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Mouse brain uptake and metabolism of stearic acid.

N. GOZLAN-DEVILLIERRE, N. A. BAUMANN et J. M. BOURRE ◇.

Laboratoire de Neurochimie, INSERM U.134 — Hôpital de la Salpêtrière, 47, bld de l'Hôpital — 75634 Paris Cedex 13, France.

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Summary. — After injection, labelled stearic acid is transported directly into the brain and incorporated into brain lipids without prior oxydation to acetate and resynthesis of fatty acids. Contamination by blood can be excluded. (The preparation contains all subcellular fractions except cytosol).

The labelled stearic acid taken up is partly metabolized in the brain either by elongation or by degradation and *in situ* resynthesis of fatty acids. The activity in oleic acid and mono-unsaturated chains is hardly detectable. The labelled acids are incorporated into lipids or subcellular particles following characteristic kinetics, which show a diminution by 24 hours. When analysing the evolution of each lipid, it is shown that this profile is followed by phospholipids, but not by cerebrosides and free fatty acids. The formers are still increasing up to 50 hours, the latters are stable (suggesting a physical binding between membranes and free fatty acids).

Thus nutrition is an important parameter for the synthesis of brain membranes as far as exogenous saturated fatty acids are needed.

The brain does not utilize fatty acids to any extent for energy, but has the ability to synthesize most lipids. However, fatty acids necessary for membrane formation can be obtained endogenously or exogenously. Dietary studies with essential fatty acids have shown that transport from blood to brain is possible [1-4]. Saturated fatty acids are synthesized in brain from endogenous or exogenous acetate ; the enzymes are located in the soluble part of the cell [5, 6], in mitochondria [7, 8] and in microsomes [9-11]. It has been shown that radioactive saturated fatty acids are taken up by brain either when fed [12] or when injected [13] ; but there is no evidence that saturated fatty acids, when incorporated into membranes, are mainly biosynthesized *in situ* or whether they come from blood. Obviously essential fatty acids are predominantly taken up directly without prior degradation to acetate, and are partly elongated [14-17]. This work was undertaken to study the fate of a saturated fatty acid (stearic acid) in brain after subcutaneous injection to know whether it is incorporated into lipids of subcellular particles as such or after being metabolized. The time of myelination was chosen for this study, as it is an active period of membrane formation. Stearic acid was used as it is the primer for very long chain fatty acids in brain [11].

Abbreviations :

PPO : 1,4-bis [2-(4-methyl-5-phenyloxazolyl)]-benzene.
POPOP : 2,5-diphenyloxazole.

◇ To whom all correspondence should be addressed.

MATERIALS AND METHODS.

1 mCi [1-^{14}C] stearic acid (51 mCi/mM) obtained from C.E.A. (France) is neutralized with equimolecular amount of NaOH (0.5 ml of a solution of 2 mg/ml of NaOH). 0.5 ml of 0.5 µM bovine albumin in 0.5 per cent NaCl is added. The mixture is stirred vigorously. 50 µCi of this clear solution is injected subcutaneously. The radio purity of the fatty acid was over 99.9 per cent (as checked by gas-liquid radio-chromatography, this acid is free from homologous fatty acids).

Animals were fed with standard diet bisquits from Extra Labo (France). Brains are excised, washed and sliced with a razor blade in isotonic solution (0.9 per cent NaCl). The tissue fragments are then spun down at 17,500 g for 10 minutes (blood cells are then discarded). The pellet is homogenized in a potter Elvejhem (30 ml of isotonic solution/g of tissue) and centrifuged at 100,000 g for 60 minutes. The pellet contains all brain subcellular particles, and the soluble fatty acids existing within the brain cells are discarded. Moreover the contamination by blood is eliminated by this method. Total lipids are extracted by chloroform-methanol (2:1 V/V) [18, 19]. The radioactivity was counted in a Packard liquid scintillator using PPO, POPOP and toluene. Lipids are isolated on thin-layer chromatograms after migration with chloroform-methanol-water (70:

30:4 V/V/V) [20]. Thin-layer chromatographies are made on silica gel Merck 60F 254 ; the various lipids are determined by migration with commercial standards. Under these conditions, sphingomyelin, inositol-phosphatides (PI) + choline phosphatides (PC) + serine phosphatides (PS), sulfatides and cholesterol and cerebrosides are quite well separated [21-23]. The spots containing PI + PC + PS from the 70:30:4 migration are scraped and extracted with 5 times 3 ml/cm² chloroform-methanol 2:1. These lipids are separated individually by phenol in water (436:100 w/v)-10.5 M NH_4OH, 99:1 V/V [24]. As free fatty acids (unesterified fatty acids) are contaminated by ceramides, another system is used : chloroform-acetic acid (90:10 V/V). This system is employed with total lipids extract.

