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Metabolism of hexacosatetraenoic acid ($C_{26:4,n-6}$) in immature rat brain

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Rat brain was recently found to contain polyenoic very-long-chain fatty acids (VLCFA) belonging to the $n-3$ and $n-6$ series with four, five and six double bonds and even-carbon chain lengths from 24 to 38 [Robinson, Johnson & Poulos (1990) Biochem. J. **265**, 763–767]. In the present paper, the metabolism *in vivo* of hexacosatetraenoic acid ($C_{26:4,n-6}$) was studied in neonatal rat brain. Rats were injected intracerebrally with [1-^{14}C]$C_{26:4,n-6}$ and the labelled metabolites were examined after 4 h. Radioactivity was detected mainly in non-esterified fatty acids, with smaller amounts in other neutral lipids and phospholipids. Radiolabelled fatty acid products included C_{28-36} tetraenoic and C_{26-28} pentaenoic VLCFA formed by elongation and desaturation of the substrate, and C_{14-24} saturated, C_{16-24} monoenoic, C_{18-24} dienoic, C_{18-22} trienoic and C_{20-24} tetraenoic fatty acids formed from released [1-^{14}C]acetate either by synthesis *de novo* or by elongation of endogenous fatty acids. The data suggest that polyenoic VLCFA are synthesized in brain from shorter-chain precursor fatty acids and undergo β-oxidation.

INTRODUCTION

Polyenoic fatty acids with carbon chain lengths greater than 22 (very-long-chain fatty acids, VLCFA) have been reported to be present in the retina of several vertebrate species (Aveldaño, 1987; Aveldaño & Sprecher, 1987; Rotstein & Aveldaño, 1988), mammalian spermatozoa (Poulos *et al.*, 1986*a*, 1987), human brain (Poulos *et al.*, 1986*b*, 1988; Sharp *et al.*, 1987; Poulos, 1989), rat brain (Robinson *et al.*, 1990) and rat testis (Bridges & Coniglio, 1970; Grogan, 1984), and to be synthesized by mouse spermatocytes and spermatids (Grogan & Huth, 1983), human endothelial cells (Rosenthal & Hill, 1984) and human skin fibroblasts (Gavino *et al.*, 1981; Street *et al.*, 1989) in culture.

In the brains of humans (Sharp *et al.*, 1987; Poulos *et al.*, 1988) and rats (Robinson *et al.*, 1990), the polyenoic VLCFA belong to the $n-3$ and $n-6$ series and have four, five and six double bonds and carbon chain lengths up to 38. These fatty acids represent a minor component of the total brain fatty acids (less than 1%). Polyenoic VLCFA are concentrated in unusual molecular species of phosphatidylcholine at the *sn*-1 position of the glycerol backbone, whereas saturated, monoenoic and polyenoic fatty acids with less than 24 carbon atoms occur at the *sn*-2 position. Sharp *et al.* (1987) and Poulos *et al.* (1988) observed that the brains of humans with Zellweger's syndrome (a rare inherited disease characterized ultrastructurally by a deficiency of tissue peroxisomes) have greatly elevated levels of $n-6$ penta- and hexa-enoic VLCFA with up to 40 carbon atoms in phosphatidylcholine. It is postulated that polyenoic VLCFA play an important role in normal brain, and accumulate in Zellweger's syndrome brain due to a deficiency in a peroxisomal system which regulates their catabolism. There is no information as yet on the synthesis of these fatty acids in brain.

The metabolism of [1-^{14}C]tetracosatetraenoic acid ($C_{24:4,n-6}$) in cultured human skin fibroblasts from normal subjects and patients with Zellweger's syndrome was examined recently in our laboratory (Street *et al.*, 1989). Radiolabelled products of the polyenoic VLCFA included CO_2, C_{14-24} saturated and monoenoic fatty acids formed from released acetate either by synthesis *de novo* or by elongation of endogenous fatty acids, C_{26-28} tetraenoic fatty acids formed by 2-carbon elongations of the added substrate, and water-soluble products (mainly amino acids found in the culture medium). Zellweger's syndrome fibroblasts showed diminished production of radiolabelled CO_2, water-soluble products and C_{14-24} fatty acids from [1-^{14}C]$C_{24:4,n-6}$. However, elongation of the fatty acid substrate in the diseased cells was enhanced. Similar results were obtained when the fibroblast cell lines were incubated with [1-^{14}C]hexacosatetraenoic acid ($C_{26:4,n-6}$) (J. M. Street & A. Poulos, unpublished work). These data provide evidence that peroxisomes in cultured skin fibroblasts are essential for the β-oxidation of polyenoic VLCFA, and that prevention of their normal catabolism in peroxisomes channels these fatty acids into elongated metabolites.

