 3). These studies indicated the homogeneity and purity of our samples. The electrophoretic profiles were similar to those reported by Agrawal *et al.* (1972) except that the DM-20 band was not detectable in our myelin preparations. Only faint bands corresponding to basic protein and proteolipid protein were detectable in the myelin-like preparation (Plate 3). The myelin protein profile (Table 1) showed, during ontogeny, a changing proportion of basic and proteolipid protein compared

Table 1. *Protein composition of developing rat brain myelin*

Brains from rats of different ages were treated as described in the Experimental section. Four rat brains were used for each extraction and the means of duplicate analyses are shown.

Age (days)	Total protein/brain (mg)	Myelin protein (mg/brain)			
		Total	Basic	Proteolipid	High-molecular-weight
32	150	10.2	2.00	3.6	2.3
51	173	14.4	2.75	4.9	2.2
72	181	17.5	3.70	6.7	2.4
116	193	23.3	4.00	9.8	2.4
220	228	23.8	4.40	9.7	2.4

Table 2. *Radioactivities in total proteins after the intraperitoneal injection of [³H]lysine and [U-¹⁴C]glucose in developing rat brain*

Radioactivity was determined in trichloroacetic acid-insoluble protein fractions as described in the text. Groups of four rats were killed at the time indicated. Each value is the result of the triplicate analysis of four pooled brains.

Time after injection	Precursor... [³ H]Lysine		[¹⁴ C]Glucose	
	Radioactivity (d.p.m./brain) in proteins of:		Radioactivity (d.p.m./brain) in proteins of:	
	Whole brain	Myelin	Whole brain	Myelin
5h	489100	18300	737800	13200
24h	504300	24400	499200	16500
4 days	467800	28900	—	—
5 days	—	—	267400	16200
9 days	394300	29000	124400	14500
14 days	358200	27000	—	—
28 days	215700	22600	—	—
30 days	—	—	34600	8700
49 days	124100	18000	25500	7100
93 days	56000	13300	10000	3800
200 days	20400	12160	5000	3150

with the uniform concentration of the high-molecular-weight proteins.

Incorporation of [^3H]lysine and [^{14}C]glucose into rat brain proteins

After injection of lysine there is an initial rapid incorporation of labelled amino acid into protein followed by a slow increase to a maximum after 24 h; the subsequent loss of radioactivity is exponential up to at least 100 days after injection (Table 2) with a calculated half-life for the mixed brain proteins of 27 days (Table 3). Since persistence and recycling of labelled lysine (see Table 4) may seriously complicate kinetic studies of this type (Garlick & Marshall, 1972; Shapira *et al.*, 1972), it was decided to compare metabolism of proteins labelled by lysine with that found by using a more readily metabolized substrate, namely [$\text{U-}^{14}\text{C}$]glucose. The radioactive amino acids formed from glucose were quickly incorporated into brain proteins and excess of amino acids (e.g. glutamate and aspartate) was rapidly metabolized. In the experiments with [^{14}C]glucose, catabolism of brain proteins was much faster with an estimated $t_{0.5}$ of 4 days (Tables 2 and 3). In addition maximum incorporation had occurred by 5 h after injection. These data suggested possible reutilization of labelled lysine in the first experiment and therefore concentrations of free amino acids in blood and brain after administration of lysine and glucose were examined.

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Free amino acids in brain and blood

Even 9 days after administration of [^3H]lysine radioactivity was detectable in the blood. In the brain, radioactivity remained at a detectable amount for up to 49 days in the free amino acid pool (Table 4).

