Conversion of α-linolenate to docosahexaenoate is not depressed by high dietary levels of linoleate in young rats: tracer evidence using high precision mass spectrometry

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Abstract The conversion of α-linolenate (18:3n-3) to docosahexaenoate (22:6n-3) in the presence of low and high dietary levels of linoleate (18:2n-6) is reported in young rats using [U-^{13}C]-α-linolenic acid (18:2n-3*) and high precision gas chromatography-combustion isotope ratio mass spectrometry (GCC-IRMS). After consuming an 18:3n-3-deficient diet for 4 weeks, dams were bred and assigned to one of three diet groups: *a)* 2 g 18:3n-3/kg diet and 17 g 18:2n-6/kg diet (Lo-18:2), *b)* 2 g 18:3n-3/kg diet and 140 g 18:2n-6/kg diet (Hi-18:2), or *c)* essential fatty acid-deficient diet (EFAD). Pups were weaned to the maternal diets. At 42 days of age, pups were gavaged with 1 mg 18:3n-3*, and killed 48 h later. Fatty acid composition of liver reflected the diets to a greater extent than did the brain, and 22:5n-6 replaced 22:6n-3 in the brain. About 80% of the label in liver, brain, and plasma was found as 22:6n-3* for the replete groups. The enrichment pattern was similar in liver and plasma except for 18:3n-3, which was higher in liver. Total label detected was 4-fold higher in the EFAD livers and 2-fold higher in the EFAD brains than in the other groups, which were indistinguishable. Conversion of 18:3n-3* to 22:6n-3* was greater in livers from the Hi-18:2 group than from the Lo-18:2 group ($P < 0.05$). Estimates of overall label recovery in liver and brain were consistent with literature values. These data indicate that high dietary levels of 18:2n-6 do not inhibit conversion of a single dose of 18:3n-3 to 22:6n-3 in young rats, and demonstrate the applicability of high precision GCC-IRMS to fatty acid tracer studies.—**Sheaff, R. C., H-M. Su, L. A. Keswick, and J. T. Brenna.** Conversion of α-linolenate to docosahexaenoate is not depressed by high dietary levels of linoleate in young rats: tracer evidence using high precision mass spectrometry. *J. Lipid Res.* 1995. **36:** 998-1008.

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The content and composition of unsaturated fatty acids in the brain changes substantially during development and is important to proper function later in life (1). The overall fatty acid content of the human brain increases 4- to 5-fold during maturation (2), to an adult level of 10% lipid by weight, with concurrent changes in fatty acid composition (3, 4). Over half of this lipid is in the form of structural glycerophospholipids that consist primarily of long-chain polyunsaturated fatty acids (LC-PUFA) (5, 6). The most abundant LC-PUFA in the brain, docosahexaenoic acid (22:6n-3), constitutes 17% of the brain by weight, followed by arachidonic acid (20:4n-6) (2, 7). The developing human fetus acquires most brain lipid during the brain growth spurt, which lasts from the beginning of the third trimester until approximately 18 months after birth (8), and is the period of greatest accumulation of 22:6n-3 (2). The rapid prenatal increase in 22:6n-3 in neural tissues slows slightly after birth, but continues to be significant until 2 years of age (3).

In adults, it is well known that 22:6n-3 can be obtained either by biosynthesis involving sequential desaturation and elongation of dietary α-linolenic acid (18:3n-3), or as preformed 22:6n-3 present in marine sources. Animal and human studies indicate that a source of n-3 fatty acids, and possibly preformed 22:6n-3 under some circumstances, is required in the perinatal period for proper neural and retinal development (9-18).

In vitro, linoleic acid (18:2n-6) and 18:3n-3 compete for desaturases and elongation enzymes during synthesis of their respective long chain functional derivatives (19). In vivo, it is known that the dietary ratio of 18:2n-6/18:3n-3, as well as the absolute levels, influences membrane n-6 and n-3 fatty acid profiles (16, 20), which are the principle storage/function sites for these compounds. High 18:2n-6/18:3n-3 can impair prostaglandin E synthesis (21, 22) and decrease 22:6n-3, while increas-

Abbreviations: GCC-IRMS, gas chromatography-isotope ratio mass spectrometry; EFAD, essential fatty acid-deficient; LC-PUFA, long-chain polyunsaturated fatty acids; FAME, fatty acid methyl esters; APE, atom percent enrichment.