The metabolism of polyenoic VLCFA in brain has not been previously studied. In the present paper, we have characterized the metabolic products of [1-^{14}C]$C_{26:4,n-6}$ in the brains of neonatal rats.

EXPERIMENTAL

Animals

Male Porton rats (*Rattus norvegicus*), aged 1 and 16 days, were used in this study. They were housed in wire mesh cages and given access to their lactating mother which was provided with a pelleted rat diet (Charlicks, Adelaide, South Australia, Australia) and water *ad libitum*.

Preparation of radiolabelled fatty acid substrate

[1-^{14}C]$C_{26:4,n-6}$ was prepared from docosatetraenoic acid ($C_{22:4,n-6}$) by four identical sequential C_1 elongation procedures involving esterification with diazomethane, reduction with lithium aluminium hydride, mesylation with methanesulphonyl chloride in pyridine, displacement with sodium [^{14}C]cyanide in dimethyl sulphoxide and hydrolysis in alkaline ethanol/water. In the fourth displacement step, pentacosatetraenylmethanesulphonate (5 mg) was reacted with sodium [^{14}C]cyanide

Abbreviations used: VLCFA, very-long-chain fatty acid(s); $C_{24:4,n-6}$, tetracosatetraenoic acid; $C_{26:4,n-6}$, hexacosatetraenoic acid.

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(0.5 mCi, 55 mCi/mmol) in hexadeuterated dimethyl sulphoxide (1 ml) at 70 °C for 4 h. The [1-^{14}C]nitrile was extracted with hexane and hydrolysed in 1 ml of ethanol/10 % aq. NaOH (2:1, v/v) at 80 °C for 16 h to produce [1-^{14}C]$C_{26:4,n-6}$, which was subjected to t.l.c. on a 10 cm × 20 cm × 0.25 mm silica gel 60 plate (E. Merck, Darmstadt, Germany) using the solvent system hexane/diethyl ether/acetic acid (40:10:1, by vol.). Reversed-phase h.p.l.c. of the [1-^{14}C]$C_{26:4,n-6}$ (dissolved in 80 μl of methanol) was performed on a 250 mm × 4 mm (internal diam.) stainless steel column with 5 μm particle size ODS 2 (C_{18}) packing (Scientific Glass Engineering, Ringwood, Victoria, Australia) coupled to an LKB Bromma 2152 LC controller, 2150 h.p.l.c. pump and 2212 fraction collector. The solvent used was acetonitrile/phosphoric acid (45 mM) (80:20, v/v) pumped isocratically at a flow rate of 1 ml/min. The purified [1-^{14}C]$C_{26:4,n-6}$ substrate (0.2 mCi, 55.9 mCi/mmol) was stored in 5 ml of toluene at −20 °C. G.l.c./m.s. of the methyl ester showed one homogeneous peak, electron impact m.s. *m/z* 404 ([1-^{14}C]-M^+, 8.8 %), 402 ([1-^{12}C]-M^+, 1.1 %), 150 (17 %), 67 (100 %).

Injection of radiolabelled fatty acid substrate into brain and analysis of lipids and water-soluble compounds

[1-^{14}C]$C_{26:4,n-6}$ substrate (2 μCi dissolved in 5 μl of 95 % ethanol) was injected using a Hamilton glass syringe into the cerebral cortex of rats. After 4 h the animals were killed by decapitation and the brains were rapidly excised. Lipids and water-soluble compounds were extracted from whole brain (approx. 0.3 g and 1.2 g for 1-day-old and 16-day-old rats respectively) according to the procedure of Folch *et al.* (1957). The lower lipid phase was taken to dryness *in vacuo* at 30 °C and the residue was redissolved in 2 ml of chloroform. The upper water-soluble phase was reduced in volume to 1.5 ml under N_2 at 30 °C. Solvents used for extraction and subsequent analyses of lipids contained the antioxidant 2,6-di-t-butyl-4-methylphenol (50 mg/l). Portions of the lipid phase (0.02 ml, evaporated to dryness under N_2) and the water-soluble phase (0.1 ml) were placed in scintillation vials along with 8 ml of scintillation fluid, and the radioactivity was measured with a Nuclear-Chicago Isocap/300 liquid scintillation counter.

