Accumulation and Turnover of the Classical Folch–Lees Proteolipid Proteins in Developing and Adult Rat Brain

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The turnover of classical Folch–Lees proteolipid proteins was studied after administration of [2,3-³H]tryptophan to both developing and adult rat brain. The animals were killed from 2 h to 250 days after subcutaneous injections of [³H]tryptophan. The measured specific radioactivity in developing brain attained maximum value 24 h after the administration of label, whereas the total radioactivity per brain reached a maximum 21 days after injection. The half-life of proteolipid protein from the measured specific radioactivity was 7–20 days, depending on the time-points used for the calculation, whereas calculation from total radioactivity between 28–77 and 91–257 days gave half-lives of 35–40 and 188 days respectively. In contrast, in animals injected at 40 days of age, the half-life from the whole-brain-radioactivity data was 188 days. The problem of the recycling of radioactivity for the synthesis of myelin proteins from either a general or a discrete amino acid pool is discussed.

Any protein that is soluble on extraction of neural or any other tissue with 19 vol. of chloroform/methanol (2:1, v/v) is classified as the classical Folch–Lees proteolipid protein (Folch & Lees, 1951). Until recently it was assumed that the chloroform/methanol (2:1, v/v)-extractable protein fraction from the whole brain was not only a homogeneous protein but also the major protein of myelin. However, recent advances in the technique of solubilizing membrane lipid–protein complexes in sodium dodecyl sulphate followed by their separation by polyacrylamide-gel electrophoresis in sodium dodecyl sulphate showed that Folch–Lees proteolipid protein is a group of proteins, the molecular weights of which ranged between 10000 and 50000 (Agrawal *et al.*, 1972; Nussbaum & Mandel, 1973; Chan & Lees, 1974; Lerner *et al.*, 1974). However, it must be emphasized here that the major protein band in 15–21-day-old rat brain migrates to the same position in sodium dodecyl sulphate/polyacrylamide gels as does myelin proteolipid protein (Plate 1). In addition, the major protein band in the chloroform/methanol (2:1, v/v)-soluble fraction from the whole brain also accounts for 90% of the total Folch–Lees proteolipid protein after 12 days of age (Plate 1).

Earlier work from both Lajtha's (Furst *et al.*, 1958) and Davison's (1961) laboratories indicated that chloroform/methanol-soluble proteins, i.e. the classical Folch–Lees proteolipid protein fraction, were metabolically stable. In contrast, Smith (1968, 1972) found that the myelin proteolipid protein in adult rats turned over with a half-life of 35–42 days.

Fischer & Morell (1974) again studied the turnover of myelin proteolipid protein and found that it is stable in developing mouse brain, but that it turned over with an apparent half-life of 102 days in the adult. Sabri *et al.* (1974) found that the 'slow component' of the rat brain myelin proteolipid protein is stable after administration of both [³H]lysine and [U-¹⁴C]-glucose. In contrast, the 'fast component' was found to have a half-life of 48 days when [U-¹⁴C]glucose was used as the precursor (Sabri *et al.*, 1974). We were interested in determining whether the turnover rate of whole brain proteolipid protein was different in animals injected at 7 days of age (just before the period of active myelination) as opposed to those injected at 40 days of age. Advantages of studying the turnover of this protein from the whole brain as opposed to proteolipid protein isolated from myelin are that we can quantitatively extract this protein from the brain at any stage of development, and determine its concentration. In the present paper we present evidence that the Folch–Lees proteolipid protein fraction is not metabolically inert (Davison, 1961), but that it has an apparent half-life of 188 days when animals are injected at 40 days of age. In contrast, in developing brain the half-life of proteolipid protein varies from 7 to 188 days depending on whether the half-life is calculated from the measured specific radioactivity or from c.p.m./brain. The significance of these results is discussed and related to the turnover of proteolipid protein in central-nervous-system myelin. A preliminary report of this work has been presented (Agrawal *et al.*, 1975).

