Metabolism of deuterium-labeled linoleic, 6,9,12-octadecatrienoic, 8,11,14-eicosatrienoic, and arachidonic acids in the rat

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Abstract Male weanling rats were fed a diet that contained 2.1% ethyl oleate, 1% ethyl linoleate, and 0.2% ethyl linolenate. After 4 weeks all the linoleate was replaced by the deuterium-labeled analog and the animals were killed 4 days later. The molar fraction of total 20:4(n-6) in liver, heart, and kidney phospholipids containing deuterium was 33.9, 8.9, and 13.3%, respectively. Second, animals were preconditioned by incorporating either 0.2% of 18:3(n-6), 20:3(n-6), or 20:4(n-6) into the above diet and again after 4 weeks all the linoleate was replaced with the labeled analog. Now the molar fraction of labeled 20:4(n-6) in liver phospholipids from these three groups of animals was reduced from 33.9 to 27.1, 23.9, and 24.1% respectively. In contrast, there was little change in the specific activity of 20:4(n-6) in heart and kidney phospholipids. The third protocol was a direct crossover study in that again unlabeled linoleate was fed during the entire period. Four days prior to killing the unlabeled 18:3(n-6), 20:3(n-6), and 20:4(n-6) were replaced with the deuterium-labeled analogs. The mole % of total esterified 20:4(n-6) in liver phospholipids was now 24.6, 32.0, and 26.2%, respectively. Even though 18:3(n-6), 20:3(n-6), and 20:4(n-6) were all fed at only 20% of the level of 18:2(n-6), it can be calculated that the molar fraction of esterified 20:4(n-6) in liver phospholipids was between 65 to 77% of that found when 18:2(n-6) was the only dietary (n-6) acid as under these conditions 33.9 mol % of the 20:4(n-6) was labeled. Interestingly, when deuterium-labeled 18:3(n-6), 20:3(n-6), or 20:4(n-6) was fed, the specific activity of esterified 20:4(n-6) in kidney and heart phospholipids was always equal to or greater than what was derived from deuterium-labeled 18:2(n-6). The results show that under steady-state dietary conditions, (n-6) dietary fatty acids are processed in different ways by liver, heart, and kidney.—**Luthria, D. L., and H. Sprecher.** Metabolism of deuterium-labeled linoleic, 6,9,12-octadecatrienoic, 8,11,14-eicosatrienoic, and arachidonic acids in the rat. *J. Lipid Res.* 1995. **36:** 1897–1904.

Supplementary key words unsaturated fatty acid metabolism • deuterium-labeled fatty acids • heart, kidney, and liver phospholipids

Dietary linoleate and linolenate are both metabolized to longer chain PUFA in the endoplasmic reticulum. De novo phospholipid biosynthesis (1, 2) and the remodeling of phospholipid fatty acids (3, 4) are metabolic processes that are also localized primarily in the endoplasmic reticulum. Although PUFA and phospholipid biosynthesis are separate metabolic events, they must be regulated in a coordinated way so that PUFA are made to meet the requirements for phospholipid biosynthesis.

When diets contain adequate amounts of linoleate, its metabolism by a 6-desaturase is the rate-limiting step in the biosynthesis of arachidonate (5, 6). When rats are raised on a diet devoid of fat, the membrane lipids accumulate 20:3(n-9). The dietary studies by Holman (7) and his colleagues have shown that when rats receive 1% or more of their calories as linoleate, 20:3(n-9) does not accumulate, and moreover, the level of esterified arachidonate in membrane lipids is independent of higher levels of dietary linoleate. The amount of arachidonate made available for membrane lipid biogenesis must then be determined by the activity of the 6-desaturase. Dietary supplements of (n-6) acids, beyond the rate-limiting 6-desaturase step, thus have the potential of increasing the amount of 20:4(n-6) made available for membrane lipid biosynthesis. When 300 mg/day of 20:3(n-6) or 20:4(n-6) was given to rats fed Purina chow, there were increases in the amounts of esterified 20:3(n-6) and 20:4(n-6) in some, but not all, tissue phospholipids (8). The addition of oils containing 18:3(n-6) to the diet has been advocated as a way to modulate various disease processes without determining how 18:3(n-6) modifies the metabolism of linoleate to arachidonate (9).

The first objective of the studies reported here was to compare how the addition of unlabeled and deuterium-labeled 18:3(n-6), 20:3(n-6), and 20:4(n-6) to the diet would modify the metabolism, respectively, of labeled and unlabeled linoleate to arachidonate and its incorporation into liver phospholipids.