Radioactivity associated with different lipid classes was determined as follows. A portion of the lipid phase (0.05 ml) was applied to a 20 cm × 20 cm × 0.25 cm silica gel 60 t.l.c. plate and developed half-way in the solvent system chloroform/methanol/acetic acid/formic acid/water (35:15:6:2:1, by vol.) to separate phospholipids. After air-drying, the plate was developed to the top in the solvent system hexane/diethyl ether/acetic acid (70:30:1., by vol.) to separate neutral lipids. Radiolabelled lipids were located by exposing high performance autoradiography film (Hyperfilm-^{3}H; Amersham Australia, North Ryde, N.S.W., Australia) to the plate for 21–28 days. The lipid zones were scraped into scintillation vials and the radioactivity was measured after adding scintillation fluid (8 ml) and water (0.5 ml). Identification of the lipids was based on a comparison of their t.l.c. mobility with that of authentic standards.

Radioactivity associated with various esterified and non-esterified fatty acids was determined by the following procedure. The remainder of the lipid phase was taken to dryness under N_2 and trans-esterified with 5 ml of 0.27 M-H_2SO_4 in methanol at 75 °C for 4 h. The liberated fatty acid methyl esters were extracted with 1 ml of water and 2 ml of hexane and applied to a silica gel 60 t.l.c. plate (20 cm × 20 cm × 0.25 mm) which was developed in dichloromethane. The zone corresponding to the fatty acid methyl esters was revealed under u.v. light after spraying the plate with 0.2 % (w/v) dichlorofluorescein in 95 % (v/v) ethanol, and eluted from the silica gel with 8 ml of chloroform/methanol/acetic acid/water (50:39:1:10, by vol.) as described by Arvidson (1968). The extract was partitioned with 2 ml of aq. 1 M-NH_3 to remove the dye and the lower phase was washed with 2 ml of methanol/water (1:1, v/v). Argentation t.l.c. was used to resolve fatty acid methyl esters according to degree of unsaturation. Concentrated portions of the purified fatty acid methyl ester sample were applied to silica gel 60 t.l.c. plates (20 cm × 20 cm × 0.25 mm) impregnated with $AgNO_3$ (Inomata *et al.*, 1982). The chromatograms were developed in chloroform/methanol (9:1, v/v) to separate hexa-, penta-, tetra- and tri-enoic species and in chloroform/methanol (99:1, v/v) to resolve dienoic, monoenoic and saturated species (Aveldaño, 1987; Aveldaño & Sprecher, 1987). Fractions were detected by spraying with dichlorofluorescein and eluted as described above. The resulting extracts were partitioned with aq. 4 M-NH_3 and washed with methanol/1 % NaCl (1:1, v/v). Fatty acid methyl esters were subsequently resolved according to carbon chain length using reversed-phase t.l.c. Concentrated argentation fractions were applied to 5 cm × 20 cm × 0.2 mm KC_{18} reversed-phase t.l.c. plates (Whatman, Clifton, NJ, U.S.A.) and developed twice in the same direction in acetonitrile-190/tetrahydrofuran (9:1, v/v). Radiolabelled fatty acid methyl esters were located by preparing autoradiographs and the radioactivity was measured as outlined above. The identity of the argentation and reversed-phase t.l.c. fatty acid methyl ester fractions was checked by g.l.c./m.s. (Poulos *et al.*, 1986*b*; Fellenberg *et al.*, 1987). Fatty acid methyl ester argentation fractions were sometimes hydrogenated before being subjected to reversed-phase t.l.c. in order to gain additional information about carbon chain length. In this case, 10 % palladium catalyst on activated carbon (0.1 g) was added to each fraction (in 5 ml of hexane) and stirred vigorously under H_2 for 6 h. The solution was applied to a column (3 cm × 0.6 cm diam.) of silicic acid and eluted with 10 ml of chloroform/methanol (2:1, v/v).

Chemicals

These were obtained as follows: docosatetraenoic acid ($C_{22:4,n-6}$) from Nu-Chek-Prep, Elysian, MN, U.S.A.; sodium [^{14}C]cyanide (sp. radioactivity 55 mCi/mmol; radiochemical purity 98 %) from Amersham Australia; 2′,7′-dichlorofluorescein, silicic acid (325 mesh) and 2,6-di-t-butyl-4-methylphenol from Sigma Chemical Co., St. Louis, MO, U.S.A.; scintillation fluid (Ready Value) from Beckman Instruments, Fullerton, CA, U.S.A.; and palladium on activated carbon (10 %, w/w) from Aldrich Chemical Co., Inc., Milwaukee, WI, U.S.A. All other chemicals were reagent grade, and solvents were distilled prior to use.

