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DISTRIBUTION OF RADIOACTIVITY IN MYELIN LIPIDS FOLLOWING SUBCUTANEOUS INJECTION OF [^{14}C]STEARATE

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Summary

Blood fatty acids are an important parameter for the synthesis of brain myelin as exogenous stearic acid is needed: after subcutaneous injection to 18-day-old mice this labelled stearic acid is transported into brain myelin and incorporated into its lipids. However the acid is partly metabolized in the brain by elongation (thus providing very long chain fatty acids, mainly lignoceric acid) or by degradation to acetate units (utilized for synthesis of medium chain fatty acids as palmitic acid, and cholesterol). These metabolites are further incorporated into myelin lipids.

The myelin lipid radioactivity increases up to 3 days; most of the activity is found in phospholipids; their fatty acids are labelled in saturated as well as in polyunsaturated homologues but sphingolipids, especially cerebrosides, contain also large amounts of radioactivity (which is mainly found in very long chain fatty acids, almost all in lignoceric acid). The occurrence of unesterified fatty acids must be pointed out, these molecules unlike other lipids, are found in constant amount (expressed in radioactivity per mg myelin lipid).

Introduction

Saturated fatty acids necessary for membrane formation are obtained endogenously or exogenously. Indeed short chain fatty acids can penetrate the blood brain barrier [1] and it has been shown that radioactive saturated long chain fatty acids are taken up by brain, either when fed (rat) [2] or injected (mice) [3], moreover we have shown in mice [4,5] that the label of stearic acid, after subcutaneous injection is incorporated into lipids of subcellular particles as such, or after being metabolized in brain (by elongation, thus providing very long chain fatty acids, or by degradation and resynthesis in situ of fatty acids). Indeed saturated fatty acids are synthesized in the brain, in the cell sap [6,7], in mitochondrial [8,10] and in microsomes [11,14] but these

organelles are not able to synthesize all the saturated fatty acids needed for elaboration of brain cell membranes, thus brain is actually dependent on exogenous fatty acids, especially during the period of myelination [14]. This work was undertaken to study the abilities of myelin membrane to incorporate a saturated fatty acid (stearic acid) after subcutaneous injection. Stearic acid was used as it is the primer for very long chain fatty acids in brain [15,16].

Materials and Methods

1 mCi [1-^{14}C]stearic acid (51 Ci/mol) obtained from C.E.A. (France) was neutralized with an equimolar amount of NaOH (0.5 ml of 2 mg/ml NaOH) and 0.5 ml of 0.5 μM bovine serum albumin in 0.5% NaCl was added. 50 μCi was injected subcutaneously into 18-day-old C_{57}B1/6J mice. The radio-purity of the fatty acid was over 99.9% (as checked by gas-liquid chromatography).

Animals were fed with standard diet biscuits from Extra Labo (France). Brains were excised, washed and sliced with a razor blade in isotonic saline. The tissue fragments were then centrifuged at 17 500 $\times g$ for 10 min. The myelin was isolated by ultracentrifugation [17]. The purity of this fraction has been checked by marker enzymes and electron microscopy [18].

Total lipids were extracted by $CHCl_3/CH_3OH$ (2 : 1, v/v) [19,20]. The radioactivity was counted in a Packard liquid scintillator using PPO (2,5-diphenyl oxazole), POPOP (1,4-bis-[2-(4-methyl-5-phenyl oxazolyl)]-benzene) and toluene. Lipids were isolated on thin-layer silica gel (Merck 60 F 254) plates developed in $CHCl_3/CH_3OH/H_2O$ (70 : 30 : 4, v/v) [21]. Under these conditions, sphingomyelin, inositol phosphatides + choline phosphatides + serine phosphatides, sulfatides and cholesterol and cerebrosides are separated [22,23]. As free fatty acids are contaminated by ceramides, another system was used ($CHCl_3$/acetic acid, 90 : 10, v/v) with total lipids extract. The spots containing lipids from the 70 : 30 : 4 migration were scraped and extracted with five times 3 ml/cm^2 $CHCl_3/CH_3OH$ (2 : 1, v/v).

The spots were visualized by iodine vapour, scraped off and counted in the presence of Cab-o-sil PPO and di-methyl-POPOP. No quench correction procedure was applied as exactly the same quantity of silica gel is scraped for each individual lipid. The efficiency of recovery through the various extractions and analytic procedures was constant (85%) as tested by standards.

Lipids from total lipid extract were methylated [24] and directly analysed by gas-liquid chromatography on an SE 30 column 175—265°C, 20°C/min in a Hewlett-Packard 5750 with automatic counting of the eluate.

