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EXOGENOUS [1-^{14}C]LIGNOCERIC ACID UPTAKE BY NEURONS, ASTROCYTES AND MYELIN, AS COMPARED TO INCORPORATION OF [1-^{14}C]PALMITIC AND STEARIC ACIDS

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Abstract—In order to compare the incorporation of several saturated fatty acids into the brain, radioactive palmitic, stearic and lignoceric acids were injected into mice. The radioactivity was measured in lipids from isolated neurons, astrocytes and myelin.

Our data indicate that specific radioactivity of lignoceric acid after its injection was very high in neurons and astrocytes when comparing with serum lignoceric acid specific radioactivity: evidence of the uptake of exogenous lignoceric acid by brain cells and myelin is provided.

The incorporation of exogenous palmitic acid into brain cells was much higher than the incorporation of exogenous stearic acid. We hypothesize that exogenous saturated fatty acid uptake is selective in relation with the acyl chain length and the intracerebral synthesis.

It has been shown that exogenous palmitic and stearic acids are taken up by the brain as such, and are incorporated into lipids of subcellular particles (Bourre *et al.*, 1979; Dhopeshwarkar *et al.*, 1973; Gozlan-Devillière *et al.*, 1976). Furthermore, exogenous stearic acid can be either directly incorporated into cerebral cells (Morand *et al.*, 1979), myelin (Gozlan-Devillière *et al.*, 1978) and synaptosomes (Bourre *et al.*, 1977) or is eventually elongated to longer chains or degraded into acetate units.

When active myelination is in process, lignoceric acid (C 24:0) is synthesized by the brain and accumulates in myelin (Bourre *et al.*, 1977). These experiments were intended to demonstrate that the uptake of exogenous lignoceric acid occurs besides its *in situ* synthesis. We also attempted to compare the level of incorporation of several saturated fatty acids: palmitic acid (C 16:0), stearic acid (C 18:0) and lignoceric acid. The determination of the specific radioactivity of fatty acids from serum and brain lipids could provide some useful information on a possible selective uptake of exogenous fatty acids by the brain.

Analyses were performed on isolated cells and myelin avoiding any contamination from blood cells and serum. It also permitted individual consideration of the metabolic activity of each compartment, regarding the accumulation of fatty acids in the developing brain.

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EXPERIMENTAL PROCEDURES

Animals

Fifteen day-old mice used for these studies were fed with standard diet biscuits from Extra-Labo (France).

Injection of labelled albumin bound fatty acids

Two hundred milligrams of bovine fatty acid free albumin (Sigma) were solubilized into 1 ml saline water (0.45% w/v). A 0.5 ml solution containing 1 mCi [1-^{14}C] palmitic acid (49 mCi/Mmole) or 1 mCi [1-^{14}C]stearic acid (51 mCi/mmol.) or 1 mCi [1-^{14}C]lignoceric acid (46 mCi/mmol.) neutralized with an equimolar NaOH solution was mixed with the albumin solution. Labelled fatty acids were purchased from C.E.A. (France), their purity was checked by radiochromatography.

Each animal was given a subcutaneous injection (in the back) of 25 μCi albumin bound [1-^{14}C]fatty acid (this way is much more physiological as compared to intracerebral injection which eliminates blood brain barrier).

Isolation of neurons and astrocytes from mouse brains

Twenty hours after fatty acid injection, mice were killed and blood was immediately taken after cutting the right brachiocephalic artery. Pipettes and tubes used for blood were heparinated to prevent coagulation. Preliminary experiments revealed that this time, i.e. 20 h, was the most

appropriate for the present study (see Morand *et al.*, 1979 for details).

Then, brains were excised (cerebellum was discarded), pooled ×10 and washed with buffer. Cells were isolated according to a previously described method (Norton *et al.*, 1973) with some modifications, as stated below. Forebrains were finely chopped with a razor blade on a glass plate over ice. The minced tissue was incubated in 0.075% acetylated-trypsin (Sigma) in HAP medium (glucose 5%, fructose 5%, albumin 1%, buffered at pH 6.0) (10 ml/g wet tissue) for 60 min at 37°C. Trypsin incubation was terminated by cooling the mixture and trypsin was removed by washing twice with HAP.

The treated tissue, resuspended in 0.9 M sucrose in HAP medium, was sieved once through nylon meshes (100 μm) and three times through steel meshes (75 μm).

