

Incorporation of ^{14}C -Docosahexaenoic Acid into Lipids of Rats (39383)R. BABCOCK, B. MEDWADOWSKI, P. MILJANICH, J. TINOCO,
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Docosahexaenoic acid (22:6n3), a member of the linolenate family, is found in membrane phospholipids of most mammalian cells. It occurs in surprisingly high concentrations in the phosphatidylethanolamines (PE) of human cerebral cortex (1), retinas of various species, including human, dog, pig, sheep, cattle, and rabbit (2), and bovine testes (3) as well as other specialized cells or organelles. Large proportions of 22:6n3 also appear in many lipids of fish. Docosahexaenoic acid cannot be totally synthesized in vertebrates so that it or its precursor, linolenic acid (18:3n3), must be obtained from the diet. Rainbow trout grow well with 22:6n3 or 18:3n3 as the only dietary unsaturated fatty acid (4), but linoleic acid (18:2n6), the most important essential fatty acid (EFA) for mammals, is of little value (5). A great decrease in 22:6n3 of tissue lipids occurred in rats fed a diet deficient in linolenic acid, but growth and reproduction remained normal (6). Despite its essentiality in trout, and its high concentrations in many vertebrate membrane phospholipids, nothing whatever is known about the specific function of 22:6n3.

Little is known about the incorporation of 22:6n3 into tissue lipids. Sinclair and Crawford (7) and Sinclair (8) administered ^{14}C -22:6n3 to rat pups and found considerable incorporation into brain and liver phospholipids after 22 hr. No investigation has been made of the incorporation of 22:6n3 into lipids at different intervals after administration, a test which would allow comparison of the behavior of 22:6n3 with that of other fatty acids. Therefore, we have measured the incorporation of 22:6n3 into rat tissue lipids at various times after administration. Normal rats were administered ^{14}C -docosahexaenoic acid prepared by biosynthesis from $^{14}\text{C}_1$ -linolenic acid in EFA-deficient rats. Incorporation of the label into lipids of

liver, heart, plasma, brain, and carcass was measured after intervals from 0.25 to 95 hr. We also investigated the distribution of radioactivity among liver lipid classes and within individual fatty acids of liver phosphatidylcholines (PC) and PE.

Preparation of ^{14}C -docosahexaenoic acid. ^{14}C -docosahexaenoic acid was isolated from liver phospholipids of EFA-deficient rats after administration of $^{14}\text{C}_1$ -linolenic acid. Just after the birth of litters, mother rats (Sprague-Dawley, Horton's Laboratories, Los Gatos, Calif.) were given an EFA-deficient diet (9). Pups were fed this diet and raised to weights of 160-180 g (8-10 weeks of age). $^{14}\text{C}_1$ -Linolenic acid (Dhom, North Hollywood, Calif., 52.5 mCi/mmmole, stated to be >99% pure) was mixed with 20% rat plasma in saline. About 30 μCi in 0.3 ml was administered (Metofane anesthesia, Pitman-Moore, Washington, Pa.) via the femoral vein. After 72 hr, the animal was anesthetized (sodium pentobarbital), bled by heart puncture, and the liver was removed and lyophilized.

Liver lipids were extracted with chloroform:methanol 2:1 (v/v), and phospholipids were isolated from the total lipid extract by column chromatography on silicic acid (Unisil, Clarkson Chemical Co., Williamsport, Pa.). Neutral lipids were eluted with chloroform, and phospholipids were collected in chloroform:methanol 1:1 and in pure methanol. Mixed phospholipids were converted to methyl esters in methanol acidified with H_2SO_4 , under nitrogen in a capped tube for 3 hr or more at 80°.

Methyl esters were separated and isolated by gas-liquid chromatography (glc) with a model 810 F&M chromatograph equipped with a Packard Tri-Carb fraction collector (Model 830). The instrument was calibrated with a mixture containing weighed amounts of the methyl esters of palmitic (16:0), pal-

mitoleic (16:1n7), stearic (18:0), oleic (18:1n9), 18:2n6, 18:3n3, arachidonic (20:4n6), eicosapentaenoic (20:5n3), and 22:6n3 acids (Hormel Institute, Austin, Minn.). Methyl esters were collected in tubes packed with glass wool which were placed in scintillation vials and counted with toluene mixture (below). Their specific activities were determined by use of ^{14}C -heptadecanoic acid as an internal standard (Nuclear Chicago). An average liver contained about 7 mg of 22:6n3, of which two-thirds was recovered as purified methyl docosahexaenoate, sp act 8.0×10^5 dpm/mg. The methyl docosahexaenoate peak contained 91.5% of the radioactivity and 88% of the mass, with the remainder distributed among several very small unidentified components that appeared both before and after 22:6n3 on the GLC trace. For administration to rats, methyl docosahexaenoate was converted to the free acid and dispersed in 20% rat plasma in saline.