Experimental Methods

Animals

Sprague-Dawley albino rats bred in our animal colony were used throughout this study. The litters were decreased to eight pups at birth and weaned at 21 days of age. For the developmental study the entire litter was killed at each time-point because of the possibility of increasing the growth rate of surviving pups when litter sizes were decreased.

Chemicals

All chemicals used were of the highest degree of purity commercially available unless otherwise specified. DL-[2,3-³H]Tryptophan (specific radioactivity 164 mCi/mmol), Omnifluor and Aquasol were obtained from New England Nuclear, Boston, Mass., U.S.A., and were used as such. NCS and Soluene were purchased from Amersham/Searle, Arlington, Ill., U.S.A. The source of reagents for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was described previously (Agrawal *et al.*, 1974). Proteins used as molecular-weight standards were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Radioactive tracer studies of Folch-Lees proteolipid proteins

Developing rats. Rats (7 days old) were injected subcutaneously with 6 μ Ci of DL-[2,3-³H]tryptophan/g body wt. and killed. The time-interval between injection and killing varied up to 250 days. Four to seven animals were used for each time-point and Folch-Lees proteolipid protein was prepared from the whole brain as described below.

Adult rats. Rats (40 days old) were injected with 1 μ Ci of DL-[2,3-³H]tryptophan/g body wt. and killed. The time-interval between injection and killing varied up to 181 days. Two to four brains per time-point were extracted individually by the technique of Folch & Lees (1951) for the preparation of chloroform/methanol (2:1, v/v)-soluble proteolipid proteins.

Analytical methods. The animals were killed by decapitation and the brains removed and weighed in a tared beaker at 0–4°C. Blood was collected from the severed neck in a heparinized glass tube and centrifuged at 0–4°C to separate the plasma. Plasma was stored at –70°C until used for the determination of free and protein-bound radioactivity. Folch-Lees proteolipid protein was extracted from the fresh whole brain with 19 vol. of chloroform/methanol (2:1, v/v) by hand homogenization in a ground-glass homogenizer at room temperature (25°C) and filtered through a sintered-glass funnel. The filtrate was partitioned with water and the lower organic phase was washed five times with synthetic upper

phase as described by Folch & Lees (1951) until the upper phase was devoid of free radioactivity. A portion (3 ml) of the washed lower phase containing both proteins and lipids was air-dried in a scintillation vial, solubilized in 1 ml of Soluene and radioactivity measured in 10 ml of toluene containing 4 g of Omnifluor/litre with a Packard Tri-Carb liquid-scintillation spectrometer, model 3375, at 4°C. The counting efficiency was 35% as determined by both external and internal standardization. Another sample was used for protein assay by the technique of Lowry *et al.* (1951), with modifications introduced by Hess & Lewin (1965). Plasma proteins were precipitated with an equal volume of ice-cold 10% (w/v) trichloroacetic acid and washed with 3 $\times$ 3 ml of 5% (w/v) trichloroacetic acid to extract free radioactivity completely. The nucleic acid was removed by heating the acid-insoluble residue in 5% trichloroacetic acid at 90°C for 15 min. Lipids from the nucleic acid-free residue were removed by successive washings with ethanol/diethyl ether (3:1, v/v) followed by chloroform/methanol (2:1, v/v) and diethyl ether as described by Agrawal *et al.* (1970). The residue was solubilized in 0.5 M-NaOH by incubating overnight at 37°C. Then 5 vol. of NCS was added to a portion of the protein solution, and radioactivity was measured in the scintillation fluid described above. Another sample was used for protein assay by the technique of Lowry *et al.* (1951). To determine the free radioactivity in plasma, the trichloroacetic acid-soluble fraction was partitioned with 5 $\times$ 5 vol. of diethyl ether to remove the acid, and the acid-free solution was freeze-dried. It was reconstituted in a known volume of water and counted for radioactivity in Aquasol.

Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate

The Folch-Lees proteolipid protein fraction was precipitated from the washed organic (lower) phase by the addition of 4 vol. of diethyl ether. It was stirred at 0–4°C in the cold-room for 1 h. The opalescent solution was centrifuged at 0–4°C for 1 h at 37000g. The supernatant was discarded and the pellet transferred and homogenized in a sample buffer containing 5 mM-sodium phosphate, pH 7.2, 8% (w/v) sucrose and 1% (w/v) sodium dodecyl sulphate. A sample was removed for protein assay by the technique of Lowry *et al.* (1951). The amount of protein was calculated against a calibration curve of bovine serum albumin which was dissolved in the sample buffer. We were able to precipitate 90–95% of chloroform/methanol (2:1, v/v)-soluble proteins from the whole brain by diethyl ether. Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate was carried out by the technique of Weber & Osborn (1969) with modifications introduced by Allison *et al.*

EXPLANATION OF PLATE I

Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate of the classical Folch-Lees proteolipid proteins of developing rat brain

Proteolipid protein (100 μ g), rat brain myelin protein (60 μ g) and each standard protein (5 μ g) were applied to 12% polyacrylamide gels. Electrophoresis was carried out in a buffer containing sodium dodecyl sulphate (0.1%, w/v) and 100mM-sodium phosphate buffer, pH7.2, as described by Allison *et al.* (1974). The gels were stained with 0.25% (w/v) Coomassie Brilliant Blue in 45% (v/v) methanol, 10% (v/v) acetic acid and water. De-staining was carried out as described by Agrawal (1974). Abbreviations: MY, rat brain myelin; WP, Wolfgram protein; PLP, proteolipid protein; DM-20 (Agrawal *et al.*, 1972); LBP, large basic protein; SBP, small basic protein; STD, standard proteins, from top to bottom; conalbumin, catalase, ovalbumin, chymotrypsinogen, myoglobin, cytochrome c. The mol.wts. are 76000, 60000, 43000, 25000, 17200 and 11700 respectively.

(1974). A sample (100 μ g of protein) was electrophoresed on polyacrylamide gels (12%, w/v) in 0.1% (w/v) sodium dodecyl sulphate. The gels were stained with 0.25% (w/v) solution of Coomassie Brilliant Blue for 24h and de-stained by diffusion into successive washes of decreasing concentrations of methanol as described previously (Agrawal, 1974).

Results

Since tryptophan has not been extensively used to study the turnover of plasma, liver or brain proteins, we first determined the logarithmic decay curve of plasma protein radioactivity and of plasma free tryptophan radioactivity in developing rats after administration of labelled tryptophan at 7 days of age. The data in Fig. 1 show that plasma proteins were rapidly labelled after the administration of [3 H]tryptophan, and its decay curve allowed calculations of a half-life of approx. 2.5 days. Acid-soluble radioactivity decreased much faster than plasma proteins, as might be expected.

When 40-day-old rats were injected with [3 H]-tryptophan, the turnover of plasma proteins was 1.9 days, whereas the free radioactivity gave the same half-life as that for developing rats (Fig. 1). Whether or not the small differences observed in plasma proteins between developing and young adult rats are real or represent only experimental variation is not known. As described by Stoner *et al.* (1975), the plasma turnover of 'free' tryptophan is a very complex phenomenon.

Turnover of proteolipid protein in developing rat brain

The concentration of the proteolipid protein fraction was 0.19mg/brain at 7 days of age and gradually increased to 16.6mg/brain at 257 days of age (Table 1). Thus there was an 88-fold increase in the concentration of this protein. Proteolipid protein accounts for 0.4% and 9.0% of the total brain protein at 7 and 257 days of age respectively. Table 1 also shows that the measured specific radioactivity was maximum at 24h after the administration of label and then showed a steady decline. When the half-life of this protein was calculated from the measured specific radioactivity, it was found to be 7–20 days, depending on the time-point used to determine the turnover rate. Since the amount of proteolipid protein was increasing (Table 1) in developing brain and we wanted to eliminate the problem of dilution of radioactive with non-radioactive proteolipid protein the results were expressed as c.p.m./brain (Table 1 and Fig. 2). The total radioactivity/brain peaked at 21 days after injection and then showed a slow and steady decline (Table 1 and Fig. 2).