The suspension of cells and debris was layered onto a first sucrose gradient (from top to bottom: 0.9 M, 1.35 M, 1.55 M, 2.0 M sucrose in HAP) and centrifuged at 3300 *g* for 10 min. Crude astrocytes were collected at the 0.9–1.35 M interface and diluted in HAP for a second purification. The 1.55–2.0 M interface contained purified neurons. Diluted astrocyte fraction was layered onto a second gradient (1.0 M, 1.35 M) and centrifuged at 3300 *g* for 20 min. After being collected from the gradients and after dilution with HAP, each cell type was concentrated by centrifugation (1200 *g* for 10 min). The cell preparations were identified by examination under phase contrast microscopy. The purity of each fraction was checked with a Malassez microscope plate. The purity of neurons and astrocytes, based upon particles counting (contamination mainly with nucleus), was respectively about 95% and 80%. Radioactivity of an aliquot of each cell suspension was counted in Biofluor scintillator (New England Nuclear). Then, suspended cells were broken by osmotic shock in distilled water and sonication (3 times, 2 min, 40 W) (Branson Sonifier). Membranes were pelleted by centrifugation (at 400,000 *g* for 20 min), lyophilized and stored at −30°C.

Lipid extraction and fatty acid methylation

Lipids were extracted by sonication (6 × 30 s) in chloroform:methanol 2:1 (v/v) (Folch *et al.*, 1957; Pollet *et al.*, 1978). Lipids from total lipid extract were methylated (Morrison *et al.*, 1964). Before GLC analysis, to each sample was added a definite quantity of tricosanoic acid (C23:0) or heptadecanoic acid (C17:0) as internal standard. Sequential azide decarboxylation was performed according to Phares (1951) and performed on the total brain fatty acids (so as to have enough material) after separation of fatty acids by GLC with fraction collection.

Gas–liquid chromatography

Fatty acid radioactive distribution was determined after analysis by gas–liquid chromatography on 3% SE 30 packed column (3 m long), temperature programming 190–280°C at 2°C/min; 10% of the affluent passed in the flame ionization detector, 90% was collected on anthracene and counted as determined by liquid scintillation. At least 5000 cpm were injected in the GLC; more than 2500 were recovered in the various fatty acids (counting efficiency is 50% in anthracene as compared to liquid scintillation; statistical precision of counting a sample was below 5%).

Table 1. Relative distribution of radioactivity and fatty acid composition of *serum* lipids from 15 day-old mice, 20 h after injection of labelled fatty acids.

	Relative distribution of radioactivity (%)			
	Injection of [1-^{14}C] C16:0 (%)	Injection of [1-^{14}C] C18:0 (%)	Injection of [1-^{14}C] C24:0 (%)	Fatty acid composition (%)
16:0	82.3 ± 6.1	2.6 ± 0.8	8.9 ± 4.3	28.6 ± 2.1
16:1	3.3 ± 2.3	1.0 ± 0.8	–	1.3 ± 0.4
18:0	6.7 ± 0.9	69.0 ± 7.5	7.0 ± 2.5	13.0 ± 0.8
18:1	3.3 ± 1.8	6.9 ± 1.7	2.8 ± 2.8	19.1 ± 1.2
18:2ω6	–	–	–	21.2 ± 1.5
18:3ω3	–	–	–	1.0 ± 0.1
20:0	1.0 ± 0.1	2.8 ± 1.1	3.2 ± 1.9	–
20:1	–	0.9 ± 0.2		0.4 ± 0.2
20:3ω6	0.8 ± 0.3	7.4 ± 3.5	3.3 ± 1.8	0.5 ± 0.3
20:4ω6				8.3 ± 1.1
20:4ω3				–
20:5ω3				1.7 ± 0.4
22:0	0.7 ± 0.3	2.3 ± 0.5	2.1 ± 1.4	–
22:1	–	0.5 ± 0.4	1.0 ± 0.7	–
22:4ω6	–	3.6 ± 4.0	–	–
22:5ω6				–
22:5ω3				–
22:6ω3				3.1 ± 1.3
24:0	–	1.0 ± 0.9	70.0 ± 10.0	2.6 ± 0.4
24:1	–	–	3.4 ± 1.0	–

Each point (% ± s.e.m.) is the mean value of 3 groups of mice (9 groups for the fatty acid composition).