Incorporation of ^{14}C -docosahexaenoic acid into rat lipids. Male Sprague-Dawley rats (Horton's; about 175 g) were given a semipurified diet for 2 weeks as described before (10), except that corn oil was substituted for cottonseed oil. They were anesthetized with Metofane, and ^{14}C -docosahexaenoic acid in diluted serum was injected via the femoral vein. Two or more rats per time period were killed after 0.25, 0.5, 1, 2, 4, 8, 10, 16, 24, 40, 48, 72, and 95 hr. The dose of radioactivity varied from about 2×10^5 dpm, for incorporation up to 24 hr, to about 9×10^5 dpm, for longer incorporations.

Rats were anesthetized and blood was taken by heart puncture. Plasma was separated and an aliquot was counted in Triton mixture (below). Brain, liver, and heart were lyophilized and their lipids were extracted into chloroform:methanol, 2:1. The remainder of the animal including intestinal contents (carcass) was digested with saturated KOH in 95% ethanol. Aliquots of carcass digests were neutralized with H_2SO_4 , and lipid-soluble material was extracted into petroleum ether for measurement of radioactivity.

Analytical procedures. Lipids in organic solvents were taken to dryness under nitrogen and counted in 0.5%, 2,5-diphenyloxazole ("PPO," Research Products International, Grove Village, Ill.) in toluene ("toluene mixture") in a Beckman LS-100 liquid scintillation counter (81% efficiency).

Liver lipid extracts were fractionated into lysolecithins, sphingomyelins, phosphatidylcholines (PC), phosphatidylinositols + phosphatidylserines, phosphatidylethanolamines (PE), and neutral lipids, by the thin-layer chromatography (tlc) system of Skipski *et al.* (11). Duplicate samples were streaked on silica gel H (Merck) plates. Fractions from one side of the plate were transferred into tubes and were eluted with methanol for determination of lipid phosphorus (12). The corresponding bands were scraped directly into scintillation vials and were counted in Triton mixture (928 ml of toluene, 544 ml of Triton X-100 (Rohm and Haas) and 4 g of PPO) (61% efficiency).

Another separation of liver lipid extracts was made for glc analysis of methyl esters from PE and PC. The two phospholipids were eluted from silica gel by the method of Arvidson (13). PE and PC were converted to methyl esters, which were separated and collected by glc as described above. With the use of the internal standard, the sp act of each individual fatty acid was measured.

All solvents and chemicals were of reagent grade.

Results and discussion. Radioactivity in lipids of heart, plasma, or brain was less than 2% of the total recovered radioactivity. These lipids were not analyzed further. The degree of incorporation did not vary in any recognizable pattern from one time period to another in these tissues.

At all times, the greatest proportion of lipid radioactivity was recovered in carcass lipids. This is not surprising since 22:6n3 accounts for 3 to 4% of the total fatty acids in skeletal muscle (14), which in turn accounts for 45% of body mass in rats. Thus the carcass contains most of the body's 22:6n3. We did not analyze the radioactivity in individual fatty acids of carcass lipids because specific activities were very low.

Liver lipid contained 11% of the total recovered radioactivity after 0.25 hr of incorporation and by 4 to 8 hr, it contained about 30% of the radioactivity recovered in the whole animal.

In liver lipids, most of the activity was

incorporated into fatty acids of PE and PC, with much smaller proportions incorporated into neutral lipids and other phospholipids. The percentages of liver radioactivity in PE and PC are shown in Fig. 1. At the beginning of incorporation, a large proportion, 60–70%, of radioactivity appeared in PE, with little in PC. As time progressed, the values converged, so that by about 16 hr, approximately 45% of liver total radioactivity was present in each fraction.

Not all of the radioactivity was located in the 22:6n3 moiety. After 0.25 and 0.5 hr of incorporation, about 55–60% of the radioactivity in PE was in 22:6n3, whereas in PC only 25% of the label was in 22:6n3 (Table I). The remainder of the radioactivity was found mainly in C-16 and C-18 chains. This indicates that part of the 22:6n3 was quickly metabolized and that the label was reincorporated into fatty acids. As time progressed, the proportion of radioactivity in 22:6n3 increased, so that by the end of the experiment, 22:6n3 accounted for 75–80% of the label in PE, and about 80–85% of the label in PC.