We have no explanation for the increase in radioactivity in developing brain until 21 days of age. However, this increase may reflect the reutilization

Fig. 1. Logarithmic decay curve of [3 H]tryptophan from plasma proteins and acid-soluble radioactivity in developing and young adult rats

Details of the methods are given in the text. The results are expressed as percentage of maximum specific radioactivity of plasma proteins and the acid-soluble pool. Δ , Plasma proteins of developing rats [100% = 46649 c.p.m./mg of protein; in rats 8 days of age and 24h after radioisotope administration]. $\blacktriangle$, Plasma proteins of adult rats [100% = 9625 $\pm$ 825 (s.d.) c.p.m./mg of protein; in rats 40 days of age and 2h after injection of [3 H]tryptophan]. $\bullet$, Plasma acid-soluble radioactivity in adult rats [100% = 89651 $\pm$ 9445 (s.d.) c.p.m./ml of plasma; in rats 40 days of age, 2h after injection]. $\circ$, Plasma acid-soluble radioactivity in developing rats [100% = 1280575 $\pm$ 15981 (s.d.) c.p.m./ml of plasma; in rats 7 days of age, 2h after injection].

Table 1. *Accumulation and turnover of whole brain proteolipid protein in developing rat*

All animals were injected subcutaneously with 6 μ Ci of DL-[2,3- 3 H]tryptophan (specific radioactivity 164 mCi/mmol)/g body weight at 7 days of age. Four to seven brains were used for each time-point for the extraction of Folch–Lees proteolipid protein fractions. The results are means $\pm$ s.d. of two to three different preparations at each age.

Age (days)	Brain weight (g)	Proteolipid protein (μ g/brain)	Radioactivity (c.p.m./brain)	Measured specific radioactivity (c.p.m./mg of proteolipid protein)
7*	0.64 $\pm$ 0.02	188 $\pm$ 13	4150 $\pm$ 30	22166 $\pm$ 1720
8	0.96 $\pm$ 0.02	220	8853	40206
10	0.96 $\pm$ 0.02	494 $\pm$ 20	16989 $\pm$ 454	34392 $\pm$ 74
14	1.28 $\pm$ 0.04	1693 $\pm$ 7	31162 $\pm$ 911	18533 $\pm$ 500
21	1.30 $\pm$ 0.07	3184 $\pm$ 271	40915 $\pm$ 1130	12923 $\pm$ 1196
28	1.39 $\pm$ 0.05	5204 $\pm$ 469	46083 $\pm$ 2454	8863 $\pm$ 94
37	1.43 $\pm$ 0.11	6667 $\pm$ 254	43975 $\pm$ 421	6601 $\pm$ 241
57	1.65 $\pm$ 0.12	8631 $\pm$ 317	34063 $\pm$ 1748	3944 $\pm$ 100
77	1.67 $\pm$ 0.05	9973 $\pm$ 552	23351 $\pm$ 42	2349 $\pm$ 126
98	1.67 $\pm$ 0.01	12089 $\pm$ 166	24260 $\pm$ 1212	2008 $\pm$ 128
116	1.81 $\pm$ 0.01	14843 $\pm$ 748	21767 $\pm$ 599	1449 $\pm$ 30
157	1.93 $\pm$ 0.04	14138 $\pm$ 292	17728 $\pm$ 1068	1252 $\pm$ 50
220	1.81 $\pm$ 0.07	16240 $\pm$ 1153	14170 $\pm$ 1009	911 $\pm$ 53
257	1.97 $\pm$ 0.06	16630 $\pm$ 787	12030 $\pm$ 328	726 $\pm$ 21

* This group of rats was killed 2h after the administration of label.

