Polyenoic acid metabolism in cultured dissociated brain cells

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Abstract The incorporation of [1-^{14}C]linolenate (18:3 n − 3) into cellular lipids of cultured dissociated brain cells was studied. During the initial phases of incubation, radioactivity was found in free fatty acids, diacylglycerols, triacylglycerols, and choline phosphoglyceride pools preferentially. Incorporation into the ethanolamine phosphoglyceride pool increased steadily and paralleled desaturation and chain elongation of 18:3 → 20:3 → 20:4 → 20:5 → 22:5 → 22:6. From pulse–chase studies it was evident that the label of the highly polyunsaturated fatty acids in ethanolamine phosphoglycerides is constantly increased while the label in the fatty acids of choline phosphoglycerides decreased. Uptake of 18:3 by the cells was reduced by lowering incubation temperature, the triacylglycerol and ethanolamine phosphoglyceride pools being mainly affected. Lowering the incubation temperatures essentially abolished conversion of labeled 18:3 to the higher polyenoic acids. At intermediate temperatures, conversion of 18:3 to 20:5 n − 3 was still active, but conversion of 20:5 n − 3 to 22:6 n − 3 was abolished, suggesting that formation of 22:6 from 18:3 proceeds by at least two reactions distinguishable by their temperature dependency.

Supplementary key words linolenic acid · fatty acids · choline phosphoglyceride metabolism · ethanolamine phosphoglyceride metabolism · fatty acid chain elongation · triglyceride biosynthesis

One of the unique features of the mammalian brain is the high content of polyunsaturated fatty acids in the phospholipid fraction. 30% of gray matter phospholipid fatty acids is derived from linoleic (18:2) and linolenic (18:3) acids (1), with over 50% of the polyunsaturated fatty acids present in adult brain accumulating during the early stages of brain development, prior to the initiation of myelin deposition (2, 3). Even though present in only trace amounts, linolenic acid, in particular, is an important precursor and is taken up directly by the brain from the blood and converted to the various n − 3 polyunsaturated acids, notably 22:5 n − 3 and 22:6 n − 3 (4, 5).

The biosynthetic pathways for these unsaturated fatty acids and their metabolic interconversions within the nervous system have not been as well studied as in other organs such as liver (6). The availability of brain cell cultures for metabolic studies (7) prompted us to examine polyunsaturated fatty acid metabolism and its regulation under these experimental conditions. Brain cell cultures can be grown in an environment that is readily controlled, and are, therefore, useful in metabolic studies conducted over longer periods and simulating a variety of pathological conditions.

MATERIALS AND METHODS

Preparation and culture of dissociated cerebral cells

Monolayered cultures were prepared from 17-day-old rat embryo cerebral hemispheres as previously described (7). In essence, the brain tissue was treated with a solution of 0.125% (w/v) trypsin for 3–4 min, and the resulting cell suspension was aspirated through a Pasteur pipette to complete the dissociation. The cells were then placed in 60-mm Petri dishes and allowed to aggregate and attach themselves to the plastic. The incubation was carried out at 37°C in an atmosphere of 95% air and 5% CO_2. The medium consisted of Eagle's MEM (Grand Island Biochemical Co., Grand Island, N.Y.) to which 20% of heat-treated fetal calf serum (Rehatuin, Reheis Chemical Corp.), 0.6% glucose, and antibiotics were added. Attachment was followed by appearance of cell processes that multiplied and enlarged and ultimately formed a network that extended throughout the Petri dish. Both neuronal and neuroglial elements could easily be distinguished after 3–4 days of culture. Medium was changed every 2–3 days, depending on the acidity of the culture. The amount of protein harvested from each Petri dish was 1–2 mg.

Incubation of cultures. Metabolic studies were carried out after cultures had reached confluency (7). By this

Abbreviations: MEM, minimal essential medium; CPG, choline phosphoglyceride; EPG, ethanolamine phosphoglyceride; TLC, thin-layer chromatography; TG, triacylglycerols; *m:n*, number of carbon atoms:number of double bonds.