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It is well established that liver has the ability to carry out all of the desaturation and chain elongation reactions required for PUFA biosynthesis. Other tissues may have no ability, or a limited capacity, to synthesize PUFA from dietary precursors (10). For example, we were unable to demonstrate that heart cardiomyocytes had the ability to carry out the desaturation and chain elongation reactions required for metabolizing linoleate to arachidonate (11). Suneja et al. (12) found that rat kidney microsomes were unable to desaturate fatty acids at positions 9, 6, and 5. Kaestner et al. (13) reported that mouse kidney contains very low levels of mRNA for both stearoyl-CoA desaturases 1 and 2. There was enhanced expression of both mRNAs when mice were raised on a diet devoid of fat. This finding suggests that the kidney should have the ability to desaturate stearoyl-CoA but the activity might be very low in animals fed chow. Other investigators have reported that rat kidney microsomes have a limited capacity to desaturate fatty acids at position 6 (14). Collectively, these studies suggest that kidney has a limited capacity to make its own PUFA and must obtain them from the circulation. This hypothesis is supported by the studies of Lefkowith et al. (15) showing that arachidonate is first taken up by the liver and then transported to heart and kidney. When linoleic acid, labeled with deuterium at the double bonds, was fed to rats it was observed that 22.8, 4.2, and 8.6 mol percent of esterified arachidonate in liver, heart, and kidney phospholipids, respectively, was deuterium-labeled (16).

The second objective of this study was to compare the specific activity of linoleate and arachidonate in liver phospholipids with those in kidney and heart, in order to provide new information as to how dietary (n-6) acids are processed in vivo by various tissues.

EXPERIMENTAL PROCEDURES

Fatty acids

The ethyl esters of oleic, linoleic, linolenic, 6,9,12-octadecatrienoic, and arachidonic acid were obtained from Nu-Chek Prep, Elysian, MN. 8,11,14-Eicosatrienoic acid was made by total organic synthesis and converted to its ethyl ester by stirring with 5% anhydrous HCl in ethanol. Linoleic acid-17,17,18,18-d_4 and 6,9,12-18:3-17,18,18,18-d_4 were made by total organic synthesis via procedures analogous to those described for the synthesis of 8,11,14-20:3-19,19,20,20-d_4 and 5,8,11,14-20:4-19,19,20,20-d_4 (17). The acids were converted to ethyl esters and purified by silicic acid column chromatography to yield a single compound that co-migrated with an authentic standard when analyzed by thin-layer chromatography using hexane–diethyl ether–acetic acid 80:20:2 (by vol) as solvent. The chemical purities of the four deuterium-labeled ethyl esters ranged from 89 to 97% (**Table 1**) and were used as such. The impurities were over-reduced compounds that were produced during Lindlars reduction of the acetylenic acid precursors. Aliquots of the free fatty acids were derivatized by reaction with N-methyl-N-(*t*-butyldimethylsilyl)trifluoroacetamide to yield tertiarybutyl-dimethylsilyl derivatives (16). The isotopic purities (Table 1) were determined by integration of the appropriate M-57 ions.

Diets

Male weanling Sprague-Dawley rats were housed individually. Rats in Group 1 were fed Purina chow. The composition of the diets used for the other groups of rats was a modified AIN-76 diet that contained 51.7% sucrose and 3.3% fat as ethyl esters rather than 5.0%. The composition of the ethyl esters of this basal diet was 2.1% oleate, 1% linoleate, and 0.2% linolenate. Rats in Group 2 were fed this diet for 4 weeks after which time all of the linoleate was replaced by 18:2(n-6)-d_4. The animals were killed on the afternoon of the fourth day. In order to determine whether dietary 18:3(n-6) modified the amount of 18:2(n-6) that was metabolized to yield esterified 20:4(n-6), rats in Group 3 were fed the modified AIN-76 diet, but the content of oleate was now reduced from 2.1% to 1.9%, and 0.2% 18:3(n-6) was included in the diet during the entire feeding period. Again, after 4 weeks on the diet all of the linoleate was replaced by 18:2(n-6)-d_4. As before, as well as in all subsequent studies, the rats were killed the afternoon of the fourth day. In the direct crossover study, (Group 4) rats were fed the same diet as Group 3 but now after 4

TABLE 1. Chemical and isotopic purity of deuterium-labeled fatty acids

Fatty Acid	Chemical Purity[a]	Isotopic Purity[b]				
		d_4	d_3	d_2	d_1	d_0
	%			%		
18:2(n-6)-17,17,18,18-d_4	96.8	94.8	3.1	2.1	0.1	–
18:3(n-6)-17,17,18,18-d_4	95.0	95.0	2.5	1.9	0.2	0.4
20:3(n-6)-19,19,20,20-d_4	93.0	95.0	3.7	1.0	0.3	–
20:4(n-6)-19,19,20,20-d_4	89.0	95.0	4.0	1.0	–	–

[a]Determined by analysis of the ethyl ester.