RESULTS AND DISCUSSION

Polyenoic VLCFA, including $C_{26:4,n-6}$, have recently been found to be natural components of rat brain (Robinson *et al.*, 1990). In the present study [1-^{14}C]$C_{26:4,n-6}$ was injected into the brains of 1- and 16-day-old rats and the radioactive products were analysed after 4 h. The total radioactivity recovered in the brains of 1-day-old rats was 44–74 % of the amount injected (range of three animals). It is likely that there were physical losses of the radiolabelled fatty acid substrate during the injection process. Of the recovered radioactivity, 98.4–99.5 % was associated with lipids and 0.5–1.6 % with water-soluble compounds. Similar incorporation of radioactivity was observed in the brains of 16-day-old animals.

Table 1 shows the distribution of radioactivity in rat brain lipids. Radioactivity was present mainly in non-esterified fatty acids, with smaller amounts in other neutral lipids and phospholipids. Phosphatidylcholine was the most highly labelled phospholipid. The relative proportion of radioactive non-esterified fatty

Table 1. Distribution of radioactivity in rat brain lipids after injection of [1-^{14}C]$C_{26:4,n-6}$

Rats were injected intracerebrally with 2 μCi of [1-^{14}C]$C_{26:4,n-6}$ and killed after 4 h. The ^{14}C radioactivity associated with brain lipids was determined as described in the Experimental section. The values represent the ranges for three animals and are expressed as a percentage of recovered total lipid radioactivity. This experiment was performed twice with similar results. Abbreviations used: PI, phosphatidylinositol; PS, phosphatidylserine; MG, monoacylglycerol; DG, diacylglycerol; UNL, unidentified neutral lipid.

Lipid	Rats...	Recovered radioactivity in lipid (% of total) 1-day-old	16-day-old
Lysophosphatidylcholine		0.14–0.31	0.22–1.02
Sphingomyelin		0.16–0.47	0.33–1.28
Phosphatidylcholine		1.75–6.24	8.91–24.88
PI + PS		0.23–1.01	0.83–2.20
Phosphatidylethanolamine		0.38–1.23	2.57–4.22
Phosphatidic acid		0.29–0.48	0.35–0.64
MG + DG		0.23–0.26	0.20–0.72
Cholesterol		0.64–1.77	2.52–4.11
Non-esterified fatty acids		80.15–93.02	37.14–75.81
Triacylglycerol		2.20–6.80	5.86–20.55
Cholesterol esters		0.68–1.40	0.91–2.98
UNL		0.25–0.50	0.78–1.05

Table 2. Distribution of radioactivity in rat brain fatty acids after injection of [1-^{14}C]$C_{26:4,n-6}$

Rats were treated as described for Table 1. The ^{14}C radioactivity associated with brain fatty acids was determined as described in the Experimental section. The values represent the ranges for three animals and are expressed as a percentage of recovered total fatty acid radioactivity. This experiment was repeated once with similar results. Abbreviations used: FA, fatty acids; N.D., not detectable.

Fatty acid (carbon atoms: double bonds)	Rats...	Fatty acid radioactivity (% of total) 1-day-old	16-day-old
14:0		0.06–0.30	0.19–0.37
16:0		0.81–5.21	5.92–17.50
18:0		0.15–1.00	1.63–4.15
20:0		0.01–0.04	0.07–0.16
22:0		0.00–0.03	0.06–0.26
24:0		0.00–0.02	0.06–0.18
16:1		0.06–0.16	0.09–0.38
18:1		0.10–0.33	0.52–3.37
20:1		0.02–0.06	0.09–0.39
22:1		0.02–0.05	0.04–0.12
24:1		0.01–0.04	0.04–0.18
18:2		0.03–0.06	0.03–0.06
20:2		0.02–0.07	0.07–0.22
22:2		0.14–0.24	0.11–0.30
24:2		0.04–0.06	0.03–0.08
18:3		0.09–0.29	0.06–0.17
20:3		0.13–0.35	0.15–0.49
22:3		0.10–0.24	0.12–0.26
20:4		0.14–0.39	0.31–0.72
22:4		0.34–0.73	0.51–1.02
24:4		0.56–0.76	0.62–1.25
26:4		88.84–96.56	67.46–87.59
28:4		0.11–0.23	0.33–0.72
30:4		0.09–0.14	0.08–0.18
32:4		0.04–0.08	0.05–0.09
34:4		0.04–0.06	0.07–0.13
36:4		0.04–0.05	0.04–0.07
22:5		0.01–0.09	0.07–0.33
24:5		0.02–0.06	0.03–0.08
26:5		0.18–0.38	0.36–0.69
28:5		0.04–0.05	0.01–0.06
Hexaenoic FA		N.D.	N.D.

acids was higher in the brains of 1-day-old than of 16-day-old rats. It is noteworthy that radiolabelled cholesterol was detected in the brain. Some of the fatty acid substrate must have undergone β-oxidation to yield [1-^{14}C]acetate, which was then used for cholesterol biosynthesis.