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time, the proportion of neuronal to glial elements was approximately 5 to 95. The nutrient medium was removed and the cells were washed once with serum-free medium, M-199 (Grand Island Biochemical Co.). The experimental medium, consisting of 2–3 ml of medium M-199 augmented with 0.6% glucose and containing [1-^{14}C]linolenic acid (52.4 mCi/mmole, Amersham/Searle) complexed to albumin (8), was then added to the cells. No antibiotics were used during the metabolic studies; the cultures were kept free from contaminating microorganisms. No visible changes in morphology were observed during the incubation period, which was maintained at various temperatures as described in the text.

For chase studies, the following procedure was performed. After completion of incubation, the radioactive medium was removed and cells were washed twice with 3 ml of M-199 medium containing 60 μM linolenic acid complexed to albumin (molar ratio 1:2). 3 ml of M-199 medium containing 6 μM linolenic acid was added, and cultures were further incubated at 37°C for selected times. The procedure was carried out under sterile conditions.

Extraction and resolution of cellular lipids

Lipids were extracted and purified as described elsewhere.[2] Neutral lipids as well as free fatty acids and phosphatidic acid and phospholipids, including glycolipids, were separated in bulk by silicic acid column chromatography (8). Neutral lipids were further separated on TLC plates by two successive developments in one direction, using the following solvent mixtures: dichloroethane–acetic acid 100:0.5 (v/v) and petroleum ether (bp 30–60°C)–diethyl ether–formic acid 50:50:1.5 (v/v/v). Identification and quantification of the acylglycerol compounds were performed as previously described (8). Choline phosphoglyceride (CPG) and ethanolamine phosphoglyceride (EPG) were isolated using a slight modification of the method of Skipski, Peterson, and Barclay (9). After the plates had been developed, they were dried under nitrogen. Lanes containing tissue phospholipids were covered with a glass plate while the markers were briefly exposed to iodine vapors. The CPG- and EPG-containing spots were then scraped off and placed in sintered glass columns 1 cm in diameter. Diethyl ether (10 ml) was added to remove any acetic acid or other impurities; methanol (20 ml) was then added to elute the phospholipids. Recovery of radioactivity was in excess of 93%. As judged by rechromatography in the above solvent system and in chloroform–methanol–7 N ammonia 65:35:5 (v/v/v), the EPG and CPG isolated by this method were free from radioactive contaminants. When the methanol eluate was methylated with diazomethane and subsequently subjected to TLC, no contamination with free fatty acids could be detected. Cerebroside remained essentially unlabeled under the present experimental conditions.

Gas–liquid chromatography was performed as previously described (8).

Reaction products determination

The various intermediates in the course of polyenoic fatty acid formation from [1-^{14}C]linolenic acid were tentatively identified by cochromatography (GLC) with the known polyenoic standards 20:3 n − 3 and 20:5 n − 3 (Hormel Institute, Austin, Minn.) and 22:5 n − 3 and 22:6 n − 3 (Supelco, Inc., Bellefonte, Pa). Intermediates were further identified by hydrogenation and quantification of the resulting saturated long-chain acids, 16:0, 18:0, 20:0, and 22:0 (10). The locations of the radioactive carbons in eicosapentaenoic (20:5 n − 3) and docosapentaenoic acids (22:5 n − 3) were determined by a micromodification of a permanganate–periodate oxidation method (11). Purified EPG from an experiment in which 94% of the radioactivity was found in 20:5 n − 3 and 22:5 n − 3 was transesterified in methanolic HCl. The methyl esters were extracted with pentane and purified by elution from a column containing 0.5 g of silicic acid (Sigma Sil) with 4% diethyl ether in pentane. The recovered radioactivity was greater than 96%. The methyl esters were then cleaved by oxidation, after which the monomethyl-dicarboxylic acids were extracted and methylated with diazomethane. Carrier dicarboxylic acid esters, a generous gift of Dr. A. Fulco, were added, and the samples were injected into a Barber-Colman gas chromatograph equipped with a Hewlett-Packard radioactivity monitor at 180°C. The recovered radioactivity corresponded mainly to penta- and heptadicarboxylic acid esters, the cleavage products of eicosapentaenoic (20:5 n − 3) and docosapentaenoic (22:5 n − 3) acids, respectively.

