Fatty Acid Chain Elongation in Rat Brain Synaptosomes†

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ABSTRACT: Rat brain synaptosomes were incubated with ^{14}C-labeled acetate, malonate, and their respective coenzyme A (CoA) esters. Only malonyl-CoA was found to be a suitable precursor for fatty acids. Acetyl-CoA was not effectively taken up by synaptosomes. Biosynthesis of synaptosomal fatty acids was stimulated by increased added concentration of ATP or by inclusion of an ATP-generating system (D-glucose). A reduced pyridine nucleotide was indispensable. Docosatetraenoic acid (22:4ω6) contained most of the fatty acid radioactivity after incubation. The most likely pathway for its formation was the one-step elongation of arachidonic acid (20:4ω6) by condensation with malonyl-CoA and release of CO_2. The fatty acid label appeared mainly in synaptosomal choline phosphoglyceride presumably by acylation of 1-acyl-lysocholine phosphoglyceride. No *de novo* biosynthesis of fatty acids occurred. After incubation, the labeled fatty acids were recovered mainly from the synaptosomal mitochondria (80%). Synaptic membranes contained 20% of the label; synaptic vesicles were not radioactive. Synaptosomes had an active malonyl-CoA decarboxylase.

Synaptosomes (Whittaker *et al.*, 1964) derive largely though not exclusively from presynaptic nerve endings (synaptic boutons) in the central nervous system. Many metabolic properties and capabilities of these anuclear bags of cytoplasm and inclusions have been described. This investigation is concerned with fatty acid biosynthesis in synaptosomes. Prior studies of lipid metabolism have indicated that synaptosomes actively incorporate precursors of common lipids such as [*Me*-^{14}C]choline and [1-^{14}C]glycerol (Lunt and Lapetina, 1970) and $^{32}P_i$ (Abdel-Latif *et al.*, 1968) into phosphoglycerides. However, no detailed studies on the incorporation of fatty acid precursors have so far been reported. Our interest in this aspect of synaptosomal lipid metabolism arose from previous observations that lipids of rat brain synaptic membranes have a distinct fatty acid composition (Koeppen *et al.*, 1971). There is disagreement (Cotman *et al.*, 1969) on the fatty acid pattern of synaptic membranes. Cotman *et al.* (1969) found a high percentage of long-chain polyunsaturates whereas Koeppen *et al.* (1971) demonstrated a tendency toward shorter and saturated fatty acids. Perhaps the local fatty acid metabolism at the synaptic ending exercises some control over the fatty acid composition of its constituents (presynaptic membranes, mitochondria, and synaptic vesicles). Synaptosomes are conceivably a suitable *in vitro* model for the fatty acid metabolism of the synaptic bouton.

Material and Methods

Subcellular Fractionation. Synaptosomes were prepared from rat cerebrum by the technique of Gray and Whittaker (1962) with a minor modification. Synaptosomes were collected from the interface of 0.8 and 1.2 M sucrose and the suspension was diluted to approximately 0.4 M sucrose. This suspension was then relayered on top of a second discontinuous gradient of 1.0 and 1.2 M sucrose. This second gradient was centrifuged at 50,000*g* for 2 hr. Synaptosomes were collected from the top of the 1.2 M sucrose layer, diluted to approximately 0.4 M sucrose, and centrifuged at 100,000*g* to form a pellet. The pellet was gently redispersed in 0.32 M sucrose, and aliquots were taken for incubation. The introduction of the second gradient was found to be necessary to remove last traces of myelin and microsomes. The success of the removal of myelin was monitored by silica gel thin-layer chromatography (tlc) of synaptosomal lipids. After the second gradient, tlc no longer showed cerebroside and cerebroside sulfate bands. Synaptosomes were examined by electron microscopy following standard techniques of fixation (Karlsson and Schultz, 1965) and embedding (Luft, 1961).

Subsynaptosomal Fractionation. After incubation with a fatty acid precursor, aliquots of the synaptosomal fraction were placed into centrifuge tubes and diluted to ten volumes (20 ml) with ice-cold distilled water to rupture the synaptosomes. After 30 min of this hypotonic exposure the suspension was centrifuged at 20,000*g* for 30 min. The pellet was dispersed in 0.32 M sucrose and layered on top of a discontinuous sucrose gradient (0.8 and 1.1 M sucrose). After centrifugation at 50,000*g* for 2 hr, two fractions were collected respectively from the top of 1.1 M sucrose (membranes) and the bottom of the tube (mitochondria). No visible band was formed at the 0.32–0.8 M interface. The supernatant was centrifuged at 100,000*g* for 60 min to obtain a synaptic vesicle fraction.