The spots are visualized by iodine, scraped and counted in the presence of Cab-o-sil PPO and dimethyl POPOP. No quench correction procedure is applied as exactly the same quantity of silica gel is scraped for each individual lipid : thus the quench is constant (iodine has been evaporated before scraping).

Lipids from total lipid extract are eventually methylated [25]. Fatty acid methyl esters thus obtained are directly analysed by gas-liquid chromatography with automatic counting of the eluate. The gas-liquid chromatography is made on SE 30 column 175-265°C 2°C/mn in a Hewlett-Packard 5750 with automatic counting of the eluate. During gas-liquid chromatography 50 per cent of the effluent gas passes through the flame ionization detector for determination of relative retention time of esters. The other 50 per cent passes through a radioactivity monitoring system with a combustion oven of 700°C and the radioactivity is measured.

Blood was eventually collected by heart puncture. It was adjusted to 1 ml by water. The mixture was saponified by 0.5 ml of 15 per cent methanolic KOH for 15 minutes at 100°C 0.5 ml of HCl (5.5 N) was added and fatty acids were extracted two times by 5 ml of petroleum ether. These acids were methylated, counted and analysed as previously described.

RESULTS.

The table I shows the uptake of radioactivity by brain total lipids and main classes of lipids of 15 day-old mice after a subcutaneous injection of [1-^{14}C] stearate. The percentage of radioactivity found in cerebrosides is alway increasing, in contrast to the free fatty acids. Little activity was found in sphingomyelin (between 1 and 4 per cent according to the time point), in sulfatides and in cholesterol. But both activities in sphingomyelin and sulfatides increase.

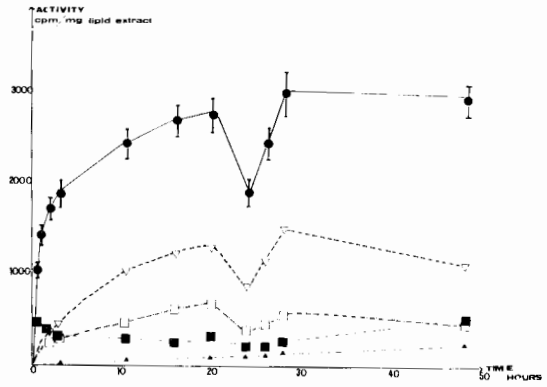

FIG. 1. — *Distribution of radioactivity in mouse brain lipids after subcutaneous injection of [1-^{14}C] stearic acid.*

● : Total lipids after Folch wash ; ▽ : PI + PC + PS ; □ : PE ; ▲ : Cerebrosides ; ■ : Free fatty acids.

The activity is expressed as the radioactivity (in cpm) per mg lipid extract (for either total lipid extract or each individual lipid). Each point is the mean value of at least three experiments. For each time point 7 mice were used. About 15,000 cpm were used at each chromatography.

The deviation is not drawn for each individual lipid as each point is a percentage of the activity found in the total lipids.

In figure 1, the activity in total lipid extract (radioactivity in cpm per mg lipid extract) increases up to 20 hours and then decreases until 24 hours to increase again and reach a plateau until 50 hours (no activity is measured at the zero time). The radioactivity is present in all major components indicating active incorporation into brain constituents. Most of the radioactivity in the lipids was found in the choline phosphoglycerides, inositol phosphoglycerides and serine phosphoglycerides followed by ethanolamine phosphoglycerides. At 20 hours after injection, the main phospholipids (choline phosphoglycerides, inositol phosphoglycerides, serine phosphoglycerides and ethanolamine phosphoglycerides) account for about 70 per cent of the total radioactivity. When PI, PC, and PS are separated individually (table II) an important part of the percentage of total phospholipid activity is present in PC ; but the type of evolution, as a function of time of injection, is the same in all phospholipids. The evolution of cerebrosides shows that the decrease in the activity at 24 hours is much less important for cerebrosides than for total

TABLE I.

Distribution of relative activity in mouse brain lipids after subcutaneous injection of [1-^{14}C] stearate.