Analytical methods

The protein content of the cultures was determined by the method of Lowry et al. (12). Radioactivity was measured using a Packard Tri-Carb liquid scintillation spectrometer, model 3003. Phosphate was determined by the Bartlett method (13).

RESULTS

Incorporation of [1-^{14}C]linolenate

The incorporation of radioactivity into total cellular lipids and the percentage distribution of ^{14}C among the various lipid fractions after cells had been incubated with [1-^{14}C]linolenic acid are given in Table 1.

After 3 hr, 89% of radioactivity was recovered from cellular lipids and proteins and from the incubation medium. Triacylglycerols (TG) and phospholipids contained

[2] Yavin, E., and J. H. Menkes. The incorporation and metabolism of fatty acids by cultured dissociated cells from rat cerebrum. *Lipids*. In press.

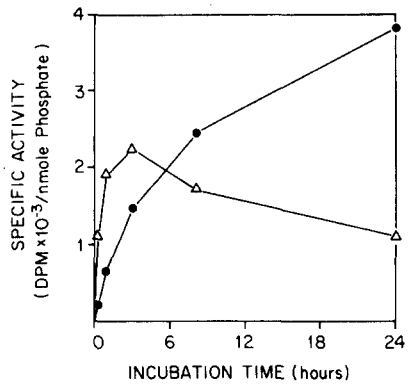

Fig. 1. Specific activities of phospholipids after incubation with [1-^{14}C]linolenic acid. Experimental conditions were the same as described for Table 1. Values for CPG (▲) and EPG (●) are expressed as dpm/nmole of phosphate.

approximately 94% of total radioactivity at the end of 3 and 24 hr of incubation.

During the initial phases of incubation, label preferentially entered choline phosphoglyceride, diacylglycerol, triacylglycerol, and free fatty acid pools. Incorporation of label proceeded in a biphasic manner, with total radioactivity in the free fatty acid pool peaking at 1 hr and total radioactivity in TG and CPG pools peaking between 1 and 3 hr. Incorporation into the EPG pool increased progressively. When incorporation of radioactivity was expressed in terms of dpm/nmole of phosphate, the maximum ratio of CPG to EPG, 2.9, was observed after 1 hr. Thereafter, this ratio decreased progressively to 0.29 by the end of 24 hr of incubation (Fig. 1).

The distribution of radioactivity among the individual fatty acids of the CPG, EPG, and TG fractions after 24 hr of incubation with [1-^{14}C]linolenate is depicted in Table 2. Incorporation of radioactivity into the higher polyenoic acids proceeded by desaturation and elongation of linolenic acid, with lesser proportions of radioactivity being derived by the recycling of two-carbon units resulting from β oxidation. The proportions of the recycled radioactivity into palmitate and stearate were particularly high in the CPG and TG fatty acids. By contrast, after a 24-hr incubation 83% of the total EPG fatty acid radioactivity was in the form of 20:5 n − 3, 22:5 n − 3, and 22:6 n − 3 (Fig. 2). The relative specific activity of some of the major polyenoic acids was felt to be unreliable, as their concentrations were too low for adequate quantification.

TABLE 1. Distribution of radioactivity among lipid fractions after incubation of cultured cells with [1-^{14}C]linolenic acid

Lipid Fraction	Incubation Time (hr) 1/3	1	3	8	24
Neutral lipids	23.1	21.2	13.4	10.8	10.0
DG[a]	4.0	3.3	3.5	2.5	1.9
TG	12.6	11.9	7.8	5.5	5.0
CE	0.1	1.7	1.4	1.6	1.5
FFA	4.5	3.4	0.4	0.8	1.0
Phospholipids	76.9	78.8	86.6	89.2	90.0
SPG[b]	11.0	11.9	10.5	12.5	16.2
CPG	56.9	53.8	53.6	38.8	19.9
EPG	8.2	12.1	21.7	34.1	48.9
Total incorporation[c]	158	323	502	528	637