Incubations. Aliquots of the synaptosomal fraction (10 mg of protein) were incubated in ordinary Warburg flasks. The medium contained in final concentration 0.32 M sucrose, 0.05 M sodium phosphate buffer (pH 7.4), 10 mM KCl, 1 mM $MgCl_2$, 1 mM ATP, 0.5 mM NADPH, and 0.1 μCi of one of the following radioactive substrates: [1-^{14}C]acetate (specific activity 56.9 Ci/mol), [1-^{14}C]malonic acid (specific activity 2.98 Ci/mol), [1-^{14}C]acetyl-CoA[1] (specific activity 48.6 Ci/mol), and [1,3-^{14}C]malonyl-CoA (specific activity 18.5 Ci/mol). The total volume was 2 ml. Synaptosomes were incubated also with the optimal concentrations of both malonyl-CoA *and* acetyl-CoA described for *de novo* fatty acid biosynthesis in developing rat brain by Volpe and Kishimoto (1972). The labeled substrate was [1,3-^{14}C]malonyl-CoA which was adjusted to a final concentration of 0.16 mM by the addition of unlabeled malonyl-CoA. Acetyl-CoA had a concentration of 0.06 mM. In contrast to the system used by these authors, the pH was held at 7.4 instead of at 6.4. In some

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[1] Abbreviation used is: CoA, coenzyme A.

experiments NADH replaced NADPH. Sodium fluoride was used as an enzyme inhibitor. The influence of the following other additions was also examined: $MnCl_2$; $KHCO_3$; citrate; D-glucose; CoA esters of stearic, palmitic, myristic, lauric, capric, and caprylic acids; unlabeled acetyl-CoA. Tables I–VI list the appropriate additions to and deletions from the complete system. The incubation temperature was 37° and the reaction was allowed to proceed for 60 min with constant agitation in a metabolic shaker. The center well of the Warburg flask contained a narrow strip of filter paper soaked in 10% aqueous KOH. Trichloroacetic acid (0.5 ml of a 10% solution) was placed into the side arm. At the end of 60 min the acid was tipped into the incubation compartment and the flasks were returned to the shaker for another 30 min. The strip of filter paper was then removed from the center well and examined for radioactivity as described below. The acidification of the reaction mixture by Cl_3CCOOH was carried out to determine $^{14}CO_2$ evolution from the labeled substrates. For extraction of total lipids, the acidification step was omitted and the reaction was stopped by addition of 19 volumes of chloroform–methanol (2:1, v/v).

Fixation of Malonyl-CoA and Acetyl-CoA to Synaptosomes. Synaptosomes (10 mg) were incubated in the complete system (Table I) with either [1,3-^{14}C]malonyl-CoA or [1-^{14}C]acetyl-CoA in a final concentration of 0.01 mM. The reaction was started by the addition of the substrates. Just before placing the reaction tubes into a 37° water bath, 0.5-ml aliquots of a total volume of 2 ml were transferred to centrifuge tubes and chilled in ice. This point in the experiment was designated as zero time. Further aliquots of 0.5 ml were removed at 15, 30, and 45 min and chilled. The collected samples were diluted with ice-cold 0.32 M sucrose containing 0.05 M sodium phosphate buffer (pH 7.4), and centrifuged at 100,000*g* for 30 min. Aliquots (1.0 ml) of the clear supernatant were transferred to liquid scintillation vials and 0.05 ml of 10 N sulfuric acid was added. The vials were heated at 37° for 30 min to remove carbon dioxide. Scintillation fluid was added, and radioactivity was determined. The obtained counts per minute were compared to the counts per minute at zero time. Disappearance of radioactivity from the supernatant was taken as a measure of substrate fixation and incorporation into synaptosomal constituents.