[b]Determined by integration of the appropriate M-57 ions after derivatizing the free fatty acids with N-methyl-N-(*t*-butyldimethylsilyl)trifluoracetamide.

TABLE 2. Fatty acid composition in mol percent of total liver phospholipid of rats fed various diets

	Group				
	1	2	4	6	8
	Dietary Component				
Fatty Acid	Purina Chow	18:2(n-6)-d_4	18:2(n-6) 18:3(n-6)-d_4	18:2(n-6) 20:3(n-6)-d_4;	18:2(n-6) 20:4(n-6)-d_4
16:0	15.9 ± 1.1	17.1 ± 0.3	17.1 ± 1.2	18.9 ± 2.4	18.3 ± 0.8
16:1	0.5 ± 0.1	2.4 ± 0.2	2.6 ± 0.2	2.6 ± 0.5	2.4 ± 0.2
18:0	20.5 ± 0.5	19.5 ± 3.5	18.0 ± 0.3	16.7 ± 1.8	16.5 ± 0.4
18:1(n-9)	4.0 ± 0.5	6.8 ± 1.3[a]	6.6 ± 0.6[a]	7.1 ± 1.1[a]	6.6 ± 0.9[a]
18:1(n-7)	2.8 ± 0.1	5.0 ± 0.8[a]	5.7 ± 0.3[a]	4.8 ± 0.2[a]	5.8 ± 0.6[a]
18:2(n-6)	14.5 ± 0.8	7.7 ± 0.1[a]	5.9 ± 0.3[a]	7.3 ± 0.2[a]	6.7 ± 0.6[a]
20:3(n-6)	1.3 ± 0.2	1.0 ± 0.1	0.8 ± 0.1	1.8 ± 0.1	0.6 ± 0.1
20:4(n-6)	22.9 ± 0.5	24.6 ± 1.2	27.3 ± 0.4	26.5 ± 1.7	26.1 ± 2.3
20:5(n-3)	1.0 ± 0.1	0.9 ± 0.2	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
22:4(n-6)	0.2 ± 0.1	0.1 ± 0.1	0.3 ± 0.2	0.4 ± 0.1	0.5 ± 0.1
22:5(n-6)	–	0.4 ± 0.4	0.5 ± 0.5	0.7 ± 0.1	0.7 ± 0.2
22:5(n-3)	2.2 ± 0.5	1.1 ± 0.2	0.9 ± 0.3	1.3 ± 0.1	1.1 ± 0.1
22:6(n-3)	10.4 ± 1.4	11.4 ± 1.7	11.3 ± 1.1	8.8 ± 1.3	8.8 ± 0.8

Male weanling Sprague-Dawley rats were fed one of the modified AIN-76 diets for 4 weeks. An equal amount of the unlabeled acid was then replaced by the deuterium-labeled analog and the animals were killed 4 days later. Purina chow was fed to one group during the entire 28 days. The results are averages from three animals ± SEM.

[a]Significantly different from rats fed Purina chow, $P < 0.03$.

weeks all of the unlabeled 18:3(n-6) was replaced by 18:3(n-6)-d_4. The variable in Groups 5 and 6 was 20:3(n-6). The modified AIN diet now contained 1.9% oleate, 1% linoleate, and 0.2% each of the ethyl esters of 18:3(n-3) and 20:3(n-6). After 4 weeks on this diet all of the 18:2(n-6) was replaced by 18:2(n-6)-d_4 (Group 5) while in Group 6 all of the unlabeled 20:3(n-6) was replaced by 20:3(n-6)-d_4. The variable in Groups 7 and 8 was 20:4(n-6). Now the modified AIN-76 diet contained 1.9% oleate, 1% linoleate, and 0.2% each of ethyl 18:3(n-3) and 20:4(n-6). After 4 weeks all of the linoleate was replaced by 18:2(n-6)-d_4 (Group 7) while in Group 8 all of the arachidonate was replaced with 20:4(n-6)-d_4.

Lipid isolation and analysis

Tissue lipids were extracted by the method of Folch, Lees, and Sloane Stanley (18). Neutral lipids were separated from phospholipids by the sequential elution of columns packed with Unisil (Clarkson Chemical Co., Williamsport, PA) with $CHCl_3$ and MeOH (16).

A portion of the total phospholipids was interesterified by stirring them overnight with 5% anhydrous HCl in MeOH. The methyl esters were recovered by extraction with hexane and analyzed by gas chromatography using a 30-m DB-WAX capillary column (J and W Scientific, Folsom, CA). The initial temperature of the oven was 200°C. After 15 min it was programmed to increase at 2°C/min to 210°C where it was held until the methyl ester of 22:6(n-3) had eluted.