Dissociated brain cells, cultured for 8 days in vitro, were incubated with 6 μM [1-^{14}C]linolenic acid (1.4–2.1 × 10^6 dpm/culture) for various periods of time. Cell lipids were extracted and separated on silicic acid columns, and lipid fractions were purified by TLC as described in Materials and Methods. Values are given as percentages of total radioactivity incorporated into the cells and represent pooled lipid extracts of three experiments. Abbreviations: DG, diacylglycerol; TG, triacylglycerol; CE, cholesterol esters; FFA, free fatty acids; SPG, serine phosphoglycerides; CPG, choline phosphoglycerides; EPG, ethanolamine phosphoglycerides.

[a] Also contains phosphatidic acid. Separation of DG and phosphatidic acid was incomplete.

[b] Also contains inositol phosphoglycerides. Due to their relatively low concentrations, these two phospholipids could not be separated effectively.

[c] Values expressed as dpm × 10^{-3}/mg protein.

Effect of temperature on [1-^{14}C] linolenic acid incorporation

Lowering the temperature at which brain cells were incubated with [1-^{14}C]linolenic acid reduced the incorporation of radioactivity into total lipids (Fig. 3). At 15°C, total uptake of labeled linolenic acid was 30% of the value at 37°C. Labeling of CPG and free fatty acid was less affected than that of the EPG, TG, or cholesterol ester frac-

TABLE 2. Distribution of radioactivity from [1-^{14}C]linolenic acid precursor into fatty acids of major labeled lipids

Fatty Acid	CPG Mass	CPG Radio-activity	EPG Mass	EPG Radio-activity	TG Mass	TG Radio-activity
			% distribution			
14:0	3.8		0.9		8.8	
16:0	26.0	17.5	6.3	1.2	18.0	18.6
16:1	9.3		2.0		5.9	
18:0	8.4	8.1	20.9	1.7	10.2	7.0
18:1	42.1	0.9	16.5		22.7	0.9
18:2	0.6		2.4		1.6	
18:3 n − 3	1.0	29.6	0.9	6.2	2.2	12.1
20:3 n − 3 / 20:4 n − 6	3.0	10.1	16.6	2.3	4.0	8.7
20:4 n − 3	0.1	4.8	0.2	4.8	2.6	11.2
20:5 n − 3	0.1	15.6	0.2	35.0	2.0	8.3
22:5 n − 3	0.3	13.3	1.7	37.0	0.9	29.3
22:6 n − 3	0.8		8.1	11.0	7.0	
16 ald[a]			7.1			
18 ald			5.5			

Experimental conditions were the same as described for Table 1. Cells were incubated for 24 hr, and the major labeled lipids were separated and purified. Fatty acid ester content was analyzed as described in Materials and Methods. Values represent percentage distribution of mass and radioactivity in three pooled experiments. Abbreviations as given for Table 1.

[a] Aldehydes.