Lipid Analysis. The procedure varied with the incubation method. The Cl_3CCOOH-treated samples were saponified overnight with two volumes of 10% methanolic KOH. The mixtures were then acidified by the dropwise addition of 10 N H_2SO_4. Fatty acids and nonsaponifiable lipids were extracted three times with 5 ml of petroleum ether (bp 30–60°). The extract was reduced to small volume by a stream of nitrogen and chromatographed by tlc on silica gel to separate impurities, cholesterol and free fatty acids in a solvent system of petroleum ether–diethyl ether–acetic acid (90:10:1, v/v). The entire cholesterol band was eluted with chloroform into counting vials for liquid scintillation spectrometry. The fatty acid band was eluted with chloroform and divided into two portions. One portion was counted directly and the other was methylated by boiling the fatty acids for 3 min in a mixture of methanol and BF_3 (14%). Methyl esters were extracted into hexane and purified by tlc in benzene.

Decarboxylation of the free fatty acids was based on the Schmidt procedure (Phares, 1951). The fatty acids were collected on the bottom of Thunberg tubes and 50 mg of sodium azide was added. Sulfuric acid was prepared by mixing two volumes of 98% H_2SO_4 (specific gravity 1.84) and one volume of fuming H_2SO_4 (20% SO_3). This mixture (0.5 ml) was placed into the side arm of the tubes and, after sealing, allowed to flow into the tube bottom. CO_2 evolving from the reaction mixture was trapped by connecting the outlet of the Thunberg tubes to liquid scintillation vials containing 0.5 ml of aqueous 10% KOH. The assembly was heated to 70° for 2 hr.

The chloroform–methanol-extracted incubation mixture was filtered and the residue was reextracted with small volumes of chloroform–methanol (2:1, v/v). The combined extracts were washed with two-tenths volume of distilled water. After separation, the lower phase was dried by a stream of nitrogen and redissolved in chloroform–methanol (1:1, v/v). Lipids were chromatographed by tlc on 250-μ-thick silica gel layers in a solvent system of chloroform–methanol–50% acetic acid (60:35:4, v/v). Phosphoglycerides separated by tlc were visualized by iodine vapor or located by appropriate standards. To study the distribution of labeled fatty acids among the phospholipids, the lipid-containing silica was scraped into culture tubes and fatty acids were transmethylated by heating the particles in 10% HCl in methanol at 100° in an atmosphere of nitrogen. After 45 min the tubes were cooled, water was added, and fatty acid methyl esters were extracted with petroleum ether. The fatty acid esters were purified by tlc in benzene, eluted with ether, and examined for radioactivity. An aliquot of the total lipid extract was similarly transmethylated and the resulting fatty acid methyl esters were studied by gas–liquid chromatography (glc). Direct methanolysis yielded results that were very similar to the procedure involving saponification and subsequent methylation with the BF_3–methanol reagent.

Phospholipase A Treatment. The positional distribution of labeled fatty acids was examined in choline phosphoglyceride only. This lipid was prepared from the total synaptosomal lipid extract by tlc as described above, and elution was done according to Skipski *et al.* (1964). Hydrolysis of the β-acyl ester linkage with commercial phospholipase A or cobra venom (*Naja naja*) followed the procedure of Lands and Hart (1964). After an incubation of 4 hr at room temperature, the ether solution was evaporated with a stream of nitrogen. The residue was redissolved in chloroform–methanol (1:1, v/v) and separated by tlc into free fatty acids, any remaining choline phosphoglycerides and lysocholine phosphoglycerides in the solvent system of Skipski *et al.* (1964). Free fatty acids were eluted directly into counting vials; fatty acids of choline phosphoglyceride and the corresponding lyso compound were obtained by methanolysis with methanolic hydrochloric acid as described.

Gas–Liquid Chromatography. Fatty acid methyl esters obtained through methylation of free fatty acids or by transmethylation of phosphoglycerides were examined by glc in a Varian 1200 or a 2720 gas chromatograph. The former instrument had a $^1/_8$-in. diameter 6-ft long column and a flame ionization detector. Temperatures for injector, column oven and detector were 225, 190, and 250°, respectively. The carrier gas was helium and had a flow rate of 50 cm^3/min. This instrument was used for analytical purposes. Peak areas were computed by automatic electronic integration. The second instrument was operated at the same settings but was equipped with a thermal conductivity detector and a heated exit port. The column diameter was 0.25 in. Fatty acid methyl esters were indicated on a recorder and collected into Pyrex tubes. The column packing of both instruments consisted of 10% diethylene glycol succinate polyester on 80–100 mesh diatomaceous earth (Gaschrom-P). An alternate liquid phase was 5% SE-30.