In some cases methyl esters were separated by HPLC by eluting a 0.46 × 25 cm Zorbax ODS column with acetonitrile–water 9:1 (by vol) at 1 ml/min. This acetonitrile was removed under N_2 and the methyl esters were recovered by extraction with diethyl ether. These methyl esters and the remaining phospholipids were saponified by stirring them overnight with 4% KOH in MeOH–H_2O 9:1 (by vol). After acidification with 6 N HCl, the free fatty acids were recovered by extracting with hexane. They were then derivatized to tertiarybutyldimethylsilyl esters by heating them with 50 µl of acetonitrile and 50 µl of N-methyl-N-(*t*-butyldimethylsilyl)trifluoroacetamide (Pierce Chemicals, Rockford, IL) for 30 min at 80°C (16). The excess derivatizing agent was removed under N_2 and the derivatives were dissolved in isooctane. Gas chromatography–mass spectrometry was carried out with a Hewlett-Packard 5790 gas chromatograph and a 5970A mass selective detector. Samples were injected in the split mode (20 to 1) on a 30 meter by 0.25 mm DB-23 column (J and W Scientific, Folsom, CA). The oven was maintained at 220°C during the entire run. The mass selective detector was programmed to monitor the mass range between 335 to 345 atomic mass units for elution of the isotopic derivatives of linoleate and then from 360 to 370 atomic mass units for the arachidonate derivatives. Results were calculated by dividing the sum of the appropriate integrated ion current in the M-57 derivatives, for the labeled derivatives, by that in the unlabeled derivative plus that of the deuterium-labeled derivatives. All data are expressed as averages ± SEM and each group contained three animals. Data were analyzed by the Student's *t*-test for unpaired data. Representative fatty acid compositional data from liver, heart, and kidney phospholipids are shown in **Table 2** and **Table 3.** The fatty acid composition of the modified AIN diets for Groups 3 and 4 was

TABLE 3. Amounts of selected fatty acids in mol percent in heart and kidney phospholipids after feeding various diets

	Group				
	1	2	4	6	8
	Dietary Component				
Fatty Acid	Purina Chow	18:2(n-6)-d_4	18:2(n-6) 18:3(n-6)-d_4	18:2(n-6) 20:3(n-6)-d_4	18:2(n-6) 20:4(n-6)-d_4
Heart phospholipids					
18:1(n-9)	3.4 ± 0.4	7.3 ± 0.1[a]	6.7 ± 0.3[a]	6.8 ± 0.5[a]	6.6 ± 0.1[a]
18:1(n-7)	3.4 ± 0.4	4.7 ± 0.1[a]	4.9 ± 0.1[a]	4.8 ± 0.6[a]	5.8 ± 0.2[a]
18:2(n-6)	19.3 ± 2.0	16.9 ± 0.7[b]	15.9 ± 2.4[b]	16.7 ± 1.2[b]	13.0 ± 0.6[a]
20:4(n-6)	16.8 ± 0.8	23.0 ± 0.7	23.1 ± 1.3	21.1 ± 0.6	24.7 ± 0.7
Kidney phospholipids					
18:1(n-9)	6.2 ± 0.4	9.2 ± 0.5[a]	8.7 ± 0.3[a]	9.3 ± 0.2	9.0 ± 0.3[a]
18:1(n-7)	2.0 ± 0.1	3.2 ± 0.2[a]	3.3 ± 0.1[a]	3.3 ± 0.1[a]	3.6 ± 0.1[a]
18:2(n-6)	13.9 ± 1.0	8.6 ± 0.1[a]	6.8 ± 1.0[a]	7.1 ± 1.1[a]	6.6 ± 0.1[a]
20:4(n-6)	30.0 ± 1.2	32.7 ± 2.0	36.9 ± 1.5	33.3 ± 1.5	31.4 ± 1.2

Male weanling Sprague-Dawley rats were fed one of the modified AIN-76 diets for 4 weeks. An equal amount of the unlabeled acid was then replaced by the deuterium-labeled analog and the animals were killed 4 days later. Purina chow was fed to one group during the entire 28 days. The results are averages from three animals ± SEM.

[a]Significantly different from rats fed Purina chow, $P < 0.03$.

[b]Not significantly different from rats fed Purina chow, $P < 0.15$.

identical, as it was for Groups 5 and 6 and for Groups 7 and 8. Values are thus compared for animals fed Purina chow (Group 1) with each dietary regimen, i.e., Groups 2, 4, 6, and 8.