To study the possibility that some precursor may be adsorbed to tissue components a solution of [^3H]lysine at 2°C was added ($1\ \mu\text{Ci}$ in $0.1\ \text{ml}$ of water) to $5\ \text{ml}$ of 5% (w/v) brain homogenate at 0°C . Protein was immediately precipitated with 10% (w/v) trichloroacetic acid, washed as described in the text and radioactivity determined by the liquid-scintillation method. Of the added radioactivity 0.005% was recovered in the washed protein fraction. This indicated that some adsorption of radioactivity may occur in the presence of high specific radioactivity lysine in the brain. For these reasons our studies were repeated with [$\text{U-}^{14}\text{C}$]glucose and the specific radioactivity in the free dicarboxylic amino acids was estimated. After injection of glucose the concentration of radioactivity in the free amino acid pool in the blood remained relatively low compared with that after lysine injection and no radioactivity could be detected 9 days after injection. Highest radioactivity in amino acids in the brain acid-soluble extract was seen 5 h after injection of glucose, but even at 28 days the radioactivity remained at 1% of the initial value (Table 4). The calculation of the specific radioactivities of amino

Table 3. *Half-lives of the proteins in the subcellular fraction of rat brain injected with [^{14}C]glucose and [^3H]lysine*

Methods are as described in the text. Rats of 22 days were injected; the experiment lasted 100 days after injection of lysine and 28 days after that of glucose. Each value is the mean of triplicate analyses of four pooled brains.

Subcellular fraction	Mean half-lives (days)	
	[^3H]Lysine	[^{14}C]Glucose
Whole homogenate	27	4.0
Nuclear fraction	33	5.0
Crude myelin	50	6.0
Synaptosomal fraction	34	6.5
Mitochondrial fraction	31	4.5
Microsomal fraction	24	4.0
Soluble fraction	24	4.0

Table 4. *HClO₄-soluble radioactivity in plasma and brain after injection of labelled lysine and glucose*

[4,5- ^3H]Lysine and [$\text{U-}^{14}\text{C}$]glucose ($1\ \mu\text{Ci/g}$ body wt.) were injected intraperitoneally into 22–23-day-old rats and radioactivity was determined in the acid-soluble extracts as described in the text. Results are means of duplicate analyses from four pooled samples separated into total amino acid fractions by chromatography.

Time after injection	Acid-soluble radioactivity after injection of:			
	[^3H]Lysine		[$\text{U-}^{14}\text{C}$]Glucose	
	(d.p.m./ml of plasma)	(d.p.m./whole brain)	(d.p.m./ml of plasma)	(d.p.m./whole brain)
5 h	56000	58000	7000	103600
1 day	9000	29000	2000	19500
5 days	3500	6000	1000	6800
9 days	700	3300	Trace	1300
28 days	Trace	1840	None	1100
49 days	None	940	None	400
93 days	None	70	None	170

Table 5. *Specific radioactivities of amino acids in acid-soluble extracts of rat brain after intraperitoneal injection of [^3H]lysine and [^{14}C]glucose*

Four rat brains were removed at the times indicated. Brains were homogenized separately and homogenates were combined. Values for the amino acid content/g of brain in developing rats were taken from the literature (Agrawal *et al.*, 1966). Radioactivity in glutamic acid and aspartic acid was determined by high-voltage electrophoresis as described in the text. HClO_4 -soluble extract was passed through a Zeo-Karb column and radioactivity eluted with NH_3 was taken as free lysine as it is subject to little metabolic change (D'Monte *et al.*, 1971). Each value is the mean of the triplicate analyses.

Time after injection	Separated amino acid	Precursor ...	Specific radioactivity (c.p.m./ μmol)		
			[^3H]Lysine	[U- ^{14}C]Glucose	
			[^3H]Lysine	[^{14}C]Glutamic acid	[^{14}C]Aspartic acid
5h			130	0.42	0.60
24h			65	0.12	0.26
5 days			22	0.05	0.14
9 days			6.2	0.02	0.04
30 days			3.3	0.01	0.02
49 days			2.0	None	None

acids derived from the two precursors showed marked differences (Table 5) since the concentration of glutamate and aspartate in the brain are respectively about 80 and 30 times that of lysine (Agrawal *et al.*, 1966).