Time	Sphingo-myelin p. cent	PC, PI, PS p. cent	Sulfatides p. cent	PE p. cent	Cerebrosides p. cent	Free Fatty Acids p. cent	Cholesterol p. cent
10 min.	0.9	13.2	0.6	8.5	0.3	59.8	4.9
1/2 h	1.2	15.2	0.9	10.3	2.5	50 2	6.5
1 h	1.4	20.4	1.0	12.2	2.8	41.0	7.0
2 h	2.1	25.5	1.2	14.1	3.0	32.9	7.8
3 h	2.1	28.5	1.2	15.9	3.0	24.3	8.0
10 h 30	2.2	42.6	1.4	17.8	3.2	14.3	8.1
16 h	2.3	44.6	1.5	20.1	3.5	9.9	8.4
20 h	2.5	44.8	2.0	20.7	3.9	11.2	9.1
24 h	2.7	45.0	2.3	20.3	4 4	12.0	10.2
26 h	3.0	46.2	2.6	19.1	4.1	8.8	11.1
28 h	3.3	48.0	3.0	17.0	4.8	10.2	11.5
48 h	4.0	37.2	3.5	14.2	7.3	16.3	13.4

Same remarks as in figure 1. The percentage refers to the activity found in the total lipid extract.
PI : Inositol phosphoglyceride ; PS : Serine phosphoglyceride ; PC : Choline phosphoglyceride ; PE : Ethanolamine phosphoglyceride.

TABLE II.

Distribution of relative activity, in mouse brain choline, serine and inositol phosphoglycerides.

Time	PI + PS + PC p. cent	PI p. cent	PS p. cent	PC p. cent
10 mn	13.2	0.7	5.2	7.3
1 h	20.4	1.4	5.1	13.9
16 h	44.6	6.2	8.1	30.3
20 h	44.8	6.1	8.3	30.4
24 h	45	2.5	8.4	34.1
26 h	46.2	5.4	11.1	29.7
28 h	48	5.6	11.7	30.7
48 h	37.2	1.3	11.2	24.7

lipids or phospholipids. Moreover activity of cerebrosides is always iscreasing during time. The activity in sulfatides is less than 5 per cent each time point, but also increasing. The activity of free fatty acids remains remarkably constant during time.

Table III shows the evolution of the fatty acid profile. The acyl groups of the lipid extract were converted to fatty acid methyl esters for gas-liquid chromatographic analysis. The lipid extract contains high amount of labelled stearic acid (94.3 per cent) 10 minutes after injection of [1-^{14}C] stearic acid. There is already 5.2 per cent radioactive palmitic acid at this time. A gradual

TABLE III.

Evolution of the metabolism of stearic acid as a function of time.

Time	Cpm/mg of total lipids after folch wash	C_{16} p. cent	C_{18} p. cent	C_{20} p. cent
10 min	650	5.2 ± 2.0	94.3 ± 2.1	0.4 ± 0.2
1/2 h	1,068	18.4 ± 3.5	74.4 ± 3.7	0.5 ± 0.3
1 h	1,462	19.3 ± 3.2	73.7 ± 5.2	1.0 ± 0.3
2 h	1,763	19.5 ± 4.1	72.3 ± 4.1	2.1 ± 0.2
3 h	1,910	21.5 ± 3.1	71.4 ± 3.9	2.5 ± 0.4
10 h 30	2,461	22.0 ± 2.9	71.1 ± 3.8	2.9 ± 0.3
16 h	2,710	23.0 ± 3.2	69.0 ± 5.3	3.3 ± 0.4
20 h	2,800	23.4 ± 4.1	66.5 ± 5.9	4.0 ± 0.4
24 h	1,912	27.0 ± 2.7	59.6 ± 4.3	3.5 ± 0.3
26 h	2,490	21.0 ± 5.6	71.1 ± 4.1	2.4 ± 0.3
28 h	3,033	19.8 ± 4.0	73.3 ± 5.8	1.5 ± 0.3

Same remarks as in figure 1. At least 10,000 cpm were injected at each gas-liquid chromatography. The percentage refers to the activity found in the total labelled fatty acid after methylation of the lipid extract.