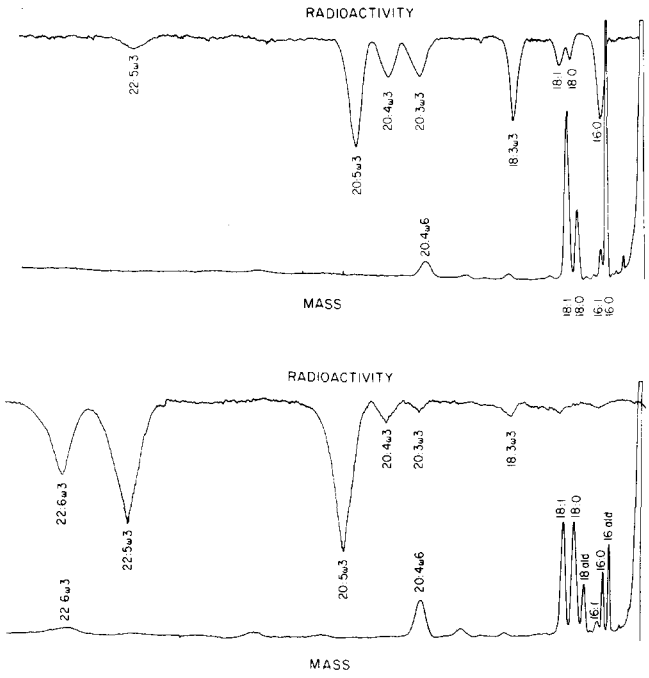

Fig. 2. Gas–liquid chromatogram tracings of the fatty acid mixtures obtained from CPG and EPG fractions. The tracings depict both the mass and the [1-^{14}C]linolenic acid. *Upper*, CPG; *lower*, EPG. (ω3 is equivalent to n − 3; ω6 is equivalent to n − 6.)

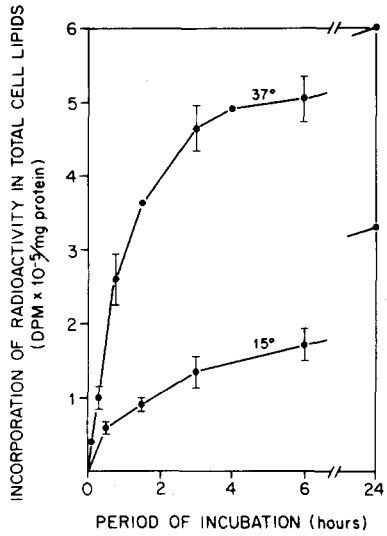

Fig. 3. Incorporation of ^{14}C into total brain cell lipids after incubation at 37°C and 15°C with [1-^{14}C]linolenic acid. Brain cells, cultured for 8 days in vitro, were incubated with [1-^{14}C]linolenic acid (6 μM) for various periods of time in an atmosphere of 100% air. Values are means ± SD (three or four Petri dishes).

tions (Table 3). At 15°C, labeling of the CPG fraction was 38% of that at 37°C, while labeling of the EPG and TG fractions was 12% and 8%, respectively, of the value at 37°C. (Table 3).

On examination of the composition of the labeled fatty acids (Table 4), it is evident that the conversion of linolenic acid to the higher polyenoates is temperature sensitive, and that this process is markedly impaired at 22°C and almost inactive at 15°C. Table 4 also shows that recycling of two-carbon units, resulting from β oxidation, into fatty acids of shorter chain length is also completely abolished at low temperatures.

When the initial incubation period with [1-^{14}C]linolenic acid was succeeded by a chase period in the presence of unlabeled linolenic acid, radioactivity (expressed in dpm/nmole of phosphate) was lost from the CPG fraction but continued to increase in the EPG fraction (Fig. 4). During the chase period, carried out at 37°C, radioactive linolenic acid was converted to the higher polyenoic fatty acids. When the initial incubation was carried out at

TABLE 3. Effect of incubation temperature on the incorporation of radioactivity into lipid classes

Lipid Fraction	Incubation Temperature					
	15°C		22°C		37°C	
	Incorporation	Radioactivity	Incorporation	Radioactivity	Incorporation	Radioactivity
	dpm	*% of total*	*dpm*	*% of total*	*dpm*	*% of total*
FFA	7.6	5.5	6.1	1.7	2.6	0.6
DG[a]	4.5	3.3	10.5	2.9	13.9	3.0
TG	2.9	2.1	28.6	7.9	36.3	7.8
CE	0.7	0.5	4.6	1.3	6.5	1.4
CPG	94.6	68.8	206.0	57.2	250.0	53.8
EPG	12.7	9.2	51.0	14.2	101.0	21.7
SPG[b]	14.2	10.3	38.0	10.6	49.0	10.5
Total incorporation	137.5		360.0		465.0	

Experimental conditions are the same as described in Table 1. Incubation was carried out for a period of 3 hr in an atmosphere of 100% air. Values for incorporation of radioactivity are expressed as dpm × 10^{-3}/mg protein and represent three pooled experiments. Abbreviations as given for Table 1.