Fig. 2. *Turnover of Folch–Lees proteolipid protein in developing and adult rat brain*

Rats [7 (●) or 40 (○) days old] were injected (marked by arrow) subcutaneously with 6 μ Ci or 1 μ Ci/g body wt. respectively of DL-[2,3- 3 H]tryptophan. The first group in both developing and adult rats was killed 2h after administration of label. Proteolipid protein was prepared and radioactivity determined as described in the text. The data for developing brain are taken from Table 1. In animals injected at 40 days of age, the half-life calculated from the whole-brain-radioactivity data was 188 days. In contrast, for animals injected at 7 days of age, two half-lives can be derived. Those between 28–77 and 91–257 days were found to be 35–40 and 188 days respectively. The half-life in all cases was calculated by the method of least squares.

of [3 H]tryptophan. A similar phenomenon was observed by Sabri *et al.* (1974) for [3 H]lysine. A gradual increase in radioactivity was not seen when 40-day-old rats were similarly injected (Fig. 2).

Computation of the half-life of proteolipid protein by the method of least squares from whole-brain radioactivity data (Table 1 and Fig. 2), gives values of 35–40 and 188 days when calculated between 28 and 77, and 91 and 257 days respectively.

Turnover of proteolipid protein in young adult rat brain

The concentration of proteolipid protein was 6mg/brain at 40 days of age and increased to 18mg/brain at 212 days of age. Therefore there was an approximate 3-fold increase in protein concentration. The fact that the concentration of brain proteolipid protein continued to increase even after 197 days of age suggests that this protein is accumulating throughout the life-span of the rat. For the reasons mentioned above, the turnover rate of proteolipid protein was not calculated from the measured specific radioactivity, but from the whole-brain proteolipid protein radioactivity (Fig. 2). The half-life calculated by the method of least squares between 77 and 181 days after injection was 188 days.

Discussion

Davidson (1961) studied the turnover of the chloroform/methanol (2:1, v/v)-soluble proteolipid protein in developing rat brain and concluded that it was metabolically stable. Smith (1968, 1972) studied the turnover of proteolipid protein isolated from myelin of adult rats after intraperitoneal and intracranial injections of [U- 14 C]glucose and [1- 14 C]leucine respectively. She found that the half-life of

myelin proteolipid protein was 35–42 days, which disagreed with the findings of Davison (1961). Fischer & Morell (1974) also studied the turnover of isolated myelin proteolipid protein in mouse brain and found that it was stable in developing brain, whereas it turned over with a half-life of 102 days in the adult. A careful examination of the data on myelin proteolipid protein in developing brain (Fischer & Morell, 1974) clearly indicates that the corrected specific radioactivity of this protein was increasing between 16 and 96 days after the administration of labelled leucine, which is in fair agreement with the findings reported in the present paper (Fig. 2). Sabri *et al.* (1974) studied the turnover of myelin proteolipid protein after administration of [^3H]lysine and [$\text{U-}^{14}\text{C}$]glucose. They found that the 'slow components' of this protein were stable, whereas the 'fast components' had a half-life of 48 days when glucose was the labelled precursor. Fig. 2 and Table 1 show that if we calculate the half-life of proteolipid protein between 28 and 77 days after administration of [^3H]tryptophan it would be 35–40 days, which would be in agreement with the findings of Smith (1968, 1972). However, when the half-life was calculated between 91 and 257 days after injection, it became 188 days. The turnover rate of proteolipid protein was also found to be 188 days when animals were injected at 40 days of age. We have thus demonstrated that the turnover of proteolipid protein in developing rat brain was (a) 7–20 days when calculated from the measured specific radioactivity, (b) 35–40 days when computed from the results of whole brain radioactivity measured between 28 and 77 days after the administration of [^3H]tryptophan, and 188 days when measured between 91 and 257 days of age. In contrast, in animals injected at 40 days of age, the half-life of this protein was 188 days. An inherent problem associated with all of these long-term studies of myelin protein turnover after a single dose of labelled precursor (Davison, 1961; Smith, 1968, 1972; Fischer & Morell, 1974; Sabri *et al.*, 1974) is the possible reutilization of radioactivity. Therefore the half-life of these proteins could be in error, unless it can be demonstrated with certainty that there was no reutilization of radioisotope either from the general metabolic pool or

from a discrete precursor pool. The problem of recycling of label for the synthesis of myelin proteins from a discrete pool is a more serious one, and one which has not yet been eliminated.

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