Fatty acids were subjected to radioactive counting or studied

TABLE I: Incorporation of Precursors into Fatty Acids of Total Synaptosomal Lipids and Evolution of CO_2.[a]

Substrate	Substrate Concn ($\times 10^{-6}$ M)	Formation of CO_2 (nmol $\times 10^{-2}$)	Formation of Fatty Acids (nmol $\times 10^{-2}$)
[1,3-^{14}C]Malonyl-CoA (11)	2.7	205.68	3.13
[1-^{14}C]Malonate + 1 mM CoA (2)	16.8	85.07	1.15
[1-^{14}C]Acetyl-CoA (5)	1.0	1.53	0
[1-^{14}C]Acetate + 1 mM CoA (2)	0.9	1.80	0

[a] Aliquots of the synaptosomal suspension (10 mg of protein) were incubated in Warburg flasks at 37° for 60 min. The complete system contained, in final concentration, the following additions in a total volume of 2 ml: 0.32 M sucrose, 0.05 M sodium phosphate buffer (pH 7.4), 10 mM $MgCl_2$, 1 mM ATP, 0.5 mM NADPH, and 0.1 μCi of radioactive substrate. CO_2 was trapped in the center well by KOH on a strip of filter paper after acidification of the incubation mixture with Cl_3CCOOH. Number of experiments in parentheses.

TABLE II: Incorporation of the Malonate Moiety of [1,3-^{14}C]-Malonyl-CoA into Synaptosomal Fatty Acids under Various Conditions.[a]

Addition or Deletion	Formation of CO_2 (nmol $\times 10^{-2}$)	Formation of Fatty Acids (nmol $\times 10^{-2}$)
Complete system[b] (11)	205.68	3.13
−ATP (6)	222.09	2.43
+100 mM NaF (6)	281.26	45.78
−Mg (4)	142.45	2.75
−Mg + 100 mM NaF (4)	141.85	5.83
+10 mM D-glucose (2)	188.32	4.62
−NADPH (2)	217.91	0
−NADPH + 0.5 mM NADH (2)	217.54	0.45
+0.1 mM malonyl-CoA + 100 mM NaF (2)	2587.06	56.41
+0.1 mM acetyl-CoA + 100 mM NaF (3)	182.78	28.00
+0.16 mM malonyl-CoA + 0.06 mM acetyl-CoA + 100 mM NaF (3)	2604.44	52.43

[a] Complete system as in Table I. Unless otherwise noted, the concentration of malonyl-CoA was 2.7×10^{-6} M. Number of experiments in parentheses. [b] From Table I.

further by hydrogenation (O'Brien and Rouser, 1964) or by oxidation to smaller fragments. For this purpose, the labeled fatty acid methyl ester was collected from the gas chromatograph and redissolved in a few drops of 1-butanol. Oxidation was carried out according to Lemieux and von Rudloff (1955). The permanganate–periodate solution (5 ml) was added to the fatty acid methyl ester, and the reaction was allowed to proceed for 20 hr at room temperature. Thereafter, the mixture was acidified by 0.5 ml of 10 N H_2SO_4 and the cleavage products (mono- and dicarboxylic acids) were extracted into diethyl ether. After evaporation of this solvent, the acids were methylated with BF_3 in methanol as described above. Carrier amounts of malonic, pimelic, and glutaric acids were added prior to methylation. Methyl esters of the cleavage products were separated by glc, collected, and examined for radioactivity. The column temperature was lowered to 170° for this purpose.

Liquid Scintillation Spectrometry. Radioactive samples (strips of filter paper with collected $^{14}CO_2$ or lipids) were placed into counting vials. Organic solvents were evaporated. A commercial liquid scintillation counting fluid (10 ml; Aquasol, New England Nuclear) was added, and radioactivity was measured in a Packard TriCarb Model 3320 liquid scintillation spectrometer.

Other Methods. Protein was determined by a biuret procedure. Malonic acid was extracted from the incubation mixture and purified as described by Numa *et al.* (1964).