Blood was collected by heart puncture and made to 1 ml with water. The mixture was saponified by heating with 0.5 ml 15% methanolic KOH for 15 min at 100°C, 0.5 ml 5.5 M HCl was added and fatty acids were extracted twice with 5 ml light petroleum (b.p. 60—80°C). These acids were methylated [24], counted and analysed as previously described. For lipid analysis, blood was lyophilized and a lipid extraction was performed.

The myelin was prepared from six animals; each experiment was performed at least three times; thus about 90 mice were injected for this study.

Results and Discussion

Table I shows the uptake of radioactivity by myelin lipids of 18-day-old mice after injection of [1-^{14}C]stearic acid. The percentage found in sphingolipids increased with the highest radioactivity in cerebrosides. The radioactivity in phospholipids also increased, in contrast to that in free fatty acids. Little activity was found in the cholesterol fraction. The upper Folch phase (which contains mainly gangliosides) contained 10% of total brain radioactivity. In total brain lipids, 0.03% of the injected acid was recovered, 0.005% in myelin 20 h after injection.

As previously shown [3], blood does not contain other labelled fatty acids except [1-^{14}C]stearic acid. Normal β-oxidation in liver would cleave [1-^{14}C]-stearate during the first cycle of oxidation to produce acetyl-CoA; this acetyl-CoA could possibly be used for synthesis of new molecules either in the liver or in the brain after transport. However, the radioactivity of acetate (located in the aqueous phase of the extract of lyophilized blood) is extremely low, thus the synthesis of very long chains within the brain is not due to elongation of fatty acids by released acetyl-CoA outside the brain.

In Fig. 1, the activity in the lipid extract (radioactivity in cpm) increases

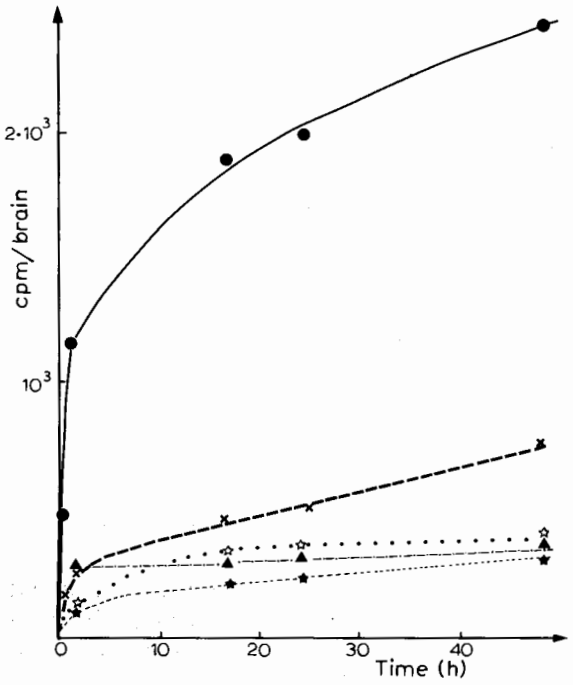

Fig. 1. Distribution of radioactivity in myelin lipids after subcutaneous injection of [1-^{14}C]stearic acid. ●, total lipids after Folch wash; X, inositol, choline and serine phosphoglycerides; ☆, ethanolamine phosphoglyceride; ★, cerebrosides; ▲, non-esterified fatty acids. The activity is expressed as the radioactivity (in cpm/brain) (for either total lipid extract or each individual lipid). Each point is the mean value of at least three experiments, for each time point 18 mice were used. About 15 000 cpm were used at each chromatography.

TABLE I

DISTRIBUTION OF RELATIVE RADIOACTIVITY IN MYELIN LIPIDS AFTER SUBCUTANEOUS INJECTION OF [1-^{14}C]STEARIC ACID

Same remarks as in Fig. 1. The percentage refers to the radioactivity found in the total lipid extract.

Time after injection	Sphingomyelin (%)	Choline-, Inositol-, Sérine-phospho-glyceride	Sulphatides (%)	Ethanolamine-phosphoglyceride	Cerebrosides (%)	Free fatty acids (%)	Cholesterol (%)
10 min	0.1	2.3	0.2	2.0	0.3	87.5	3.0
20 min	0.5	6.4	0.5	2.9	1.7	83.0	6.0
30 min	0.6	14.6	0.7	3.8	5.8	55.4	8.9
1.45 h	0.5	25.9	0.7	10.7	9.4	26.3	10.4
17 h	2.3	26.3	1.0	15.5	12.7	15.3	12.1
24 h	2.9	26.2	1.7	17.6	12.2	15.4	13.4
48 h	3.8	30.6	2.8	12.1	14.3	17.9	16.8

TABLE II

PROFILE OF LABELLED FATTY ACIDS IN SOME PURIFIED LIPIDS

The animals being killed 20 h after the injection of [1-^{14}C]stearic acid. C_{16}, palmitic acid; C_{18}, stearic acid; C_{20}, arachidic acid; C_{22}, behenic acid; C_{24}, lignoceric acid; C_{20} : p and C_{22} : p, polyunsaturated acids. tr, trace amount.