[a] Also contains phosphatidic acid.

[b] Also contains inositol phosphoglycerides.

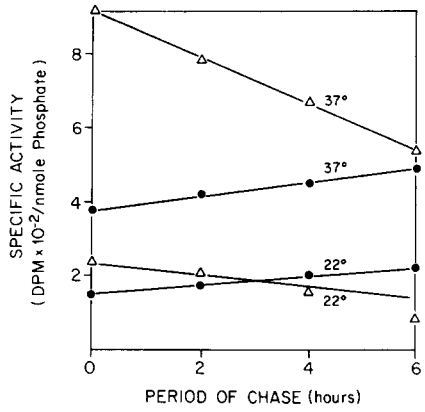

Fig. 4. Pulse-chase studies of CPG and EPG fractions after labeling with [1-^{14}C]linolenic acid. Dissociated rat brain cells, cultured for 10 days in vitro, were incubated with [1-^{14}C]linolenic acid for 1 hr at 37°C or at 22°C. After the radioactive medium was removed, all cells were further incubated with nonradioactive linolenic acid at 37°C as described in Materials and Methods. At each specified time, two or three Petri dishes were removed, and the CPG (△) and EPG (●) fractions were purified and analyzed for radioactivity.

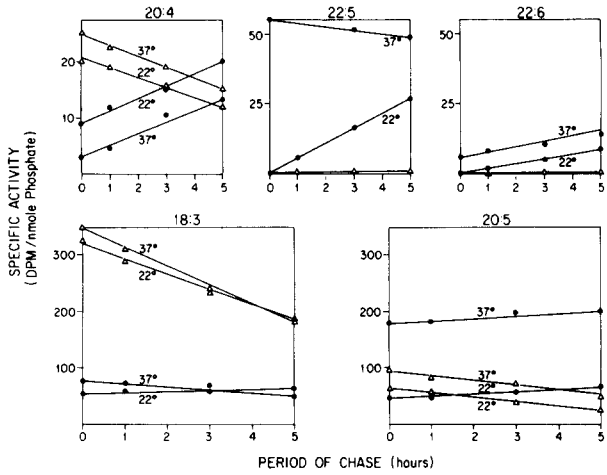

Fig. 5. Pulse-chase studies of labeled fatty acids in the CPG and EPG fractions after labeling with [1-^{14}C]linolenic acid. Experimental conditions are the same as described for Fig. 4 except that incubation was carried out for 2 hr with cells grown for 13 days in vitro. Each figure represents the changes in the radioactivity in the various fatty acids of the CPG (△) and EPG (●) fractions. The dpm for each fatty acid was calculated from its relative distribution.

37°C, 20:5 n − 3 and 22:6 n − 3 were formed, but when the initial incubation temperature was 15°C, there was no labeling of polyenoic fatty acids higher than 20:5 n − 3 during the chase period carried out at 37°C (Table 4). It is possible that cell damage produced by prolonged cooling might have affected the conversion of 20:5 n – 3 to the higher polyenoates.

On examining alterations in fatty acid labeling in the CPG and EPG fractions, we found that during the chase period labeled polyenoic acids in CPG became diluted, while the radioactivity entering the EPG fraction was enhanced. As a rule, changes in labeling after incubation at 22°C and 37°C proceeded in parallel. In the EPG fraction the amount of labeled 18:3 remained constant, while that of 20:5, 22:5, and 22:6 increased (Fig. 5).

