Turnover of Myelin Proteins in Mouse Brain *in vivo*

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The incorporation of tyrosine into proteins was measured after the subcutaneous implantation of a pellet of [^{14}C]tyrosine in mice. This method keeps the specific radioactivity of free tyrosine fairly constant and makes it possible to follow incorporation up to a 10-day period. At the end of 10 days most of the protein-bound tyrosine was replaced (i.e. most protein turned over) in lung, liver, heart, kidney and spleen; about half was replaced in brain, one-quarter in muscle. The rate of protein turnover in myelin was approx. 40% of that of whole brain proteins; at 10 days one-fifth of the myelin proteins were replaced. All protein components of myelin measured were in a dynamic state; incorporation decreased in the following order, Wolfgram > DM-20 > basic > proteolipid proteins. The incorporation of tyrosine into each protein fraction was greater in the 0–5-day than in the 5–10-day period, indicating heterogeneity of metabolic rates. The results show that after myelination at least a portion of each protein component of myelin is undergoing significant metabolic turnover. In the adult, myelin components are not stable, but turnover is heterogeneous, and each protein may be compartmentalized. Turnover can be influenced by a variety of factors.

The measurement of metabolism of proteins of myelin in the brain presents a number of technical difficulties in addition to those inherent in measuring the metabolism of any protein component of the brain. For example, in measuring the incorporation of amino acids, the degree of access and the local reutilization of the labelled amino acids present special problems in a structure like the myelin sheath.

Because of obstacles to clear interpretation even of the numerous interesting reports from many laboratories, a number of questions remain. We would like to know, for example: (a) are some of the proteins of myelin stable?; (b) is the metabolism different at various layers of the myelin sheath?; (c) do changes in proteins participate in demyelination and other pathologies?; (d) what is the relationship of myelination to myelin protein metabolism?

Previous work has indicated that the rate of incorporation of amino acids into myelin proteins seems to be lower than into the bulk of brain proteins (Furst *et al.*, 1958; Davison, 1961), although the degree of stability of myelin proteins has not been established. Synthesis of myelin proteins was found in the adult as well as in the immature brain (Smith, 1968; Agrawal *et al.*, 1976), but the actual values differ, depending on the method of measurement used. Heterogeneity of incorporation was also indicated (D'Monte *et al.*, 1971; Smith, 1972; Fischer & Morell, 1974; Sabri *et al.*, 1974), and great increase in protein synthesis (Smith & Rauch, 1974) and

breakdown (Smith *et al.*, 1974) was noted in experimental allergic encephalomyelitis.

Methods used in most studies involved short-term pulse-labelling and the measurement of the release of label with time. The pulse dose would be preferentially contained in a metabolically active fraction that is not representative of the bulk of proteins. The release of label from proteins also may not be representative of true metabolic rates, since the initial pulse may not label all proteins in a uniform manner, and reutilization of label may occur extensively in myelin. We have reported measurements of rates of protein turnover in the brain (Lajtha *et al.*, 1976). Our method of subcutaneously implanting a labelled tyrosine pellet in mice kept the specific radioactivity of precursor amino acid constant over several days, thus avoiding many of the problems of pulse-labelling and the compartmentation of the label, while allowing the measurement of incorporation over a sufficient time for a significant portion of tissue proteins to become labelled. These measurements indicated that brain proteins could be divided into two pools: a small pool, which comprised 6% of the total, was rapidly metabolized, with an approximate average half-life of 15 h, and the remaining 94% turned over with an average half-life of 10 days (Lajtha *et al.*, 1976). These measurements also indicated that there was an approx. 50% reincorporation of label in experiments measuring decay or radioactivity, and that in short-term labelling of under 1 h most of the radioactivity is

in the small more active pool comprising only 5% of proteins.

In the present paper we report our measurements of rates of turnover of myelin proteins in adult mouse brain under conditions in which the incorporation of tyrosine into proteins could be followed over a 10-day period. A preliminary account of some of the results has appeared (Lajtha *et al.*, 1975).