The radioactive fatty acid profile in rat brain is presented in Table 2. $C_{26:4,n-6}$ was the principal radioactive fatty acid. Traces of radiolabelled C_{28-36} tetraenoic fatty acids were observed (Table 2 and Fig. 1) which were probably formed by 2-carbon elongations of the original fatty acid substrate. Small amounts of radioactive pentaenoic fatty acids ($C_{26:5}$ and $C_{28:5}$) were present, suggesting that some of the [1-^{14}C]$C_{26:4,n-6}$ was desaturated and

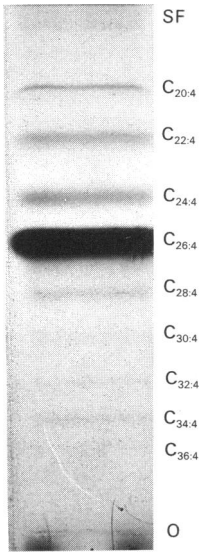

Fig. 1. Radiolabelled tetraenoic fatty acids in rat brain after injection of [1-^{14}C]$C_{26:4,n-6}$

Rats were treated as described for Table 1. The ^{14}C radioactivity associated with brain fatty acids was determined as described in the Experimental section. The Figure shows an autoradiogram produced from a reversed-phase t.l.c. chromatogram of the brain tetraenoic fatty acid fraction. Abbreviations used: O, origin; SF, solvent front.

then elongated. No labelled hexaenoic fatty acids were detected. Table 2 indicates that radiolabelled C_{14-24} saturated fatty acids were observed, with $C_{16:0}$ and $C_{18:0}$ being particularly prominent. Low levels of radiolabelled C_{16-24} monoenoic, C_{18-24} dienoic, C_{18-22} trienoic and C_{20-24} tetraenoic fatty acids were also found. These labelled shorter-chain fatty acids presumably reflect β-oxidation of the [1-^{14}C]$C_{26:4,n-6}$ substrate and recycling of labelled acetate moieties in synthesis *de novo* or chain elongation of endogenous fatty acids. A minor portion of the [1-^{14}C]acetate released by the β-oxidation process was available for the synthesis of water-soluble compounds. There was greater conversion of [1-^{14}C]$C_{26:4,n-6}$ into radioactive fatty acid products in brains from 16-day-old than 1-day-old rats (Table 2).

It was previously demonstrated that cultured human skin fibroblasts can elongate, desaturate and β-oxidize the polyenoic VLCFA $C_{24:4,n-6}$ and $C_{26:4,n-6}$ (Street *et al.*, 1989; J. M. Street & A. Poulos, unpublished work). The results of the present work indicate that rat brain can also metabolize $C_{26:4,n-6}$ by corresponding pathways. Rat brain has the capacity to elongate $C_{26:4,n-6}$ to $C_{36:4,n-6}$. Conversely, elongation of $C_{24:4,n-6}$ and $C_{26:4,n-6}$ in fibroblasts appears to be restricted to a maximum of C_{28} chain length. It is reasonable to speculate that arachidonic acid ($C_{20:4,n-6}$) is the ultimate precursor of tetraenoic VLCFA. Elongation and desaturation of fatty acids with less than 24 carbon atoms has been found to occur mainly in the endoplasmic reticulum of brain (Naughton, 1981; Yoshida *et al.*, 1988; Saitoh *et al.*, 1988) and fibroblasts (Tsuji *et al.*, 1984), and presumably this also applies to polyenoic VLCFA. There is evidence that β-oxidation of polyenoic VLCFA is confined to peroxisomes in these tissues (Sharp *et al.*, 1987; Poulos *et al.*, 1988; Street *et al.*, 1989). The enzymes involved in the metabolism of polyenoic VLCFA in these organelles need to be characterized. In addition, it remains to be determined whether polyenoic VLCFA can be converted into more polar oxygenated derivatives similar to prostaglandins and leukotrienes with the potential to modify cell function.

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