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ing tissue 22:5n-6 levels (10-12, 23-25). In animals, the dietary 18:2n-6/18:3n-3 ratio and absolute 18:3n-3 levels (0-0.4%) influence brain and retinal 22:6n-3 levels (10, 11, 26, 27). In humans, term and preterm infants fed formulas with high 18:2n-6/18:3n-3 levels had reduced erythrocyte 22:6n-3 levels (16, 28, 29). This work suggests that the dietary 18:2/18:3 ratio, as well as the absolute 18:3n-3 level, affects the accretion of 22:6n-3 in developing neural tissues. However, there are no published tracer investigations of the effect of dietary 18:3n-3 and 18:2n-6 levels on organ-specific incorporation and conversion of 18:3n-3 to 22:6n-3.

Over recent years, we (30, 31) and others (32) have developed a novel approach to stable isotope tracers based on labeling with ^{13}C and analysis by gas chromatography (GC) coupled to isotope ratio mass spectrometry (IRMS) via a combustion interface. This approach has several attractive features for biomedical studies, including high sensitivity and compound-independent detection. We have applied this method to investigate the relative conversion of 18:3n-3 to 22:6n-3 in young rats maintained on purified diets with constant 18:3n-3 levels and low or high 18:2 levels, or an essential fatty acid-deficient diet (EFAD) to detect the physiological response to EFA stress. We report here the results of this investigation along with some of the issues specific to the application of this methodology to biotracer experiments.

MATERIALS AND METHODS

Diet formulation

All diets were based on the non-fat AIN-76A formulation (Dyets, Inc, Bethlehem, PA). The 18:3n-3 and 18:2n-6 contents for the three experimental diets were as follows: *a)* 2 g 18:3n-3/kg diet and 17 g 18:2n-6/kg diet (18:2/18:3 = 9; Lo-18:2), *b)* 2 g 18:3n-3/kg diet and 140 g 18:2n-6/kg diet (18:2/18:3 = 69; Hi-18:2), or *c)* essential fatty acid-deficient diet (EFAD). Hydrogenated coconut oil, safflower oil (source of 18:2n-6), and linseed oil (source of 18:3n-3) were blended to achieve a constant 36% en (200 g/kg), typical of a western diet. Each diet was analyzed after $CHCl_3$-MeOH extraction followed by saponification/methylation as described below. **Table 1** shows the oil composition (in g/kg) and fatty acid profile of each diet. The sum "18:2n-6 + saturated fatty acids" was held constant to maintain identical fat levels among all three groups. A fourth diet with 18:2n-6 levels identical to the Lo-18:2 diet but with no detectable 18:3n-3, was prepared for depletion of dams prior to breeding. All diets included 100 IU α-tocopherol/kg and 0.02% *t*-butylhydroquinone (TBHQ) as antioxidants.

The constant 18:3n-3 content of 2 g/kg diet (0.36% en) in the two replete diets is near the lower level required to prevent development of deficiency symptoms (33, 34), and was chosen to provide adequate n-3 without excess 18:3n-3 available for general energy or as a C source. The 18:2n-6 levels corresponded to about 3% and 26% of energy for the Lo-18:2 and Hi-18:2 diets, respectively.

TABLE 1. Fat blends (g/kg) and quantitative fatty acid profiles (% of total fatty acids) for Lo-18:2, Hi-18:2, and EFAD diets

Composition	Lo-18:2	Hi-18:2	EFAD
		g/kg	
Safflower oil	24.99	195.52	0
Linseed oil	4.08	4.08	0
Hydrog. coconut oil	170.93	0	200
Fatty acid		%	
10:0	1.3	0.0	4.8
12:0	33	1.1	26
14:0	31	1.4	24
16:0	16	10	26
18:0	7.4	2.4	19
18:1	1.8	11.5	0.25
18:2n-6	8.3	72	ND
18:3n-3	0.92	1.05	ND
18:2/18:3	9	69	

ND, not detectable.