RESULTS

All of the diets fed in this study contained 1% by weight of linoleate, which corresponds to about 2.2% of the total caloric intake. It was not possible to detect esterified 20:3(n-9) in tissue phospholipids under conditions where 0.1% of this compound would have been detected. The compositional data in Tables 2 and 3 show that the level of esterified arachidonate in all phospholipids was similar to that found when animals were fed a Purina chow diet. The amount of linoleate in liver and kidney phospholipids of rats fed the modified AIN-76 diets was statistically less ($P < 0.05$) than from rats fed Purina chow. In general, there was also a reduced level of esterified linoleate in heart phospholipids from rats fed the AIN-76 diets versus Purina chow, but these differences were not all statistically significant. The reduced level of esterified linoleate in all phospholipids was always accompanied by increased levels of both 18:1(n-9) and 18:1(n-7), i.e., $P < 0.05$.

When methyl esters of 18:2(n-6)-d_4 and 18:2(n-6) were analyzed by gas chromatography there was baseline separation of these two compounds. This observation allowed us to compare the gas chromatography-mass spectrometry data with that obtained by gas chromatography. The results in **Table 4** show that 56.8 ± 4.2% of the linoleate in liver phospholipids was labeled, when the analysis was carried out by gas chromatography-mass spectrometry. This value was 56.3 ± 1.8% when the analysis was carried out by gas chromatography. The specific activity of esterified linoleate in heart and kidney phospholipids was similar, but in both cases statistically lower than in liver phospholipids. As was the case with liver, the addition of unlabeled (n-6) acids to the diet did not alter the specific activity of esterified linoleate.

When rats were fed 18:2(n-6)-d_4, the data in **Table 5** show that 33.9 ± 1.1 mol % of the 20:4(n-6) in liver phospholipids was labeled. When unlabeled 18:3(n-6), 20:3(n-6), or 20:4(n-6) were included in the diet, there was a reduction in the molar fraction of labeled 20:4(n-6) in liver phospholipids. These values were 27.1 ± 0.5, 23.9 ± 1.4, and 24.1 ± 1.1 mol %, respectively, versus 33.9 mol % when 18:2(n-6)-d_4 was the only dietary

TABLE 4. Molar fraction in percent of total esterified linoleate in liver, heart, and kidney phospholipids that is deuterium-labeled as influenced by feeding rats 18:2(n-6)-d_4 with or without other (n-6) acids

Dietary Acids	Liver	Heart	Kidney
18:2(n-6)-d_4	56.8 ± 4.2	40.8 ± 0.9[a];	44.5 ± 2.0[a]
18:2(n-6)-d_4 18:3(n-6)-d_0	52.9 ± 2.9	43.7 ± 4.7[a]	43.7 ± 3.2[a]
18:2(n-6)-d_4 20:3(n-6)-d_0	55.9 ± 4.1	42.6 ± 1.2[a]	43.7 ± 3.5[a]
18:2(n-6)-d_4 20:4(n-6)-d_0	60.2 ± 1.2	41.1 ± 2.4[a]	44.5 ± 1.2[a]

Male weanling Sprague-Dawley rats were fed the experimental diet for 4 weeks. An equal amount of the unlabeled acid was then replaced by the deuterium-labeled analog and the animals were killed 4 days later. The results are averages from three animals ± SEM.

[a]Statistically different from liver, $P < 0.03$.

TABLE 5. Molar fraction in percent of total esterified arachidonate in liver, heart, and kidney phospholipids that is deuterium-labeled as influenced by feeding rats labeled and unlabeled (n-6) acids

Dietary Acids	Liver	Heart	Kidney
18:2(n-6)-d_4	33.9 ± 1.1	8.9 ± 0.3	13.3 ± 0.6
18:2(n-6)-d_4 18:3(n-6)-d_0	27.1 ± 0.5	9.9 ± 1.1	12.6 ± 2.0
18:2(n-6)-d_0 18:3(n-6)-d_4	24.6 ± 1.2	8.5 ± 0.6	10.6 ± 1.2
18:2(n-6)-d_4 20:3(n-6)-d_0	23.9 ± 1.4	12.5 ± 1.4	12.4 ± 0.8
18:2(n-6)-d_0 20:3(n-6)-d_4	22.0 ± 1.2	10.6 ± 0.6	12.6 ± 1.6
18:2(n-6)-d_4 20:4(n-6)-d_0	24.1 ± 1.1	6.8 ± 1.3	8.8 ± 1.2
18:2(n-6)-d_0 20:4(n-6)-d_4	26.2 ± 2.3	13.0 ± 0.9	16.8 ± 1.5

Male weanling Sprague-Dawley rats were fed the experimental diet for 4 weeks. An equal amount of the unlabeled acid was then replaced by the deuterium-labeled analog and the animals were killed 4 days later. The results are averages from three animals ± SEM.