Methods

Administration of [¹⁴C]tyrosine

Adult male Swiss mice (28–30g) bred in our own animal colony were implanted subcutaneously with a [¹⁴C]tyrosine pellet at the beginning of the experiment (Lajtha *et al.*, 1976). For preparation of the pellets, 500mg of L-tyrosine (Cyclo Chemical Corp., Los Angeles, CA, U.S.A.) was dissolved in 100ml of water at 80°C, 250 μ Ci of [¹⁴C]tyrosine was added, and the combined solution was concentrated to 50ml in a vacuum desiccator at room temperature. The resulting suspension was then pipetted into five centrifuge tubes and centrifuged at 0°C and 100000g for 90min in the SW39 head of the Spinco centrifuge. The pellet formed (100mg) did not break apart on implantation.

Preparation of myelin

Myelin from individually frozen brains was isolated as described previously (Agrawal *et al.*, 1972).

*Isolation of myelin proteins by preparative polyacrylamide-gel electrophoresis in SDS**

Myelin proteins for SDS/polyacrylamide-gel electrophoresis were prepared and separated from one another by a scaled-up version of the analytical SDS/polyacrylamide-gel system previously described (Agrawal *et al.*, 1972). Acrylamide was polymerized in a glass column with an internal diameter of 22mm and a total length of 344mm. Briefly, 18ml of 10% (w/v) acrylamide was added to 20ml of 50mM-sodium phosphate buffer, pH 7.2, containing 0.2% (w/v) SDS. This was followed by the addition of 60 μ l of NNN'-tetramethylethylenediamine and 2ml of a freshly prepared solution of ammonium persulphate (8mg/ml). The solution was mixed, 37ml was pipetted into the glass column, and water was immediately layered over the gel solution. After polymerization, the surface of the gel was washed with the gel buffer (diluted 1:1 with water). The total length of the polymerized gel was 110mm. Myelin proteins (from one mouse brain) from which lipids had been removed were dissolved in a sample buffer (5mM-sodium phosphate, pH 7.2) containing 8% (w/v) sucrose, 1% (w/v) SDS, 1.5% (w/v) dithiothreitol and 100 μ l of an aqueous solution of Bromo-

phenol Blue (0.05%, w/v), and heated for 2min in a boiling-water bath. The protein was layered on the gel and overlaid with gel buffer (pH 7.2) diluted 1:1 (v/v) with water. The diluted gel buffer was used to fill the lower (anode) and upper (cathode) compartments of the electrophoresis apparatus. The current was maintained at 3.0mA for 3h, increased to 5mA for 5.5h, then to 8mA for 15h, and then gradually increased to 20mA. Electrophoresis was stopped 2h after the Bromophenol Blue had migrated out of the gel. The gel was then removed from the column and left at 4°C for 15h. After this period, the white bands of flocculated SDS-protein complex of all myelin proteins could be readily seen within the gel. These bands correspond exactly to the respective proteins stained with Coomassie Brilliant Blue. Appropriate sections of the gel containing the proteins were sliced and crushed, and each protein was eluted with 0.1% (w/v) SDS containing 0.1% (w/v) dithiothreitol (20ml/gel section) by incubation overnight at 25°C. After centrifugation (1000g) to sediment the gel particles, the clear solution was filtered through glass wool. A small portion was freeze-dried and used to determine the homogeneity of eluted myelin proteins by analytical SDS/polyacrylamide-gel electrophoresis (Allison *et al.*, 1974). The purity of each myelin protein (Fig. 1) having been ascertained, the remainder of the extract was concentrated by freeze-drying. The bands were also tested by co-chromatographing purified proteins with the mixed myelin. In each case the migration of our 'marker' was identical with that of the myelin component.