	C_{16} (%)	C_{18} (%)	C_{20} (%)	C_{20} : p (%)	C_{22} (%)	C_{22} : p (%)	C_{24} (%)
Cerebrosides	tr	19	10	34	—	37	
Phosphatidylcholine, -serine, -inositol	37	41	—	22	—	—	—
Phosphatidyletanolamine	12	65	—	22	—	tr	—
Free fatty acids	19	80	—	—	—	—	—

drastically up to 10 h, then levelling off slowly although still positive 48 h after injection. The radioactivity of each individual lipid fraction continuously increased, but the activity of free fatty acids remained constant with time. These free fatty acids are very tightly bound to myelin: incubation of a suspension of 0.4 mg/ml myelin in the presence of 1 mg/ml delipidated albumin did not extract any activity. Moreover, when pure myelin (from animals which have not been treated with radioactive acids) was incubated in the presence of [1-^{14}C]stearic acid, the uptake was very low. Thus, the uptake of fatty acids by myelin membrane is made in vivo through active metabolism (not simply physical non-specific binding), and fatty acids embedded inside the myelin sheath are not available for albumin extraction. The myelin free fatty acids must have a high specific radioactivity, as they are found in minute amounts in brain.

Table II shows the radioactive fatty acid profile in each lipid isolated 20 h after injection. It should be noted that cerebrosides contain a high amount of radioactive very long chain fatty acids, especially at the level of lignoceric acid. The smallest amount of radioactivity is found in stearic acid. Non-esterified fatty acid fractions contain mainly stearic acid and palmitic acid; the same acids are found in phospholipids, but the radioactivity found in C_{20}-polyunsaturated fatty acid is quite high.

TABLE III

PROFILE OF LABELLED FATTY ACIDS IN MAJOR MYELIN LIPIDS

The animals being killed 2 days after the injection of [1-^{14}C]stearic acid. Legend as in Table II.

	C_{16} (%)	C_{18} (%)	C_{18} : 1 (%)	C_{20} (%)	C_{20} : p (%)	C_{22} (%)	C_{22} : p (%)	C_{24} (%)
Cerebrosides	4	16	tr	4	—	11	—	64
Sulphatides	7	27	tr	3	—	9	—	53
Sphingomyelin	tr	51	tr	6	—	29	—	14
Phosphatidylcholine, -serine, -inositol	29	68	tr	—	3	—	—	—
Phosphatidylethanolamine	11	56	7	3	16	1	6	—
Free fatty acids	23	57	19	tr	—	—	—	—

Table III shows the fatty acid profile in each lipid isolated 2 days after injection. Sphingolipids still contain labelled very long chain fatty acids. But cerebrosides contain nearly the same amount of lignoceric acid at 2 days and at 20 h; this is similarly true for sulphatides, which also contain large amounts of radioactive lignoceric acid. (This is expected as sulphatides are synthesized by sulphation of cerebrosides [25]). The activity found in palmitic acid in all lipids when labelled stearic acid is injected is due to degradation to radioactive acetate and subsequent biosynthesis of medium chain fatty acids through a de novo procedure (this radioactive acetate is probably used in elongation systems). Moreover, this acetate is also used for synthesis of cholesterol; thus explaining the radioactivity found in this compound (see Table I). In phospholipids, the radioactivity in oleic acid was very low; the radioactivity in very long chain monounsaturated fatty acids in sphingolipids was hardly detected: this is unexpected, as brain microsomes are able to desaturate stearate to oleate, this latter molecule being further elongated to longer monunsaturated chains [26]. However, activity is detected in the polyunsaturated fatty acids involved in phospholipids, thus stearic acid when injected is desaturated and elongated.

Conclusions

During nutritional deprivation myelin synthesis is lowered [27] and dietary studies with essential fatty acids have shown that transport from blood to brain is possible [28—31]. If radioactive essential fatty acids are taken up by brain [32—35], those polyunsaturated fatty acids are poorly incorporated into myelin lipids, as this membrane contains large amount of sphingolipids containing saturated and monounsaturated fatty acids. This work demonstrates that a saturated fatty acid is taken up by brain and further incorporated into myelin lipids. The form under which it penetrates into brain is unknown, either in the free form or in the complex lipid form or in another form. This acid is either directly incorporated into myelin, or is elongated inside brain (thus providing arachidic and behenic acids) or is metabolized to acetate units (used for synthesis of medium chain fatty acids as palmitic acid or elaboration of cholesterol). These products of metabolism are partly incorporated into myelin lipids.