Fatty acid composition was analyzed on an open tubular capillary column coated with Carbowax 20 M (0.25 mm diameter, 80 m long): detection was made by flame ionization. Integration of fatty acid peaks was performed using an integrator ICAP 10 (LTT). Distribution of radioactivity and fatty acid composition, using internal standard, permitted calculation of fatty acid specific radioactivity of serum, astrocytes, neurons and myelin.

RESULTS AND DISCUSSION

In the whole brain, 20 h after injecting [1-^{14}C]stearic acid, the isolated stearic acid contained 82% of its label in the carboxyl end; only 26% of the label was found in the carboxyl end of the palmitic acid. This demonstrated that the injected acid was largely incorporated as such, but it was also partly degraded and the degradation products were reutilized for fatty acid synthesis. When [1-^{14}C]lignoceric acid was injected, the isolated lignoceric acid from the whole brain contained 63% of the label in the carboxyl end. Very similar results were obtained in the myelin.

After injecting [1-^{14}C]stearic acid, fatty acid analysis in the blood showed that about 70% of the total radioactivity was in stearic acid (as shown in Table 1), and 91% of the label was in its carboxyl end.

Thus, under our conditions, the acid found in blood as well as in brain was still largely original according to azide decarboxylation. In the brain, the original acid was partially degraded and the degradation products were reutilized.

Considering the distributions of radioactivity in fatty acids from serum lipids (Table 1), one can see that the radioactivity was mainly detected in the corresponding injected fatty acid. The specific radioactivity of serum lignoceric acid (Table 5), after its injection, was high, indicating a large uptake of this fatty acid in serum lipids although it represents only 2.6% of total serum fatty acids. Radioactivity was also present in other saturated, monounsaturated and polyunsaturated fatty acids. Furthermore, after injection of stearic acid or lignoceric acid, serum lipids contained radioactive palmitic acid (Table 1), thus it is very probable that those fatty acids have been degraded producing acetate units used for *de novo* synthesis.

In respect with the uptake of lignoceric acid, one can see that specific radioactivity of this fatty acid

Table 2. Relative distribution of radioactivity in fatty acids and fatty acid composition of *astrocyte* lipids from 15 day-old mouse brains, 20 h after injection of labelled fatty acids

	Relative distribution of radioactivity (%)			
	Injection of [1-^{14}C] C16:0 (%)	Injection of [1-^{14}C] C18:0 (%)	Injection of [1-^{14}C] C24:0 (%)	Fatty acid composition (%)
16:0	71.6 ± 0.4	24.2 ± 1.6	28.5 ± 3.8	28.6 ± 0.4
16:1	4.1 ± 2.5	–	1.9 ± 1.3	2.4 ± 0.2
18:0	5.2 ± 2.5	47.5 ± 4.2	23.0 ± 1.2	20.7 ± 1.4
18:1	2.8 ± 1.7	16.4 ± 1.2	12.1 ± 0.1	22.2 ± 2.2
18:2ω6	–	–	–	2.1 ± 0.2
18:3ω3	–	–	–	0.5 ± 0.1
20:0	2.5 ± 1.8	–	–	0.3 ± 0.1
20:1	1.4 ± 1.0	2.0 ± 0.8	1.9 ± 1.2	0.1 ± 0.05
20:3ω6	1.9 ± 0.8	4.2 ± 1.0	6.9 ± 2.5	0.8 ± 0.2
20:4ω6				9.9 ± 0.4
20:4ω3				1.1 ± 0.7
20:5ω3				0.3 ± 0.1
22:0	–	1.9 ± 0.8	1.3 ± 0.7	0.2 ± 0.1
22:1	–	–	1.8 ± 1.6	0.1 ± 0.04
22:4ω6	6.1 ± 0.7	2.1 ± 0.8	2.7 ± 1.4	1.1 ± 0.3
22:5ω6				–
22:5ω3				0.3 ± 0.2
22:6ω3				8.4 ± 0.7
24:0	1.3 ± 0.3	1.6 ± 1.3	12.7 ± 3.7	0.1 ± 0.02
24:1	–		6.5 ± 2.7	–

Each point (% ± s.e.m.) is the mean value of 3 groups of mice (9 groups for the fatty acid composition).