Subcellular fractions

The distribution of the radioactivity between the proteins of the subcellular fractions was similar in rats injected with [^3H]lysine and [^{14}C]glucose. Maximum incorporation was seen in the soluble proteins after injection of both precursors, and subsequent turnover of labelled protein was exponential. However, with lysine the calculated half-lives (Table 3) were about 4–5 times longer. Little difference was seen in catabolism of the proteins of individual subcellular fractions except that crude myelin labelled with lysine appeared more stable than other fractions. The myelin prepared here by the method of Gray & Whittaker (1962) is not pure and had a significant amount of contamination from microsomal fractions and nerve-ending particles. So it was decided to extend the investigation by using purified myelin and to examine the specific radioactivity of the individual myelin proteins.

Incorporation and persistence of radioactivity in purified myelin proteins

Myelin was isolated by flotation on 0.88M-sucrose and then purified by repeated osmotic shock and washing by the method described by Norton (1971). Incorporation of the labelled precursors into purified myelin proteins reached a maximum 1 and 9 days after injection of [^{14}C]glucose and [^3H]lysine respectively. To calculate turnover rates labelling at these times was taken as 100%. The decay curve did not appear to be first-order for the duration of the experiment, but to have two components (Fig. 1). It was possible

Fig. 1. *Turnover of total myelin proteins in rat brain: graphical plot of log (% radioactivity/brain) against number of days after maximum incorporation into total myelin proteins of rat brain*

Developing rats (22–23 days old) were injected intraperitoneally with [^3H]lysine and [^{14}C]glucose (1 $\mu\text{Ci/g}$ body wt.). Maximum incorporation into the myelin proteins (16500 d.p.m./brain) after [^{14}C]glucose ($\bullet$) and (29000 d.p.m./brain) after [^3H]lysine ($\circ$), which occurred 1 day and 9 days after the injection respectively, was taken as 100% at zero time. Each point was based on pooled brains from four rats. The semi-logarithmic plots showed the presence of two components turning over at different rates. Corrected decay curves were plotted for [^3H]lysine (Δ) and for [^{14}C]glucose ($\triangle$) from the values obtained by extrapolation. Half-lives for the fast components were calculated from the corrected decay curves, and for the slow components from the lines A–B for [^3H]lysine and C–D for [^{14}C]glucose, obtained by back extrapolation. The half-lives for fast components were $t_{0.5} = 24$ and 22 days for glucose and for lysine precursors respectively. Slow components were stable with either of the precursors used (50% in lysine compared with 27% with glucose).

Table 6. Radioactivity in the isolated myelin proteins of developing rat brain (23 days old) after intraperitoneal injection of [^3H]lysine and [^{14}C]glucose

Each value is the result of the triplicate analysis of four pooled brains at the times indicated. Isolation and determination of radioactivity in myelin proteins were carried out as described in the text. Radioactivities are given as d.p.m./brain. Samples were lost in (—) cases during extraction procedure.

Time after injection (days)	Radioactivity (d.p.m./brain)							
	Total radioactivity		In basic protein		In proteolipid protein		In high-molecular-weight protein	
	[^3H]Lysine	[^{14}C]Glucose	[^3H]Lysine	[^{14}C]Glucose	[^3H]Lysine	[^{14}C]Glucose	[^3H]Lysine	[^{14}C]Glucose
9	29000	14500	6280	1730	12800	3870	3780	6060
28	22600	8700	4560	1250	9120	3070	1600	2600
49	18000	7100	3540	810	9600	2880	640	1470
93	13000	3800	2670	960	7660	1060	210	720
200	12100	3100	2280	640	—	620	—	—