Measurement of incorporation of [¹⁴C]tyrosine

To test whether the specific radioactivity of plasma tyrosine was constant during the incorporation period, two blood samples were taken from each animal during the experiment, and a third when it was killed. The specific radioactivity of the free tyrosine in brain at the end of the experiment was also determined. For determination of free tyrosine, portions of plasma or whole-brain homogenate were frozen, weighed, homogenized in 2% (w/v) HClO₄, kept at 0°C for 1h, then centrifuged at 30000g for 15min. Portions of the supernatant were run on a Technicon amino acid analyser. The purified myelin protein fractions were hydrolysed under N₂ for 20h at 110°C in 2M-HCl and dried under vacuum. The hydrolysates were then taken up in a small amount of water and put on the amino acid analyser. One part of the tyrosine peak from the analyser was used for the determination of the amount of tyrosine with ninhydrin and another for the determination of radioactivity in a scintillation spectrometer (Teller *et al.*, 1974). The changes in the specific radioactivity (d.p.m./ μ mol) of protein-bound tyrosine in relation to the average specific radioactivity of free tyrosine in brain in each time interval were used to estimate metabolic rates.

* Abbreviation: SDS, sodium dodecyl sulphate.

Fig. 1. Purity of the myelin protein fractions
A fraction of separated myelin proteins used for determining the specific radioactivity of protein-bound tyrosine was subjected to polyacrylamide-gel electrophoresis as described in the Methods section. Results of a typical run with each of the four fractions Wolfgram (WP), proteolipid (P-P), DM-20 and basic proteins (BP) are shown.

Results

Specific radioactivity of the free amino acid pool

The specific radioactivity of the tyrosine in the plasma rose rapidly, remained constant in the 6–48 h period, and then declined. In the plasma the value at 4 days was 95% of that at day 1, 79% at 7 days and 61% at 10 days (Fig. 2). Implantation of a second pellet at 5 days to avoid the decrease in specific radioactivity gave unreliable results and was abandoned. The specific radioactivity of free tyrosine in brain was approx. 80–85% of that in the plasma throughout the experiment. The closeness of specific radioactivities of plasma and brain free amino acids avoids the possibility that an external high-specific-radioactivity precursor can be incorporated that does not mix completely with the internal pool. In each animal, blood samples were taken throughout the experiment. In most animals the specific radioactivity of tyrosine was maintained as indicated in Fig. 2; the few animals in which this was not achieved were discarded. At the end of the experiment most of the tyrosine pellet remained at the implantation site. The decline in plasma specific radioactivity was probably

due to a decreased rate of absorption caused by scar-tissue formation around the pellet.

Incorporation of tyrosine into tissue proteins

As reported by Lajtha *et al.* (1976), in 5 days about one-third of protein-bound tyrosine is replaced in brain and about three-quarters in liver and kidney. The replacement is greater in heart, lung and spleen than in brain (Table 1); in muscle, it is less than in brain. At the 10-day period most protein-bound tyrosine had been replaced in the organs measured, but in brain only about half and in muscle only one-quarter had been replaced.

These results agree with our earlier report based on

Fig. 2. Changes in specific radioactivity of free tyrosine in the plasma with time

The specific radioactivity (d.p.m./ μ mol) of isolated tyrosine in plasma is shown as a percentage of the specific radioactivity at 1 day after the implantation of [14 C]tyrosine pellet. Changes in brain free tyrosine were somewhat smaller, since the specific radioactivity of free tyrosine in the 2–5-day period was about 90%, and in the 5–10-day period 96%, of that in plasma.

Table 1. Replacement of tyrosine in proteins of organs

The specific radioactivity of free and protein-bound tyrosine was measured after the subcutaneous implantation of [14 C]tyrosine in adult Swiss mice. The amino acid was separated on the amino acid analyser. Replacement is expressed by the specific radioactivity of protein-bound tyrosine as a percentage of the specific radioactivity of free tyrosine in that organ for each time period. Averages of five experiments are given; the S.D. was within 6% of the mean.