(n-6) acid. When rats were fed 18:2(n-6)-d_4 and unlabeled 20:3(n-6), 20:4(n-6) was also isolated by HPLC. When it was derivatized and analyzed by gas chromatography-mass spectrometry, the molar fraction of labeled 20:4(n-6) was 22.0 ± 1.2%. This value is in close agreement with the value of 23.9 ± 1.4 that was obtained when an aliquot of the entire sample was analyzed directly by gas chromatography-mass spectrometry. The results show that addition of (n-6) acids to the diet, beyond the rate-limiting 6-desaturase step, was equipotent in depressing the amount of 18:2(n-6)-d_4 that was metabolized to yield esterified 20:4(n-6). In the direct crossover studies, where unlabeled 18:2(n-6) was fed with 18:3(n-6)-d_4 or 20:3(n-6)-d_4, it was found that the molar fraction of labeled 20:4(n-6)-d_4 was the same as when 20:4(n-6)-d_4 was included directly in the diet. The results suggest that the metabolism of 18:3(n-6) and 20:3(n-6) to 20:4(n-6), followed by its esterification into liver phospholipids, is a tightly coupled metabolic process.

As these are isotopic crossover feeding studies, it is possible to calculate the molar fraction of labeled 20:4(n-6) in phospholipids when rats were fed only 18:2(n-6)-d_4 versus the combined amount of esterified 20:4(n-6)-d_4 derived from 18:2(n-6)-d_4 plus that produced from the other labeled (n-6) acids. The implicit assumption of the calculations in **Table 6** is that the amounts of labeled and unlabeled (n-6) acids metabolized to yield esterified 20:4(n-6)-d_4 are the same. For example, when 18:2(n-6) was replaced by 18:2(n-6)-d_4, the data in Table 5 show that 33.9 mol % of all the esterified 20:4(n-6) in liver phospholipids was labeled. When animals were preconditioned by including 18:3(n-6) in the diet, followed again by replacing the 18:2(n-6) with 18:2(n-6)-d_4, the molar fraction of deuterium-labeled 20:4(n-6) was 27.1%. When 18:3(n-6) was replaced by 18:3(n-6)-d_4, it was found that 24.6 mol % of esterified 20:4(n-6) in liver phospholipids was labeled. The combined amount of labeled 20:4(n-6) in liver phospholipids is thus equal to that derived from 18:2(n-6)-d_4 (27.1%) plus that produced from 18:3(n-6)-d_4 (24.1%), which is 51.2 mol %. When this value is divided by 33.9 mol %, it can be calculated that the addition of 18:3(n-6) to a diet containing a constant amount of linoleate increased the amount of labeled 20:4(n-6) by a factor of 1.5, i.e., 51.2/33.9 = 1.5. The other values in Table 6 were obtained via an identical calculation using the data from Table 5. The results thus show that addition of 18:3(n-6), 20:3(n-6), or 20:4(n-6) to the diet increased by a factor of from 1.4- to 1.5-fold the molar fraction of labeled 20:4(n-6) without altering the actual amount of 20:4(n-6) in liver phospholipids, i.e., Table 2.

When 18:2(n-6)-d_4 was fed to rats, 40.8 and 44.5 mol %, respectively, of esterified 18:2(n-6) in heart and kidney phospholipids was labeled versus 56.8 mol % in liver phospholipids, i.e., Table 4. Although these values for heart and kidney are less than for liver it appears that an adequate amount of dietary 18:2(n-6) is available in these tissues for metabolism to 20:4(n-6). However, only 8.9 and 13.3 mol %, respectively, of the 20:4(n-6) in heart and kidney phospholipids was labeled versus 33.9 mol % for liver. When unlabeled 18:3(n-6) or 20:3(n-6) were included in the diet, there was little or no reduction in the specific activity of esterified 20:4(n-6), i.e., Table 5. These results, unlike those for liver, show that dietary

TABLE 6. Effects of adding 18:3(n-6), 20:3(n-6), or 20:4(n-6) to the diet on the specific activity of esterified arachidonate in liver, heart, and kidney phospholipids

	Liver	Heart	Kidney
$\frac{18:2(n-6)\text{-}d_4 + 18:3(n-6)\text{-}d_4}{18:2(n-6)\text{-}d_4}$	1.5	2.1	1.7
$\frac{18:2(n-6)\text{-}d_4 + 20:3(n-6)\text{-}d_4}{18:2(n-6)\text{-}d_4}$	1.4	2.6	1.9
$\frac{18:2(n-6)\text{-}d_4 + 20:4(n-6)\text{-}d_4}{18:2(n-6)\text{-}d_4}$	1.5	2.2	1.9