Fig. 2. Turnover of myelin basic proteins in rat brain

Basic proteins were isolated from the purified myelin after intraperitoneal injection of [^{14}C]glucose (○) and [^3H]lysine (●) and the values at 9 days were taken as 100% at zero times. Each value is based on pooled samples from four brains. The plots show the basic proteins turning over at two rates. Corrected decay curves were plotted for [^{14}C]glucose (Δ) and for [^3H]lysine (▲) from the values obtained by extrapolation. Half-lives for the fast components were calculated from corrected decay curves ($t_{0.5} = 22$ days for [^{14}C]glucose and $t_{0.5} = 19$ days for [^3H]lysine) and for slow components from the lines AB for [^{14}C]glucose and CD for [^3H]lysine obtained by back extrapolation. (a) [^{14}C]Glucose; (b) [^3H]lysine.

to calculate the true turnover rates of the fast component by extrapolation back from the slowly metabolizing fraction and correcting for radioactivity for this contribution (see Fig. 1). Very slow turnover was noted of about half the myelin protein from the animals injected with lysine and about 27% from animals injected with glucose. The fast-turning-over components of myelin proteins decayed with similar half-lives of 22–24 days for both precursors.

Decay of radioactivity in individual myelin proteins

Three major myelin protein components, e.g. pro-

teolipid protein, basic protein and high-molecular-weight (Wolfgram, 1966) proteins, were separated and their purity was checked by gel electrophoresis and the radioactivity determined as described above (Table 6). Myelin basic proteins extracted with cold 0.1 M-HCl (Martenson *et al.*, 1969) gave quantitative recovery of relatively pure basic proteins as judged by polyacrylamide-gel electrophoresis. To check for the presence of labelled lipid, isolated basic proteins labelled with [^3H]lysine and [^{14}C]glucose were extracted three times with chloroform-methanol (2:1, v/v) and the washings were combined, evaporated and

Table 7. Turnover of myelin proteins of rat brain

Developing rats (22 days old) were injected intraperitoneally with [^3H]lysine and [^{14}C]glucose (1 $\mu\text{Ci/g}$ body wt.). Purified myelin was isolated by flotation on 0.88M-sucrose from four pooled brains starting 9 days after the precursor injection. Proteins were separated from myelin and radioactivity was determined in them by the method described in the text. Fast and slow components were calculated from the decay curves by back extrapolation. (See legend for Fig. 2 for the method of calculation for basic proteins.)

	Calculated protein (component %)		Half-lives (days)	
	[^3H]Lysine	[^{14}C]Glucose	[^3H]Lysine	[^{14}C]Glucose
Total { fast component	50	73	22	24
{ slow component	50	27	Stable	Stable
Basic { fast component	50	50	19	22
{ slow component	50	50	Stable	Stable
Proteolipid { fast component	0	50	Not present	48
{ slow component	100	50	Stable	Stable
High-molecular-weight (Wolfgram, 1966) fast component	100	100	17	22

Table 8. Half-lives for myelin basic proteins of rat brain

Data from the literature are cited.

Isotope precursor	Route of injection	Duration of experiment (days)	Calculated half-life (days)	Reference
[^3H]Leucine	Subcutaneously	120	21	Wood & King (1971)
[^{14}C]Lysine	Intracisternal	0.25	14–21	D'Monte <i>et al.</i> (1971)
[^{14}C]Leucine	Intracisternal	56	42	Smith (1972)
[^{14}C]Tryptophan	Intracisternal	27	Rapid	Sammeck <i>et al.</i> (1971)
[^3H]Leucine	Intraperitoneal	80	100	Fischer & Morell (1973)
[^3H]Leucine	Not mentioned	43	Stable	Shapira <i>et al.</i> (1972)
[^3H]Lysine	Intraperitoneal	180	Fast component, 19; slow component, stable	This paper
[^{14}C]Glucose	Intraperitoneal	210	Fast component, 22; slow component, stable	This paper

radioactivities determined in a liquid-scintillation counter. No significant radioactivity was detected in the chloroform-methanol extract, suggesting the absence of the chloroform-methanol-extractable labelled lipid in our isolated basic proteins. Decay curves of the label from the isolated myelin basic proteins (Fig. 2) show two components of the first order. By extrapolation back a stable component comprising about half the basic protein was found and on correction a fast component for both precursors with a half-life of 19–22 days was calculated. After injection of [^{14}C]glucose, labelled proteolipid protein similarly appeared to have two components (Table 7). The half-life for the fast component comprising half of the proteolipid protein was 48 days and the other half was stable. In animals injected with [^3H]lysine data for the proteolipid protein did not clearly demonstrate the presence of a fast component and most of the proteolipid was in the stable form. Decay of the label from the high-molecular-weight (Wolfgram, 1966) proteins was exponential and faster than for the basic or proteolipid proteins. The calculated

half-lives were 16 and 22 days for rats injected with lysine and glucose respectively.