Organ	Replacement (%)	
	5 days	10 days
Brain	32	53
Liver	78	89
Kidney	74	80
Muscle	11	23
Heart	41	84
Lung	67	98
Spleen	49	72

a 5-day experiment (Lajtha *et al.*, 1976), but the extension of incorporation to 10 days indicates the presence of additional small more slowly metabolized protein compartments in the liver and kidney. These experiments also indicate that any stable protein present must comprise only a very small portion of the total in most organs. However, from the present data, the presence of a significant stable protein compartment in muscle cannot be excluded. Our previous studies (Lajtha & Toth, 1966) indicated that less than 2% of the proteins could be metabolically stable in brain. Since significant amounts of tyrosine were implanted in the experimental animals, the effect of increased free amino acid in the tissue has to be considered. We have previously shown that brain protein metabolism is insensitive to fairly large increases of a free amino acid (Dunlop *et al.*, 1975). In experiments with implanted tyrosine we have shown that our method produces less than 10% increase in control free tyrosine, and does not affect the measured average turnover rate of brain proteins (Lajtha *et al.*, 1976). In these experiments the implant increased free tyrosine in liver and kidney (Lajtha *et al.*, 1976), and possibly also in other organs, which may influence the rates of turnover of proteins in these tissues.

Incorporation into myelin proteins

There was less replacement of protein-bound tyrosine in myelin proteins than in the total brain proteins (Table 2), with the specific radioactivity in brain protein 2.5–3 times that in myelin. Thus an average half-life of approx. 25–30 days is indicated, which is an average of separate heterogeneous metabolic rates. The heterogeneity is shown by the decrease in rates of incorporation in later time periods. The incorporation in the 7–10-day period was considerably lower than in the 3–5-day period, indicating that short-term experiments measure primarily the more rapidly metabolized fraction,

whereas the longer-term experiments measure the more slowly metabolized protein fractions. Even after 10 days of incorporation, only 20% of protein-bound tyrosine was replaced. Therefore it is possible that a significant portion of myelin proteins is fairly stable, or not readily accessible to the labelled amino acid. In these calculations we equate the rate of replacement of protein-bound tyrosine with the rate of protein turnover. This may not be so if the tyrosine content of various proteins differs greatly and turnover rates vary in parallel with tyrosine content. Most myelin proteins have been isolated in a high degree of purity, and several amino acid analyses for each are available (Eng *et al.*, 1968; Gonzalez-Sastre, 1970; Nussbaum *et al.*, 1974; Agrawal *et al.*, 1977). These show an average tyrosine content, except for a higher one in the proteolipid proteins. It is unlikely that, in mixed-organ proteins, turnover would parallel tyrosine content; therefore we consider that the replacement rate of protein-bound tyrosine is a suitable measure of the average rate of tissue protein turnover, especially if incorporation is measured over a long period of time, as was done in the present study.

Turnover of myelin components

Although at the end of 10 days only 20% of myelin proteins were replaced, no component appeared stable, since incorporation occurred into each protein component tested (Table 3). Incorporation into the Wolfgram protein (Wolfgram & Kotorii, 1968) was the fastest; the proteolipid protein and basic proteins were labelled at similar but lower rates; the DM-20 component (Agrawal *et al.*, 1972) was slightly more active than these two.

The rate of incorporation of tyrosine into each protein fraction was higher in the 5–7-day period than in the 7–10-day period; the decrease on the average was 30%; it was smallest with basic proteins, and largest with the Wolfgram-protein fraction. From our

Table 2. *Incorporation of tyrosine into myelin protein*
The specific radioactivity of protein-bound tyrosine was measured in isolated myelin. Incorporation (increase in specific radioactivity in protein as percentage of the specific radioactivity of free tyrosine of that time period) was calculated for each time period. Averages of four experiments $\pm$ s.d. for each time point are given.