TABLE 4. Distribution of radioactivity in fatty acid esters of the phospholipid fraction

	Incubation Temperature					
	15°C		22°C		37°C	
Hours after pulse	0	4	0	4	0	4
16:0					5.0	5.0
18:0					2.0	2.0
18:3 n − 3	95.2	64.7	54.1	24.3	8.0	7.0
20:3 n − 3	1.8	3.4	1.8	1.2	1.5	1.2
20:4 n − 3		6.1	10.1	6.1	1.8	1.9
20:5 n − 3	2.9	25.7	33.9	46.8	27.0	24.0
22:5 n − 3				20.4	31.0	33.0
22:6 n − 3				1.1	23.6	25.8

Experimental conditions are similar to those described for Table 2. Cells were incubated with the radioactive medium for 24 hr. Nonlabeled linolenic acid was then added, and cells were incubated at 37°C for a period of 4 hr as described in Materials and Methods. Values represent percentage distribution of radioactivity in total phospholipid fatty acids in three pooled experiments.

DISCUSSION

The fatty acid compositions of the CPG and EPG fractions in cultured brain cells (Table 2) resemble those found by others in the immature nervous system. When the fatty acid composition of the CPG fraction is compared with that obtained by Svennerholm et al. (14), the only significant difference is a somewhat lower concentration of C_{16} fatty acids in cultured brain cells. Like brain, but unlike liver, there is little 18:2 and 20:4 n − 6 in cultured nerve cells, and the concentration of 18:1 is greater than that of 18:0. Comparing the fatty acid composition of the EPG fraction with that published by White, Galli, and Paoletti (15), we find that our values closely resemble those obtained by these workers for human fetal brain. Differences in fatty acid composition, as, for instance, the absence of 22:4 n − 6 in the EPG fraction of brain cell cultures contrasted with its presence in brain, may reflect the spectrum of fatty acids in the culture medium, which influences to some extent the fatty acid composition of cellular lipids (16). As was previously demonstrated,[2] labeled linolenic acid added to brain cell cultures is effectively converted to higher polyenoic acids. This conversion can be shown to proceed as follows: 18:3 → 20:3 → 20:4 → 20:5 → 22:5 → 22:6. We have been unable to detect any labeled 18:4.

When linolenic acid is incubated with brain cell cultures, it is taken up by the cells and esterified to TG in temperature-sensitive reactions. In this respect linolenic acid behaves like stearic acid (8). Turnover of radioactivity in these two fractions is rapid, and after a 1-hr incubation there is no further increase in the total radioactivity in each of these fractions. These findings suggest that a

significant portion of the labeled linolenic acid entering the cells is stored in the TG fraction of the cells. The gradual appearance of labeled higher polyenoic acids in TG (Table 2) indicates that TG may also store these higher polyenoic acids either after acylation to their CoA derivatives or in the form of a labeled diacylglycerol moiety released from CPG. Incorporation into the EPG fraction proceeded at a slower rate due to the fact that most of the label, at the initial time points studied, was in the form of linolenic acid and not the higher polyunsaturated fatty acids.

Lowering the incubation temperature decreased the uptake and esterification of labeled linolenic acid and essentially abolished conversion of labeled linolenic acid to the higher polyenoic fatty acids. At intermediate temperatures the conversion of 18:3 to 20:5 n − 3 was still active, while conversion of 18:3 to 22:6 proceeded by at least two distinct steps, having different temperature sensitivities.

This finding, which has not been shown up to now in the whole brain, demonstrates the versatility of brain cultures in the study of lipid metabolism and suggests a number of further studies using incubation temperature as one of the parameters.

Our data on pulse and pulse–chase studies (Figs. 1, 4) strongly indicate that the increase in the EPG specific activity is due to an increase in the labeled polyenoic fatty acid content (Fig. 5).

Labeled palmitic and stearic acids, derived from recycling of two-carbon units, enter the CPG and TG but not the EPG fractions, although the latter has a relatively high content of palmitic and stearic acids. It is well known that brain phospholipids contain an appreciable amount of phosphatidal- and phosphatidylethanolamines into which the higher polyunsaturated fatty acids are specifically incorporated.

The entrance of the higher polyenoic acids into the EPG fraction depends on the chain elongation and desaturation of essential fatty acids available to the brain and would therefore be affected in nutritional deprivation or hypothermia (14, 17). Further investigations into the role of essential fatty acids in the metabolism of brain cell cultures under these experimental conditions are in order.

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