Discussion

Various experimental methods have been used for studying the turnover of brain structural proteins and a whole range of different isotopic precursors, routes of injection and times of experiment have been used (e.g. Table 8). This has led to contradictory data appearing in the literature for myelin proteins (Smith, 1972; Wood & King, 1971; De Lores Arnaiz *et al.*, 1971; Fischer & Morell, 1973), and it is the object of the present paper to clarify at least some of the complicating factors.

Purity of membrane and subcellular fractions

In the present study brain homogenates were separated into crude subcellular fractions by using a well-established method. No attempt was made to fractionate organelles further. The homogeneity of myelin-like and highly purified myelin fraction was

assessed by electron microscopy, by marker-enzyme assay and by polyacrylamide-gel electrophoresis. The protein profile of the purified myelin fraction (Plate 3) was similar to that reported by Waehndt & Mandel (1972). The 'myelin-like' membrane fraction had minor bands corresponding to basic and proteo-lipid proteins (see Plate 3), these could, however, be accounted for by slight contamination from myelin membrane.

Quantitative recovery of whole membrane and specific proteins

During subcellular fractionation of neonatal rat brain there is considerable loss of organelle and soluble protein, but with increasing age these losses are decreased. Our experiments began with rats about 30 days postnatal because recoveries at this age are more reliable. The analysis of radioactivity from polyacrylamide gels gave low counting efficiency and therefore we estimated radioactivity in isolated myelin proteins by the method described above. Since in development, brain protein content increases as a result of growth our results have been expressed as radioactivity per brain rather than per mg of protein thus avoiding the problem of correcting for dilution of the radioactivity (see Table 9 and Smith, 1972) by unlabelled protein due to growth. Some authors have not attempted such corrections (e.g. Wood & King, 1971; D'Monte *et al.*, 1971) which could possibly account for the rapid turnover rates reported by them for myelin proteins.

Presence of labelled lipid and adsorbed amino acid

It was considered possible that labelled lipid might be present in separated myelin proteins since lipids are known to be bound to isolated brain proteins (Uda & Nakazawa, 1973; Saito *et al.*, 1972). However, labelled lipid could not be extracted from our isolated basic protein preparations even after repeated extraction with organic solvents. These experiments

suggest either the absence of labelled lipid bound to the basic proteins or very tightly bound lipid which could not be extracted with chloroform-methanol. In preliminary experiments when [^{14}C]acetate was injected into developing rat and 24 h afterwards basic proteins were isolated from the purified myelin, 95% of the label incorporated could be extracted by chloroform-methanol (2:1, v/v) (M. I. Sabri & A. N. Davison, unpublished work). More work along these lines is needed to confirm the presence of lipid in isolated basic protein preparations. Adsorption of labelled amino acids in protein preparation was also considered, since adsorption of high-specific-radioactivity amino acids may be a problem in short-term experiments, particularly after intracranial injection (McEwen & Hyden, 1966). In our experiments after intraperitoneal injection the specific radioactivity of the free amino acids was considerably lower than one would get by using intracranial injection and therefore the problem of adsorption of labelled amino acids is minimized; moreover radioactive precursor in blood becomes quite low 9 days after the intraperitoneal injection (see Table 4).