Time (days)	Incorporation	
	(%)	(%/day for period)
1	3.7	3.7 $\pm$ 0.3
3	9.5	2.9 $\pm$ 0.3
5	14.3	2.4 $\pm$ 0.2
7	18.1	1.9 $\pm$ 0.2
10	20.5	0.8 $\pm$ 0.1

Table 3. *Turnover of separate myelin proteins*
Proteins from myelin were separated by gel electrophoresis and hydrolysed, and tyrosine was isolated and determined with an amino acid analyser. Turnover of proteins is expressed as described in the legend to Table 2, and is related to the specific radioactivity of free tyrosine in the brain for each time period. Averages of four experiments are given; the s.d. was within 9% of the mean.

	Turnover (%)		
	5 day	7 day	10 day
Basic protein	7.5	9.9	13
DM-20	9.0	12	15
Proteolipid	7.0	9.4	12
Wolfgram	20	25	29

results we cannot state what incorporation rates would be in time periods beyond 10 days. If incorporation continued linearly at the rates observed in the 7–10-day period, the half-life of protein components would be between 25 and 50 days. Freezing the brain before myelin isolation may enhance the degradation of myelin basic protein during subsequent preparation of the purified proteins. We think that this did not occur in our case, confirming similar previous reports (Ansari *et al.*, 1975). In a set of control experiments, the gel band pattern of myelin proteins prepared from fresh brain, then stained with Coomassie Brilliant Blue, was identical with the band pattern of proteins prepared from brain.

Discussion

Turnover of myelin proteins

We consider that our method of labelling myelin proteins minimizes, as much as possible, the problems encountered with other methods of measuring incorporation. Especially with a structure that is not rapidly accessible to precursor, long-term incorporation studies are easier to interpret than pulse-labelling, or decay after pulse-labelling. In spite of keeping the specific radioactivity of the tissue-free pool relatively stable over several days, we do not know the extent of equilibration of the free amino acid pool of myelin with the free pool in the rest of the brain, especially in the inner portions of the myelin sheath. Any lack of equilibration, combined with local reincorporation of liberated non-equilibrated amino acids, would mask some of the metabolic activity. Our measurements give at least minimal values for the protein components and are in agreement with most recent studies (Smith, 1968, 1972; D'Monte *et al.*, 1971; Fischer & Morell, 1974; Sabri *et al.*, 1974; Agrawal *et al.*, 1976) in that the rates of turnover are considerable, though lower than that of average brain proteins. Since all the proteins in myelin are metabolically active, the capacity for breakdown and for synthesis *in vivo* is present for each. At least a portion of each myelin protein must be degraded and resynthesized several times over a 1-year period. The proteins of myelin have been extensively studied, and there is general agreement that the protein components that we studied represent almost all the proteins contained in myelin. The characteristics of our bands are also well established (Chan & Lees, 1974); among others, we have compared them with molecular-weight markers (Agrawal *et al.*, 1972). Each of the proteins was purified, and its amino acid composition has been established; for basic protein, the amino acid sequence is known (Agrawal, 1974; Carnegie & Dunkley, 1975). The protein composition was similar in the various species analysed, including man; it is likely that turnover rates are also similar in the various species. We found that the turnover rate of

brain proteins of fish, if extrapolated to 37°C, was similar to that in mice and rats (Lajtha & Sershen, 1975).

Our finding that there are differences in turnover rates among the various protein components confirms similar previous observations (Smith, 1968; D'Monte *et al.*, 1971; Figlewicz & Druse, 1976; Benjamins *et al.*, 1976). The indication that the same protein may have portions with different turnover rates is probably related to structural heterogeneity in myelin (Sabri *et al.*, 1974; Poduslo & Braun, 1975; Zimmerman *et al.*, 1975). The turnover rate of the part of a myelin protein more accessible to the metabolic machinery could be considerably higher. Iodination studies indicated that basic protein is less exposed to the outer surface than proteolipid protein is (Poduslo & Braun, 1975). Atypical incorporation into Wolfgram proteins specifically has also been observed (Johnson *et al.*, 1976). The heterogeneity of protein metabolism is similar to the heterogeneity of the metabolism of the lipid components of myelin (Smith, 1968; Hayes & Jungalwala, 1976).