For at least the first 10 hr of incorporation, we found more radioactivity in PE than in PC. This prompt incorporation of 22:6n3 into PE and its delayed incorpora-

tion into PC is quite unlike the incorporation behavior of 16:0, 18:0, 18:1n9, 18:2n6, or 20:4n6, the other major fatty acids in rat liver phospholipids. For example, when 16:0 was administered intravenously to rats, PC had more than twice the radioactivity present in PE (15) at 10 min after the dose and at 20 min after the dose,

TABLE I. RADIOACTIVITY IN FATTY ACIDS OF RAT LIVER PHOSPHATIDYLETHANOLAMINES (PE) AND PHOSPHATIDYLCHOLINES (PC) AFTER ADMINISTRATION OF ^{14}C -DOCOSAHEXAENOIC ACID.^a

Time (hr)	Number of rats	Percentage as 22:6n3 in phosphatidylethanolamines	Percentage as 22:6n3 in phosphatidylcholines
0.25	2	55	27
0.5	2	61	24
1	2	69	46
2	2	68	49
4	4	67	56
8	2	76	52
10	2	65	63
16	4	66	61
24	2	54	57
40	2	79	73
48	2	77	79
72	2	80	80
95	2	76	85

^a Percentage of total radioactivity in fraction recovered as 22:6n3.

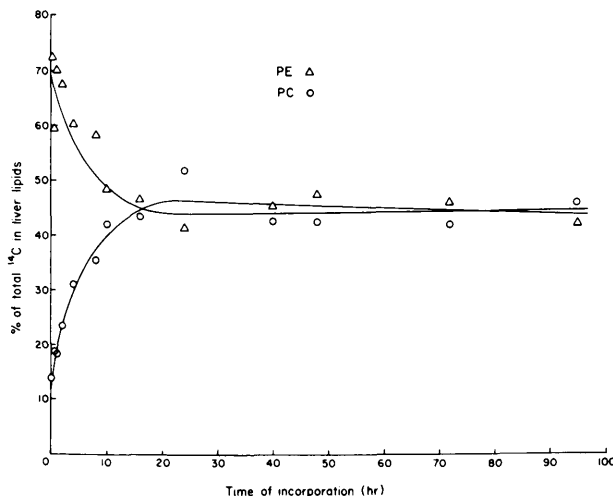


FIG. 1. Radioactivity in phosphatidylethanolamines (PE) and phosphatidylcholines (PC) of rat liver after administration of ^{14}C -docosahexaenoic acid. Percentage of total radioactivity recovered in liver total lipids. Each point represents the mean of two analyses for each of two animals, except for points at 4, 16, and 48 hr, which represent four animals. Agreement between values for replicate animals was usually within $\pm 5\%$ of the mean, with a maximum range of $\pm 11\%$.

PC had three times the amount of radioactivity that PE did (16). Similarly, 18:0 and 18:1n9 were incorporated in greater amounts into PC than into PE of rat liver microsomes, at 1, 2, 4, and 24 hr after administration of these fatty acids by stomach tube (17) to rats deficient in EFA. Clouet and colleagues (18) administered 16:0, 18:2n6, or 20:4n6 via the jugular vein to male Wistar rats, and more activity appeared in liver PC than in PE after 10, 30, 60, and 180 min of incorporation.

The selective incorporation of 22:6n3 into liver PE is consistent with observations made *in vitro* with rat liver microsomal systems. Kanoh and Ohno (19) showed that CDP 1,2-¹⁴C-ethanolamine was converted selectively into PE molecules that contain 22:6n3, an indication that the PE-synthesizing system in rat liver selects precursors (presumably diglycerides) that contain 22:6n3. In contrast, the system that forms PC from CDP choline preferentially forms 20:4n6-PC.

Once the PE is labeled with ¹⁴C-22:6n3, these molecules can be converted to PC by sequential methylation with *S*-adenosylmethionine. It has been observed that ¹⁴CH₃-methionine is incorporated quickly and selectively into rat liver 22:6n3-PC *in vivo* (20, 21). The combined actions of phospholipases and transacylases (22) could also cause the transfer of ¹⁴C-22:6n3 from PE to PC.

The rat liver evidently selects 22:6n3-containing precursors for incorporation into PE rather than PC or the other lipid classes, and the label later appears in PC. Docosahexaenoic acid thus behaves distinctly differently from the other important fatty acids in rat liver. These observations suggest that the function of 22:6n3 also is different from those of the other fatty acids.

Summary. ¹⁴C-docosahexaenoic acid, prepared by administration of ¹⁴C₁-linolenic acid to essential fatty acid-deficient rats, was isolated from liver phospholipids and converted to methyl docosahexaenoate, sp act 8 × 10⁵ dpm/mg, purity about 90%. This was converted to the free acid and injected into

rats. ¹⁴C-docosahexaenoic acid was incorporated mainly into carcass and liver. In liver, radioactivity appeared most rapidly in PE and later in PC. This behavior is unlike the incorporations of 16:0, 18:0, 18:1n9, 18:2n6, or 20:4n6, which are all incorporated most quickly into PC. Most of the radioactivity was recovered as 22:6n3, although considerable activity was present in C-16 and C-18 chains.

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