decrease of labelled stearic acid and conversely an increase of labelled palmitic acid occurs until 20 hours with a maximum at 24 hours. The level of labelled arachidic is low but not negligible. Longer labelled chains (behenic and lignoceric acid) are detectable. Unsaturated fatty acids were not detected except trace amounts of oleic acid (less than 3 per cent of the total radioactivity). On the other hand, in blood we find only labelled stearic acid and no longer or shorter chains. This indicates that the administered stearic acid was predominantly taken up directly without prior degradation to acetate. The total radioactivity was determined at each time point in blood and brain lipids. For instance, one hour after injection of 70×10^6 cpm, the blood still contains 60,890 cpm and the whole brain subcellular particles 9,780 cpm. Twenty hours after the same injection, 43,560 cpm are found in the blood and 19,670 cpm in brain subcellular particles : at this time point 0.03 per cent of injected radioactivity was recovered in the brain.

DISCUSSION.

Transport of stearic acid across the blood-brain-barrier was studied by subcutaneous injection, as feeding involves also metabolism inside the digestive tract and direct injection into brain excludes « blood-brain-barrier ».

It has been reported that fed radioactive essential fatty acids are elongated by brain [14, 15] but the possibility of trapped blood contributing a substantial portion of the radioactivity in fatty acids could not be ruled out. Moreover, the liver for instance could elongate some injected fatty acids, those newly biosynthesized fatty acids being released into blood and then taken up by brain. Our technique excludes any contamination by blood for we only isolate subcellular particles which are washed to discard any contamination from cytosol. This work demonstrates that there is transport of stearic acid as such from blood to brain. Gas-liquid radio-chromatography of blood fatty acids shows that there are only trace amounts of fatty acids other than stearic acid. Injected fatty acid penetrates in brain intact and is further metabolized. In brain, some activity is found in fatty acids with 20, 22 and 24 carbon atoms, indicating that stearic acid is partly elongated. The activity found in palmitic acid when stearic acid is injected is probably due to degradation to radioactive acetate and subsequent biosynthesis or elongation of endogenous short chains within brain. Moreover, this radioactive acetate is also used for synthesis of cholesterol, thus explaining the radioactivity found in this compound. Unexpected is the low activity found in mono-unsaturated fatty acids, mainly oleic acid, as it is known that brain microsomes desaturate stearate to oleate and this latter compound is further elongated to longer mono-unsaturated chains [26].

Gas-liquid analysis of blood fatty acids shows that there is only trace amount of any fatty acid excepting C_{18}. Stearic acid is incorporated into brain membrane lipids. The decrease at 24 hours in total lipid activity and in phospholipid activity and the rapid increase thereafter would indicate the presence of two pools for stearic acid in membranes, the nature of which remains to be determined. The table I and the figure suggest that incorporation of fatty acids in myelin (as cerebrosides are myelin markers) increases with time. Activity in free fatty acids is constant even when the total lipid activity is low, either at 24 hours or at very short times. These free fatty acids must have a high specific activity as they are found in minute amount in brain ; their biochemical and physiological role is unknown. Moreover unesterified fatty acids associated to proteolipids have a possible role in the process of synaptic transmission [27].

This work definitely demonstrates that there is a transport from blood to brain of stearic acid as well as an incorporation into lipids of subcellular particles. The importance of nutrition compared to *in situ* biosynthesis remains to be determined. Further work will localize the uptake of injected stearic acid into the different subcellular fractions.

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RÉSUMÉ.

Après injection sous-cutanée, l'acide stéarique radioactif est transporté dans le cerveau où il est incorporé dans les lipides cérébraux, sans avoir été au préalable dégradé en unités acétates permettant la resynthèse des acides gras. La contamination de la préparation « membranaire » par le sang est exclue. (Le culot « membranaire » contient l'ensemble des structures subcellulaires à l'exception du cytosol).

L'acide stéarique capté est en partie métabolisé par le cerveau, soit par allongement, soit par dégradation en unités acétate et resynthèse *in situ* d'acides gras. L'acide oléique et les acides mono-insaturés sont difficilement détectables. Les acides gras radioactifs sont incorporés dans les lipides « membranaires » selon

une évolution temporelle qui montre une diminution à la 24e heure. L'analyse de chaque lipide montre que cette évolution est suivie par les phospholipides mais pas par les cérébrosides ou les acides gras libres : les premiers augmentent régulièrement jusqu'à 50 heures au moins, les seconds sont stables (suggérant une liaison physique entre les membranes et les acides gras libres non estérifiés).

Ainsi, la nutrition intervient dans la composition en acides gras saturés des membranes cérébrales.

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