Source of Chemicals. All radioactive substances were purchased from New England Nuclear, Inc., Boston, Mass. Phospholipase A, snake venom (*Naja naja*) and free CoA were obtained from the Sigma Chemical Co., St. Louis, Mo. Acetyl-CoA, caprylyl-CoA, capryl-CoA, lauryl-CoA, myristyl-CoA, palmityl-CoA, and stearyl-CoA were purchased from P-L Biochemicals, Inc., Milwaukee, Wis. Solvents for glc were of Spectrograde quality. Tlc plates with layers of silica (250 μ) had been prepared commercially (Brinkmann Instruments, Westbury, N. Y.).

Results

Electron Microscopy. Synaptosomes were of variable size and many did not contain visible mitochondria. Recognizable contaminants were some free mitochondria and membrane fragments.

Incorporation of Fatty Acid Precursors. Data on the fate of [1,3-^{14}C]malonyl-CoA, [1-^{14}C]malonate, [1-^{14}C]acetyl-CoA, and [1-^{14}C]acetate are summarized in Tables I–VI.

The label of [1,3-^{14}C]malonyl-CoA and [1-^{14}C]malonate appeared in fatty acids and carbon dioxide evolving from the reaction mixture. Free CoA was required for the incorporation of free malonate into fatty acids and for conversion of the malonate to CO_2. There was some oxidation of acetyl-CoA to CO_2 but the label failed to appear in fatty acids. Free labeled acetate was activated in the presence of added CoA and oxidized to CO_2 (Table I). Added $KHCO_3$, $MnCl_2$, and citrate did not result in fatty acid labeling from [1-^{14}C]acetyl-CoA. No labeled malonate could be recovered after incubation with [1-^{14}C]acetyl-CoA and $KHCO_3$.

The discrepancy between $^{14}CO_2$ evolution and ^{14}C label in fatty acids shown in Table I for [1,3-^{14}C]malonyl-CoA as the substrate is explained by malonate decarboxylation at the C-3 position due to malonyl-CoA decarboxylase (malonyl-CoA carboxy-lyase, EC 4.1.1.9.).

Biosynthesis of fatty acids from [1,3-^{14}C]malonyl-CoA was stimulated by the addition of ATP (Figure 1). In the absence of this nucleotide fatty acid labeling was reduced (Figure 1 and Table II). Fluoride greatly enhanced labeling of fatty

TABLE III: Fixation of Malonyl-CoA and Acetyl-CoA to Synaptosomes.[a]

Incubn Time (min)	% Retention [1,3-^{14}C]Malonyl-CoA	[1-^{14}C]Acetyl-CoA
0	100.0	100.0
15	89.0	99.2
30	78.3	102.1
45	72.2	97.5

[a] Synaptosomes (10 mg of protein) were incubated with 0.01 mM concentration of [1,3-^{14}C]malonyl-CoA or [1-^{14}C]-acetyl-CoA. At intervals aliquots were removed, diluted, and centrifuged. Radioactivity was determined in the supernatant. Results are expressed as per cent retention of label in the supernatant compared to label at zero time. Average of three experiments.

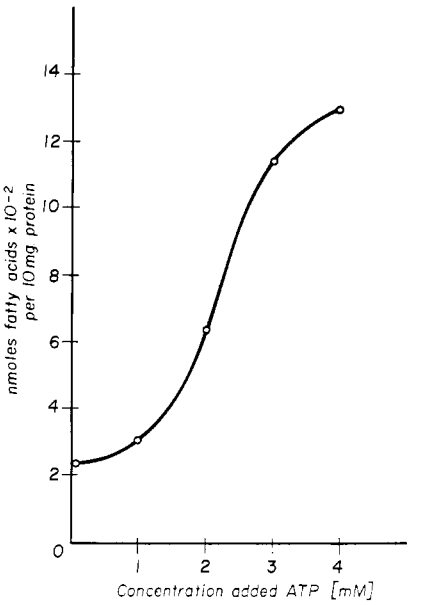

FIGURE 1: Dependence of fatty acid biosynthesis on ATP. Complete system as in Table I except for the amount of added ATP.

acids (Table II). In the presence of this inhibitor of ATPase, the evolution of $^{14}CO_2$ was increased commensurately with greater label in fatty acids.