Table 3. Relative distribution of radioactivity and fatty acid composition of *neuron* lipids from 15 day-old mouse brains, 20 h after injection of labelled fatty acids

	Relative distribution of radioactivity (%)			
	Injection of [1-^{14}C] C16:0 (%)	Injection of [1-^{14}C] C18:0 (%)	Injection of [1-^{14}C] C24:0 (%)	Fatty acid composition (%)
16:0	45.4 ± 13.5	28.7 ± 1.0	48.1 ± 1.1	25.6 ± 0.5
16:1	4.0 ± 0.8	–	2.5 ± 1.2	2.9 ± 0.1
18:0	27.0 ± 0.5	46.5 ± 2.3	20.9 ± 0.2	21.2 ± 0.5
18:1	4.0 ± 3.6	14.2 ± 1.3	8.0 ± 2.6	19.8 ± 1.2
18:2ω6	–	–	–	1.5 ± 0.06
18:3ω3	–	–	–	0.4 ± 0.07
20:0	0.6 ± 0.5	1.6 ± 0.8	1.3 ± 0.8	0.2 ± 0.05
20:1	2.1 ± 0.4	1.8 ± 1.5	2.7 ± 0.8	0.2 ± 0.06
20:3ω6	8.1 ± 1.9	3.3 ± 0.1	3.3 ± 0.9	0.8 ± 0.2
20:4ω6				16.5 ± 0.3
20:4ω3				0.8 ± 0.4
20:5ω3				–
22:0	1.1 ± 0.5	–	–	0.1 ± 0.04
22:1	2.0 ± 1.6	–	1.2 ± 0.5	–
22:4ω6	3.4 ± 1.5	–	2.9 ± 2.3	1.5 ± 0.1
22:5ω6				–
22:5ω3				0.1 ± 0.04
22:6ω3				8.3 ± 0.2
24:0	1.6 ± 0.6	–	5.3 ± 2.7	0.1 ± 0.07
24:1	0.2 ± 0.1	–	3.1 ± 2.7	–

Each point (% ± s.e.m.) is the mean value of 3 groups of mice (9 groups for the fatty acid composition).

Table 4. Relative distribution of radioactivity in fatty acids and fatty acid composition of *myelin* lipids from 15 day-old-mouse brains, 20 h after injection of labelled fatty acids

	Relative distribution of radioactivity (%)			
	Injection of [1-^{14}C] C16:0 (%)	Injection of [1-^{14}C] C18:0 (%)	Injection of [1-^{14}C] C24:0 (%)	Fatty acid composition (%)
16:0	22.4 ± 9.0	22.0 ± 1.6	24.2 ± 5.1	20.9 ± 0.5
16:1	2.9 ± 1.3	1.1 ± 0.5	1.5 ± 0.3	2.0 ± 0.2
18:0	29.6 ± 6.0	26.6 ± 3.5	17.5 ± 3.3	20.7 ± 0.4
18:1	10.3 ± 3.1	21.2 ± 2.7	14.0 ± 4.0	25.1 ± 0.3
18:2ω6	–	–	–	1.5 ± 0.05
18:3ω3	–	–	–	0.4 ± 0.1
20:0	3.0 ± 0.7	3.8 ± 0.8	5.2 ± 2.9	1.7 ± 0.05
20:1	3.8 ± 0.3	4.2 ± 1.1	4.8 ± 2.1	2.0 ± 0.3
20:3ω6	4.7 ± 1.1	6.1 ± 1.5	4.8 ± 1.2	1.7 ± 0.04
20:4ω6				7.9 ± 0.3
20:4ω3				–
20:5ω3				0.1 ± 0.04
22:0	3.1 ± 0.8	3.0 ± 0.6	2.9 ± 1.0	1.7 ± 0.3
22:1	1.9 ± 0.7	1.4 ± 0.3	2.5 ± 1.3	0.4 ± 0.1
22:4ω6	4.5 ± 1.5	3.0 ± 1.2	3.1 ± 0.5	2.3 ± 0.1
22:5ω6				–
22:5ω3				0.2 ± 0.1
22:6ω3				4.9 ± 0.4
24:0	4.0 ± 1.4	4.7 ± 1.1	12.8 ± 4.7	3.5 ± 0.3
24:1	5.6 ± 1.2	3.4 ± 1.5	6.3 ± 0.4	3.4 ± 0.4

Each point (% ± s.e.m.) is the mean value of 3 groups of mice (9 groups for the fatty acid composition).