The values in the numerator for 18:2(n-6)-d_4 represent the molar fraction of deuterium-labeled 20:4(n-6) in phospholipids when the diets contained unlabeled 18:3(n-6), 20:3(n-6), or 20:4(n-6). The values for 18:3(n-6)-d_4, 20:3(n-6)-d_4, and 20:4(n-6)-d_4 are the molar fraction of deuterium-labeled 20:4(n-6) in phospholipids when unlabeled 18:2(n-6) was fed along with the deuterium-labeled 18:3(n-6), 20:3(n-6), or 20:4(n-6). The value for 18:2(n-6)-d_4 in the denominator is the molar fraction of labeled 20:4(n-6) in phospholipids when 18:2(n-6)-d_4 was the only dietary (n-6) acid.

(n–6) acids do not depress the mol % of 20:4(n–6)-d_4 esterified into the two extrahepatic tissues. When labeled 18:3(n–6), 20:3(n–6), or 20:4(n–6) were now included in the diet along with unlabeled 18:2(n–6), the mol % of labeled 20:4(n–6) was always approximately equal to, or greater, than when 18:2(n–6)-d_4 was the only dietary (n–6) acid, i.e., Table 5. Indeed, the results in Table 6 show that the addition of (n–6) acids to the diet increased the specific activity of 20:4(n–6)-d_4 from 1.7- to 2.6-fold above that when 18:2(n–6)-d_4 was the only dietary (n–6) acid. As with liver phospholipids, this higher specific activity was not due to an increase in the amount of esterified 20:4(n–6) in either heart or kidney phospholipids, i.e., Table 3.

DISCUSSION

The animals in these studies were all fed a defined diet for 4 weeks prior to the incorporation of a deuterium-labeled fatty acid into the diet. As the deuterium-labeled compounds simply replaced an equal amount of the unlabeled acid, the observed compositional and isotopic changes represent whole-body metabolism under steady-state conditions. With all diets, the amount of arachidonate in tissue phospholipids was similar to that in rats fed chow. The absence of esterified 20:3(n–9), in phospholipids shows that these animals are not essential fatty acid-deficient. All the experimental diets contained 1% by weight of linoleate. When we extracted Purina chow it was found to contain 5% fat on a weight basis. Fatty acid compositional analysis showed that it contained 33% linoleate. The chow-fed animals thus received about 1.6% by weight of linoleate versus 1% in the experimental groups. The higher levels of esterified 18:2(n–6) in tissue phospholipids of rats fed chow, versus the experimental animals, may be due to a somewhat higher dietary level of linoleate in chow. The reduced level of 18:2(n–6) in phospholipids is accompanied by increases in both 18:1(n–9) and 18:1(n–7).

When 18:2(n–6)-d_4 was fed the specific activity of esterified 18:2(n–6) in liver phospholipids was 1.7-times greater than for 20:4(n–6). These differences in specific activity could be explained in two possible ways. If de novo phospholipid biosynthesis, and the various pathways for remodeling phospholipids collectively were slow, relative to the rate of 20:4(n–6) biosynthesis, it is possible that the specific activity of the intracellular 20:4(n–6) free fatty acid pool might be similar to that of 18:2(n–6). This model assumes that dietary 18:2(n–6)-d_4 mixes with endogenous unlabeled 18:2(n–6) to establish a specific activity of linoleate. This common pool of linoleate is then used both for phospholipid biosynthesis and for the production of 20:4(n–6) followed by its incorporation into phospholipids. According to this model the specific activity of esterified linoleate should be similar to that of arachidonate, which obviously was not the case. The second model again assumes that dietary 18:2(n–6)-d_4 mixes with endogenous 18:2(n–6) to establish a specific activity of intracellular 18:2(n–6), which again is used directly for phospholipid synthesis and the production of 20:4(n–6). If production of arachidonate, via the 6-desaturase-mediated reaction, is slower than are the reactions used to incorporate 20:4(n–6) into phospholipids, the specific activity of esterified 18:2(n–6) would exceed that of arachidonate. This type of labeling pattern was observed in this study, as well as when linoleic acid, labeled with deuterium at the double bonds, was fed to rats (16). The implication of this model is that, under steady-state conditions, there must be considerable recycling of arachidonate, i.e., decylation–reacylation, in order to maintain the amount of esterified arachidonate at a constant level. When rats were maintained on a fat-free diet, the total liver lipids contained 1.4% of 18:2, but it was all 18:2(n–7). The liver lipids still retained 4.2% 20:4(n–6) (19). As there is little, if any, 18:2(n–6) available for 20:4(n–6) biosynthesis, these compositional studies suggest that this level of esterified 20:4(n–6) was maintained by recycling. Rates of turnover of 18:2(n–6) and 20:4(n–6) in phospholipids differ from one molecular species to another but overall rates of turnover of these two fatty acids are similar (20). It does not appear that a slower rate of turnover of 20:4(n–6) versus 18:2(n–6) explains the observed differences in specific activity for esterified 18:2(n–6) versus 20:4(n–6). Obviously additional experiments are required to systematically address this question.