Omission of Mg^{2+} from the incubation mixture led to reduced CO_2 evolution and impaired fatty acid labeling (Table II). The effect on CO_2 production was much more striking than on fatty acid biosynthesis. Fatty acid label increased when NaF was included in the mixture without added Mg^{2+}. However, the enhancing effect was much less pronounced than with NaF *and* added Mg^{2+} (Table II).

Synaptosomes-Oxidized Glucose. Glucose oxidation led to a minor increase of fatty acid labeling. Carbon dioxide evolution from the incubation mixture was reduced when glucose was present (Table II).

A reduced pyridine nucleotide (NADPH) was indispensable for malonate incorporation into fatty acids. NADH could make up only partially for the NADPH requirement (Table II).

Higher substrate concentration (0.1 mM malonyl-CoA) in the presence of 100 mM NaF led to a striking increase of CO_2 evolution but only modest enhancement of fatty acid labeling. Acetyl-CoA had a depressing effect on both CO_2 evolution and fatty acid labeling. The combination of malonyl-CoA and acetyl-CoA in higher concentrations had effects similar to the inclusion of malonyl-CoA alone (Table II).

The disappearance of labeled substrate from the incubation medium as a function of incubation time is summarized in Table III. Malonyl-CoA showed a progressive decrease of radioactivity in the high-speed supernatant. The acetyl-CoA label did not change significantly in the supernatant throughout the incubation period. The removal of $^{14}CO_2$ from the supernatant was essential because of the activity of malonyl-CoA decarboxylase in synaptosomal mitochondria. Carbon dioxide returned to the external incubation medium and thus masked the entry of [1,3-^{14}C]malonyl-CoA into the interior of the synaptosomes.

Distribution of Fatty Acid Label in Synaptosomal Lipids. Table IV indicates the distribution of the labeled fatty acids in the phospholipids of synaptosomes. When the incubation mixture was extracted promptly and the lipid extract was subjected to tlc, no radioactivity was recovered from free fatty acids and cholesterol. Fatty acids in choline phosphoglycerides showed the strongest labeling, followed by ethanolamine phosphoglycerides and serine phosphoglycerides. Some radioactivity with the sphingomyelin band probably represents contamination by lysocholine phosphoglyceride.

Phospholipase A treatment of choline phosphoglyceride after elution from the silica gel released 70–80% of the radioactivity from the lipid. The balance of 20–30% was recovered from the resulting lysocholine phosphoglyceride. This result was taken to represent that most of the label occurred in polyunsaturated fatty acids (see below).

Distribution of Radioactive Label among Fatty Acids. Table V lists the peak area distribution of the total fatty acids and their degree of labeling from [1,3-^{14}C]malonyl-CoA. The fatty acid with the strongest label was identified as 22:4ω6 (Δ 7) as follows. After hydrogenation and glc on an SE-30 column, the radioactivity moved to the 22:0 peak. Oxidation of the collected 22:4 with $KMnO_4$ and periodate produced radioactive pimelic acid. Hexanoate that resulted from the same oxidation procedure was not radioactive. Restriction of the radioactivity to pimelate also suggested that the label resided in the carboxyl carbon of 22:4. This assumption was con-

TABLE IV: Distribution of Labeled Fatty Acids in Synaptosomal Phospholipids after Incubation with [1,3-^{14}C]Malonyl-CoA.[a]

Phospholipid	% Total Radioactivity
Cardiolipin and phosphatidic acid	2.3
Ethanolamine phosphoglyceride	12.6
Inositol phosphoglyceride	3.7
Serine phosphoglyceride	9.4
Choline phosphoglyceride	71.4
Sphingomyelin (and lysocholine phosphoglyceride)	0.6

[a] Complete system as in Table I. Average of five experiments.

TABLE V: Radioactivity in Total Synaptosomal Fatty Acids after Incubation with [1,3-^{14}C]Malonyl-CoA.[a]

Fatty Acid	% Peak Area	% ^{14}C
14:0	0.4	0
16:0	24.4	10.4
16:1	0.1	0
18:0	22.8	12.3
18:1	19.5	4.9
18:2	1.3	0
18:3 and 20:1	1.0	1.0
20:4	10.6	0
22:4	2.8	69.0
22:6	16.5	0
Other	0.9	2.3

[a] Complete system as in Table I. Average of ten experiments. Results expressed as per cent peak area and per cent total radioactivity, respectively.