Table 5. Specific radioactivity of fatty acids from serum, astrocyte, neuron and myelin total lipids 20 h after injection of labelled fatty acids (expressed in cpm/mg fatty acid). Mean values from 3 experiments

		16:0	18:0	18:1	24:0	24:1
	Serum	143,200	25,900	8,000	–	–
Injection of	Astrocytes	22,500	2,800	1,200	(86,000)	
[1-^{14}C] C16:0	Neurons	14,600	10,700	1,000	(128,000)	–
	Myelin	7,100	6,900	2,100	5,600	6,500
	Serum	6,700	940,000	23,000	79,000	–
Injection of	Astrocytes	6,200	15,700	6,700	(39,000)	–
[1-^{14}C] C18:0	Neurons	5,900	11,100	4,100	–	–
	Myelin	6,200	7,500	5,500	8,800	5,200
	Serum	7,100	10,500	2,900	620,000	–
Injection of	Astrocytes	10,200	10,500	6,700	650,000	–
[1-^{14}C] C24:0	Neurons	14,700	7,500	3,500	420,000	–
	Myelin	1,900	1,300	850	4,500	3,000

remained markedly high in neurons and astrocytes (Table 5) as compared to serum after its injection. This fact should be related to the low content of lignoceric acid in those cells (less than 0.5%) (Tables 2 and 3). However, it must be pointed out that, for instance, astrocyte lignoceric acid contained 13% of the total fatty acid radioactivity after injection of [1-^{14}C]lignoceric acid, instead of 1.3 and 1.6%, respectively after injection of labelled palmitic and stearic acids (Table 3). Considering also the specific radioactivity of serum lignoceric acid, it means that this very long chain fatty acid is taken up as such by brain cells from serum lipids.

Values of relative radioactivity in lignoceric acid of myelin indicate an accumulation of this fatty acid from exogenous origin (Table 4). But, as lignoceric acid is synthesized in the brain (Bourre *et al.*, 1977), exogenous radioactive lignoceric acid represents only a part of the total amount of lignoceric acid which is incorporated into myelin lipids. It is here clearly demonstrated that lignoceric acid in the brain originates simultaneously from *in situ* synthesis and from exogenous uptake. This fact correlates with other findings (Kishimoto *et al.*, 1980) indicating that some very long chain fatty acids which accumulate in brains of patients with adrenoleukodystrophy are of exogenous origin. It is also known that intracerebrally injected lignoceric acid is metabolized to complex lipids as such or degraded to acetate units (Gatt, 1963; Seidel *et al.*, 1975).

The high specific radioactivity of serum stearic acid after its injection was not followed by a corresponding high specific radioactivity of astrocyte, neuron and myelin stearic acid (Table 5), indicating a low uptake of this fatty acid into the brain as compared to palmitic acid. Stearic acid specific radioactivity in astrocyte lipids after injection of labelled stearic acid was 60 times less than in serum lipids (Table 5); on the other hand, the ratio was only 6 when considering palmitic acid specific radioactivity after its injection. However, even if *in situ* synthesis of stearic acid is predominant, one cannot consider its uptake from serum to be negligible as stearic acid presents the highest value of specific radioactivity (expressed in cpm/mg fatty acid) in each fraction (astrocytes, neurons or myelin) after its injection. It appears that the incorporation of exogenous palmitic acid into brain cells is more important than the incorporation of stearic acid (Table 6). Occurrence of radioactivity in polyunsaturated fatty acids indicates a degradation

Table 6. Radioactivity of neurons and astrocytes from 15 day-old mouse brains, 20 h after injection of labelled fatty acids

	Injection of [1-^{14}C] C16:0	Injection of [1-^{14}C] C18:0	Injection of [1-^{14}C] C24:0
Neurons	310 ± 34	131 ± 23	137 ± 10
Astrocytes	1213 ± 97	897 ± 80	441 ± 18

Each point (expressed in cpm/million cells ± s.e.m.) is the mean value of 3 groups of mice.

of the injected labelled fatty acids to [1-^{14}C]acetate units which are reutilized for elongation of polyunsaturated fatty acids.

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