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References

1 Oldendorf, W.H. (1973) Am. J. Physiol. 224, 1450—1453
2 Dhopeshwarkar, G.A. and Mead, J.F. (1969) Biochim. Biophys. Acta 187, 461—467
3 Bourre, J.M., Bouchaud, C. and Baumann, N. (1974) Arterial wall 1—58
4 Gozlan-Devillierre, N., Baumann, N. and Bourre, J.M. (1976) Biochimie 58, 1129—1123
5 Gozlan-Devillierre, N., Baumann, N. and Bourre, J.M. (1976) C.R. Acad. Sci. 282, 1825—1826
6 Brady, R.O. (1960) J. Biol. Chem. 235, 3099—3103
7 Pollet, S., Bourre, J.M. and Baumann, N. (1969) C.R. Acad. Sci. 268, 2146—2149

8 Boone, S.C. and Wakil, S.J. (1970) Biochemistry 9, 1470—1479
9 Paturneau-Jouas, M., Baumann, N. and Bourre, J.M. (1976) Biochem. Biophys. Res. Commun. 71, 1326—1334
10 Paturneau-Jouas, M., Baumann, N.A. and Bourre, J.M. (1976) Biochimie 58, 341—349
11 Aeberhard, E. and Menkes, J.M. (1968) J. Biol. Chem. 243, 3834—3840
12 Bourre, J.M., Pollet, S.A., Daudu, O. and Baumann, N. (1975) Biochem. Biophys. res. Commun. 63, 1027—1034
13 Bourre, J.M., Pollet, S., Daudu, O. and Baumann, N. (1971) C.R. Acad. Sci. 27, 1534—1537
14 Bourre, J.M., Paturneau-Jouas, M., Daudu, O. and Baumann, N. (1977) Eur. J. Biochem. 72, 41—47
15 Bourre, J.M., Pollet, S., Chaix, G., Daudu, O. and Baumann, N. (1970) C.R. Acad. Sci. 271, 1221—1223
16 Bourre, J.M., Pollet, S., Chaix, G., Daudu, O. and Baumann, N. (1973) Biochimie 55, 1473—1479
17 Norton, W.T. and Poduslo, S.E. (1973) J. Neurochem. 21, 749—757
18 Baumann, N., Bourre, J.M., Jacque, C. and Harpin, M.L. (1973) J. Neurochem. 20, 753—759
19 Folch, J., Ascoli, I., Lees, M., Meath, J.A. and Lebaron, F.N. (1951) J. Biol. Chem. 191, 833—841
20 Folch, J., Lees, M. and Sloane-Stanley, G.M. (1957) J. Biol. Chem. 226, 497—509
21 Rouser, G., Bauman, A.J., Kritchevsky, G., Heller, D. and O'Brien, J.S. (1961) J. Am. Oil Chem. Soc. 38, 544—555
22 Baumann, N.A., Jacque, C.M., Pollet, S.A. and Harpin, M.L. (1968) Eur. J. Biochem. 4, 340—344
23 Bourre, J.M., Pollet, S.A., Daudu, O. and Baumann, N.A. (1973) Brain Res. 51, 225—239
24 Morrisson, W.R. and Smith, L.H. (1964) J. Lipid Res. 5, 600—608
25 Mac Khann, G.M. and Ho, W. (1967) J. Neurochem. 14, 717—724
26 Bourre, J.M., Daudu, O.L. and Baumann, N.A. (1976) Biochim. Biophys. Acta 427, 1—7
27 Wiggins, R.C., Mollet, S.L., Benjamins, J.A., Krigman, M.R. and Morell, P. (1976) Brain Res. 107, 257—273
28 Alling, C., Bruce, A., Karlsson, I., Sapia, O. and Svennerholm, L. (1972) J. Nutr. 102, 773—782
29 Galli, C., Trziciak, H.I. and Paoletti, R. (1972) J. Neurochem. 19, 1863—1867
30 Mohrhauer, H. and Holman, R.T. (1953) J. Neurochem. 10, 523—530
31 Sun, G.Y. (1972) J. Lipid Res. 13, 56—61
32 Dhopeshwarkar, G.A., Subramanian, C. and Mead, J.F. (1971) Biochim. Biophys. Acta 231, 8—14
33 Dhopeshwarkar, G.A., Subramanian, C. and Mead, J.C. (1971) Biochim. Biophys. Acta 239, 162—167
34 Illingworth, D.R. and Glover, J. (1973) J. Neurochem. 21, 1403—1414
35 Sinclair, A.J. (1975) Lipids 10, 175—184