Nature of precursor, its metabolism and the problem of reutilization

In the present study we have used [^3H]lysine and [$\text{U-}^{14}\text{C}$]glucose as precursors. The former is only slowly metabolized by brain (D'Monte *et al.*, 1971) whereas glucose is rapidly converted mainly into dicarboxylic acids. Table 5 shows that whereas there is persistence of labelled lysine for up to 49 days after injection only small amounts of radioactive amino acids are present in the acid-soluble pool of the brain after injection of glucose on a specific radioactivity basis. Thus after lysine injection radioactive precursor is available for reutilization for a number of days thereby influencing the apparent turnover rates of labelled protein. In short-term experiments of 3-4 h Garlick & Marshall (1972), who used [^{14}C]tyrosine,

Table 9. *Specific radioactivities of purified myelin and total proteins of rat brain after intraperitoneal injection of [^3H]lysine*

Each value is based on pooled samples from four brains. Total protein and radioactivity per brain were calculated for each time. Values at 9 days are taken as maximum incorporation. Corrected values were obtained by dividing the total radioactivity per brain by that of the total proteins at 9 days.

Days after injection	(d.p.m./mg of protein)			
	Total myelin proteins		Total brain proteins	
	Observed radioactivity	Corrected radioactivity	Observed radioactivity	Corrected radioactivity
9	2900	2900	2700	2700
14	2670	2700	2350	2460
28	1680	2220	1250	1480
49	1130	1840	680	850
93	570	1330	290	380
Calculated mean half-life (days)	30	70	18	27

have already demonstrated up to 40% recycling of precursor. On correction their half-life for total brain-proteins was 4 days; identical with our result after [^{14}C]glucose injection (see also Austin *et al.*, 1972). The large differences in the apparent turnover rates of brain proteins obtained with lysine and glucose precursors might also be due to the fact that [^3H]lysine would preferentially be incorporated in the basic proteins (nuclear histones) which turn over slowly (Piha *et al.*, 1966). The radioactivity from [^{14}C]glucose would be incorporated into the brain acidic proteins which turn over faster than the basic proteins. To confirm this, brain homogenates labelled with [^3H]lysine and [^{14}C]glucose were extracted with 0.1 M-HCl. Basic proteins being soluble in 0.1 M-HCl were separated from acid insoluble proteins which were mainly acidic in nature (M. I. Sabri & A. N. Davison, unpublished work). A 15% higher incorporation in basic proteins after lysine injection was observed, whereas with glucose as precursor acidic proteins had more radioactivity than basic proteins. This could in part explain the differences found on turnover of brain subcellular fraction proteins; those labelled with lysine being apparently more stable than those where glucose was precursor. It therefore appears that turnover of brain organelle protein is more rapid than has been considered hitherto.

Heterogeneity of decay curves

Our data for whole myelin proteins and isolated basic and proteolipid proteins illustrate the possible error of assuming biological decay to be first order. Thus after injection of lysine half of the total myelin protein apparently undergoes relatively fast turnover whereas the remainder is stable. This latter stability may be ascribed to considerable reutilization of labelled lysine particularly during the first week after injection (Table 5) when accumulation of [^3H]lysine in the slowly metabolizing proteolipid and basic proteins may occur. After [$\text{U-}^{14}\text{C}$]glucose injection, only 27% is incorporated into the more stable protein, for here the specific radioactivity of the amino acids rapidly decreases, preventing the continuous uptake into the more slowly synthesized protein.

It is apparent that the more stable myelin proteins can only be detected by experiments of more than 100 days' duration. Calculations assuming first-order kinetics will give misleading results particularly when different precursors and different routes of injection are used (see Table 8). In this type of experiment glucose has an advantage over other precursors in that persistence of labelled amino acids is not a serious complicating factor (Garlick & Millward, 1972). The high-molecular-weight proteins described by Wolfgarm (1966) undergo rapid metabolism and may therefore be considered to be possibly localized on the membrane surface. It may be concluded that proteolipid protein and some of the myelin basic pro-

tein has a high degree of metabolic stability providing an inert structural framework for the membrane lipids.

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