TABLE VI: Fatty Acids and Their ^{14}C Radioactivity in Synaptic Membranes and Synaptosomal Mitochondria.[a]

	Synaptic membranes		Synaptosomal Mitochondria	
Fatty Acid	% Peak Area	% ^{14}C	% Peak Area	% ^{14}C
14:0	2.2	5.8	0.7	0
16:0	27.2	23.6	13.1	17.7
16:1	Trace	0	1.0	0
18:0	21.6	10.3	23.7	7.0
18:1	19.7	Trace	12.8	0
18:2	2.1	0	1.6	0
18:3 and 20:1	1.1	0	0.6	0
20:4	7.6	0	19.8	0
22:4	0.5	60.3	5.4	75.3
22:6	14.8	0	18.9	0
Other	3.2	0	2.4	0

[a] Intact synaptosomes were incubated with [1,3-^{14}C]-malonyl-CoA in the complete system given in Table I. After incubation, synaptosomes were fractionated as described in Material and Methods. Average of two experiments. Results are expressed as per cent peak area and per cent total radioactivity, respectively.

firmed by azide decarboxylation. The total ^{14}C-labeled fatty acid:$^{14}CO_2$ ratio of 22:4 (and of all other labeled fatty acids) was 1.20 to 1.25. Accordingly, 22:4ω6 was presumed to be a single-step elongation product of 20:4ω6 (arachidonic acid).

The relative distribution of the radioactive label among the fatty acids was not significantly altered with any of the additions or deletions listed in Table II. Docosatetraenoic acid (22:4ω6) remained the fatty acid with the strongest radioactivity at all times. There was no evidence of *de novo* fatty acid synthesis. Added acyl-CoA (8:0–18:0) were not elongated by synaptosomes from malonyl-CoA.

Subsynaptosomal Fractionation. Following incubation with [1,3-^{14}C]malonyl-CoA, the fatty acid radioactivity was not equally distributed between the subsynaptosomal constituents. Synaptosomal mitochondria contained 80% of the radioactivity, synaptic membranes 20%. Synaptic vesicles contained no labeled fatty acids. The percent distribution of the label among the fatty acids of synaptic membranes and mitochondria is listed in Table VI. Docosatetraenoic acid (22:4ω6) had the strongest label in both subsynaptosomal fractions. However, in synaptic membranes a shift to higher labeling of 16:0 and 18:0 was apparent (Table VI). Docosatetraenoic acid (22:4ω6) was only poorly represented in synaptic membranes but constituted 5.4% of the total mitochondrial fatty acids.

Discussion

Fatty Acid Biosynthesis in Rat Brain Synaptosomes. The enzyme system in synaptosomes that achieved incorporation of the malonate moiety into fatty acids was similar to the extramitochondrial or soluble cytoplasmic system described for yeast (Lynen *et al.*, 1962), liver (Ganguly, 1960; Brady *et al.*, 1960; Wakil, 1961) and rat brain (Brady, 1960; Volpe and Kishimoto, 1972) in that it required malonyl-CoA and NADPH for maximum activity (Table II). The failure of synaptosomes to utilize acetyl-CoA was somewhat surprising since rat brain mitochondria have been demonstrated to incorporate [1-^{14}C]acetyl-CoA into fatty acids (Aeberhard and Menkes, 1968; Boone and Wakil, 1970), and mitochondria are present in synaptosomes. The lack of acetyl-CoA utilization by synaptosomes could be due to absent transport (Table III). However, acetyl-CoA may be generated indirectly within synaptosomes. The active malonyl-CoA decarboxylase in the synaptosomal interior produces acetyl-CoA from malonyl-CoA which, in turn, could serve as a primer for the *de novo* synthesis of fatty acids. The site of malonyl-CoA decarboxylation is mitochondrial (Scholte, 1969; Landriscina *et al.*, 1971), and it is probable that acetyl-CoA cannot exit from the synaptosomal mitochondria in sufficient amounts to allow *de novo* fatty acid biosynthesis. Labeled citrate ([1,5-^{14}C]-citric acid) was used in an effort to generate labeled acetyl-CoA in the synaptosomal cytosol (Koeppen *et al.*, unpublished observation). ATP:citrate lyase (EC 4.1.3.8.) has been found in synaptosomes and was shown to produce acetyl-CoA for the biosynthesis of acetylcholine (Tuček, 1967). We could not demonstrate incorporation of the citrate label into synaptosomal fatty acids.