Alterations of turnover

During myelination, when there is a net deposit of myelin proteins, greater incorporation of amino acids was observed (Sabri *et al.*, 1974; Gaitonde & Martenson, 1970); the greater release of label with time from immature brain (Agrawal *et al.*, 1976) indicates that in addition to net deposition the turnover is also higher. This is analogous to studies with whole brain: we found in young brain higher rates of turnover, that is during growth (net deposition of proteins) not only synthesis but also protein breakdown was elevated (Lajtha & Dunlop, 1974; Dunlop *et al.*, 1975). Changes in turnover may also reflect developmental changes in myelin protein composition (Morell *et al.*, 1972), since various proteins are deposited at different rates (Detering & Wells, 1976), but changes in metabolism of individual components (Benjamins *et al.*, 1975) may also be involved.

Changes in myelin protein metabolism were observed under a number of conditions. Starvation caused decreased protein synthesis, probably by decreasing oligodendroglial proliferation (Wiggins *et al.*, 1974), and hexachlorophene poisoning decreased myelin proteins, particularly glycoprotein (Matthieu *et al.*, 1974). The changes were especially large with experimental allergic encephalomyelitis (Smith & Rauch, 1974), where synthesis and breakdown were greatly increased. Other pathological changes in myelin protein turnover have been noted (Figlewicz & Druse, 1976). Endocrine influence on myelinogenesis has also been observed (Valcana *et al.*, 1975). Diphtheria toxin inhibited myelin protein synthesis in a heterogeneous manner; synthesis of Wolfgram protein was inhibited by 21%, that of proteolipid and basic proteins by 79–88% (Pleasure

et al., 1973). These results indicate that both synthesis and the breakdown of myelin proteins can be altered under a number of conditions.

Breakdown of myelin proteins

By the end of the 10-day period the extent of incorporation is so large that it cannot be due chiefly to myelination. Protein turnover thus indicates local breakdown of proteins. Although initial studies indicated the resistance of myelin proteins, especially proteolipids, to breakdown, the tryptic digestion of myelin proteins is well established; other extracellular proteinases also seem to be active (Raghavan *et al.*, 1973; Banik & Davison, 1974; Lees & Chan, 1975). Lipid-protein interactions (Banik & Davison, 1974; Lees & Chan, 1975; Banik *et al.*, 1976) may influence digestibility. The lower content of proteolipid in spinal-cord myelin may result in weaker cohesion of lipids and proteins, increasing instability (Smith & Sedgewick, 1975). Of special importance in support of myelin protein turnover are the findings of breakdown of myelin proteins by brain proteinases, as shown *in vitro* in purified preparations (D'Monte *et al.*, 1971; Raghavan *et al.*, 1973) and by autolysis *post mortem* (D'Monte *et al.*, 1971). We found that incubation of myelin at physiological pH resulted in significant endogenous breakdown of basic proteins; incubation with cathepsin D resulted in a 30–60% loss of all myelin proteins as determined by gel electrophoresis, but without significant liberation of free amino acids (Marks *et al.*, 1976b). More detailed analysis of the breakdown of basic proteins showed that cathepsin D split the protein at two bond sites into three polypeptides; some of these (but not the intact protein) are substrates for cathepsin A for further breakdown (Marks *et al.*, 1976a). These findings indicate that the protein composition of myelin is governed not only by synthesis but also by breakdown. The role of protein breakdown caused by endogenous or exogenous enzymes in demyelination is of great importance and has often been studied in experimental allergic encephalitis (Smith *et al.*, 1974; Buletza & Smith, 1976).

Our studies, combined with numerous previous findings, show significant metabolic activity in at least a part of myelin involving all protein components. This metabolic activity is heterogeneous and is likely to be affected by many circumstances.

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