When 18:3(n–6), 20:3(n–6), or 20:4(n–6) were included in the diet there was always a reduction in the amount of 18:2(n–6)-d_4 that was metabolized to esterified 20:4(n–6)-d_4 in liver phospholipids. The addition of these acids to the diet did not increase the amount of esterified 18:2(n–6) nor did it increase the specific activity of esterified 18:2(n–6). An increase in the specific activity of esterified 18:2(n–6) might be expected if (n–6) acids, beyond the 6-desaturase step, inhibited the biosynthesis of 20:4(n–6) to make more 18:2(n–6)-d_4 available for esterification. The addition of polyunsaturated fatty acids to the diet down-regulates the amounts of both fatty acid synthetase (21) and the 9-desaturase (22). It is possible that the addition of (n–6) acids also down-regulated 6-desaturase activity. It is also possible that the activity of this enzyme was not affected by low levels of dietary (n–6) acids but that the combined synthesis of 20:4(n–6) from 18:2(n–6)-d_4 plus that produced from the other (n–6) acids resulted in the production of more 20:4(n–6). Indeed it was observed, in the direct cross-over studies, that the addition of 18:3(n–6)-d_4, 20:3(n–6)-

d_4, and 20:4(n–6)-d_4 to the diet resulted in the production of from 1.4- to 1.5-fold more esterified labeled 20:4(n–6) versus when rats were fed only 18:2(n–6)-d_4. These isotopic changes did not alter the actual amount of esterified 20:4(n–6) in liver phospholipids. These findings suggest that dietary (n–6) acids, beyond the 6-desaturation step, have the potential of modifying the total amount of 20:4(n–6) made in liver, but they cannot be detected by measuring the amounts of esterified fatty acid.

When (n–6) acids beyond the 6-desaturase step are added to the diet, one of their effects may be to make more 20:4(n–6) available for transport to extrahepatic tissues, versus when 18:2(n–6) is the only dietary (n–6) acid. When 18:2(n–6)-d_4 was fed, the relative specific activity of esterified 18:2(n–6)-d_4 in heart and kidney phospholipids was 72 and 78%, respectively, of that in liver phospholipids. It is not known whether this 18:2(n–6) is taken up directly by these tissues or whether it is processed by liver for subsequent transport to heart and kidney. In any case, it appears that adequate 18:2(n–6) is available in heart and kidney for 20:4(n–6) biosynthesis. However, the relative specific activity of esterified 20:4(n–6) in heart and kidney phospholipids was only 26 and 39%, respectively, of that in liver phospholipids. The results suggest that 18:2(n–6) may be metabolized to 20:4(n–6) in liver which is then transported to heart and kidney. When labeled 18:3(n–6), 20:3(n–6), or 20:4(n–6) were included in the diet there was an increase in the specific activity of esterified 20:4(n–6) in liver phospholipids of from 1.4- to 1.5-fold above that when 18:2(n–6) was fed alone. The molar fraction of esterified labeled 20:4(n–6) in heart and kidney phospholipids increased by a factor of from 1.7 to 2.6 when labeled 18:3(n–6), 20:3(n–6), or 20:4(n–6) were included in the diet versus when 18:2(n–6)-d_4 was fed alone. These studies thus show that dietary (n–6) acids, beyond the 6-desaturation step, modify the specific activity of esterified 20:4(n–6) in various tissues in different ways. If (n–6) acids must be metabolized to 20:4(n–6) in liver, our results suggest that newly synthesized 20:4(n–6) may be preferentially exported to extrahepatic tissues rather than being used for liver membrane lipid biosynthesis. None of the dietary manipulations described in these studies altered the actual amount of arachidonate esterified in membrane phospholipids. The addition of 18:3(n–6), 20:3(n–6), and 20:4(n–6) to the diet resulted in the synthesis of more 20:4(n–6) than when 18:2(n–6) was fed alone. It remains to be determined whether this type of dietary intervention has the potential of mediating physiological processes via pathways that are modified by possible increases in the level of 20:4(n–6) in the free fatty acid pool.

These studies were supported by National Institutes of Health grant DK20387.

Manuscript received 7 December 1994, in revised form 14 March 1995, and in re-revised form 8 June 1995.

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