Our data do not rule out activity of acetyl-CoA carboxylase because of the described difficulty to achieve acetyl-CoA concentrations in the synaptosomal cytosol and the presence of malonyl-CoA decarboxylase. The latter enzyme would destroy any malonyl-CoA formed from acetyl-CoA.

The reason for the remarkable enhancement of fatty acid labeling by NaF (Table II) is not entirely clear but is perhaps attributable to inhibition of synaptosomal ATPase (Kurokawa *et al.*, 1965). Webster and Alpern (1964) studied the acylation of lysolecithin by rat brain crude mitochondrial fractions in the presence of [^{14}C]oleic acid and found an 11-fold higher incorporation when NaF (109 mM) was present. Added ATP and Mg^{2+} also strongly promoted the acylation of lysolecithin to lecithin.

Glucose was selected as an ATP-generating system (Table II) because Bradford (1969) had previously shown that this substrate allows the accumulation of ATP when sufficient

phosphate buffer is present. The glucose-supplemented system also permits the assessment of an intact glycolytic pathway in the cytosol of synaptosomes (Bradford, 1969) . The effect of ATP is believed to be activation of precursor fatty acids that are present in synaptosomes as the free compounds. A small pool of available ATP in synaptosomes was probably responsible for the limited fatty acid biosynthesis when no ATP was added (Table II and Figure 1). This assumption is also supported by the observation that the total amount of ATP (either endogenous or added) had little influence on the *distribution* of the label in the fatty acids after incubation with [1,3-^{14}C]malonyl-CoA (Table V). Our results are in strong contrast to the labeling of rat brain microsomal fatty acids from [1,3-^{14}C]malonyl-CoA after incubation without ATP (Aeberhard and Menkes, 1968). These authors found biosynthesis of short-chain fatty acids rather than elongation of longer chain fatty acids when the nucleotide was absent.

Nature of the Biosynthesized Synaptosomal Fatty Acids. The origin of the precursor arachidonic acid for biosynthesis of docosatetraenoic acid is not entirely clear. The only synaptosomal constituents that have an abundance of 20:4ω6 are the mitochondria (Table VI). Utilization of free mitochondrial arachidonic acid for elongation would require activation and thus explain the ATP dependence (Table II and Figure 1). The similarity between the synaptosomal system and soluble fatty acid synthetase from other tissues suggests a cytoplasmic location of the biosynthetic pathways. Thus, arachidonic acid would have to be transported out of the mitochondria into the synaptosomal cytoplasm.

Incorporation of Labeled Fatty Acids into Synaptosomal Phospholipids. The most attractive explanation for the strong labeling of choline phosphoglyceride during incubation with [1,3-^{14}C]malonyl-CoA is acylation of lysocholine phosphoglyceride within synaptosomes. Webster (1967) has postulated that long-chain fatty acids are incorporated into phospholipids of respiring rat brain slices by this mechanism. In our experiments, a 1-acyl lysophospholipid would accept 22:4 at the 2 position of glycerol. Labeling of synaptosomal lipids through long-chain fatty acids differs from [^{32}P]orthophosphate incorporation (Abdel-Latif *et al.*, 1968). These authors found that the ^{32}P label was preferentially incorporated into phosphatidic acid and inositol phosphoglycerides rather than choline phosphoglycerides.

Fatty Acid Incorporation into Subsynaptosomal Constituents. Mitochondria proved to be better acceptors for the biosynthesized fatty acids than synaptic membranes. The shift toward incorporation of saturated labeled fatty acids (16:0 and 18:0) that occurred in synaptic membranes (Table VI) may be significant for our previous observation that these membranes have fatty acids of shorter chain length and of a low percentage of polyunsaturation (Koeppen *et al.*, 1971). Compared to mitochondria, synaptic membranes may have a smaller concentration of lysophospholipids that would be capable of accepting 22:4 biosynthesized in the synaptosomal cytoplasm.

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