Tracer doses

Tracer doses of [U-^{13}C]18:3n-3 (18:3n-3*) were purified from a U-^{13}C-labeled algal oil (Martek, Columbia, MD) in a two-step method. First, fatty acid methyl esters (FAME) were separated by degree of unsaturation using a scaled-up version of the method of Christie (35). Briefly, solid phase extraction columns containing Ag^{2+}-loaded benzenesulfonic acid sorbent (Varian, Harbor City, CA) are loaded with the FAME mixture. FAME were eluted by degree of unsaturation using mobile phases of graded polarity, made up of mixtures of dichloromethane, acetone, and acetonitrile. The particular algal oil used for these studies contained substantial quantities of 16:3n-3, so a second step based on preparative high performance liquid chromatography (HPLC) was used to separate fatty acids based on chain length. An SSI (State College, PA) HPLC equipped with a 4.6 cm (I.D.) ODS-Hypersil preparative column (Keystone Scientific, Bellefonte, PA) was used with 100% acetonitrile (isocratic) mobile phase. Dose purity of 99+% was verified by capillary GC. Purified methyl-18:3n-3* was hydrolyzed to the free fatty acid and sonicated into 10% lipid emulsion at about 1 mg 18:3n-3*/ml. Overall dose isotopic enrichment was determined to be 95% ^{13}C.

Animals

A timeline for the experimental protocol is shown in **Fig. 1.** Five-week-old female Sprague-Dawley rats were obtained from Charles River Breeding Laboratories (Wilmington, MA) and housed individually in a temperature-

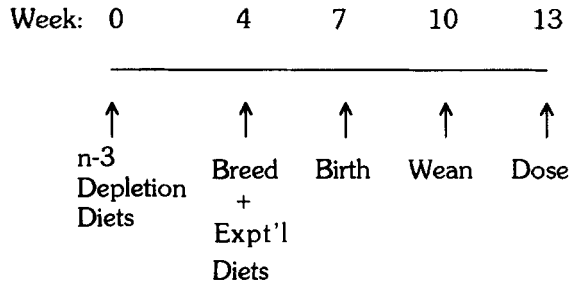

Fig. 1. Experimental time line for the three diet groups. All dams were placed on an n-3-deficient diet at week 0. At 4 weeks, dams were bred and placed on one of three experimental diets: two repletion diets Lo-18:2 and Hi-18:2, or the essential fatty acid-deficient diet (EFAD). Pups were weaned to the diet of their dam and killed at 44 days of age.

and humidity-controlled room with a 12-h light/dark cycle. The fatty acid composition of chow diets consumed by the dams prior to shipment is variable, and thus a lead-in diet was provided to deplete body stores of excess n-3 fatty acids. For 4 weeks, dams were fed the 18:3n-3 depletion diet ad libitum, which maintained adequate levels of 18:2n-6 to insure reproductive capacity. It is unlikely that 4 weeks on a low n-3 diet is sufficient to produce evidence of deficiency in adult rats (33). Dams were bred with chow-fed male Sprague-Dawley rats and conception was verified by the presence of sperm plugs, weight gain, and vaginal smears. At conception, dams were randomly placed into one of three dietary treatments, with six rats in each group. One group was placed on the EFAD diet, while the other groups received one of two essential fatty acid repletion diets, Lo-18:2 or Hi-18:2. The pups were weaned to the diet of the dam and were provided food and water ad libitum. At 42 days of life and after a 4-h fast, 1 mg [U-^{13}C]18:3n-3 sonicated into about 1 ml 10% Intralipid carrier was administered by gavage to four pups of each group of six. The remaining two pups were gavaged with unspiked Intralipid and served as baseline isotope controls. Forty-eight hours later, the pups were weighed, anesthetized with CO_2, and killed by decapitation. Liver, brain, and plasma from all six pups in each group were harvested, weighed, and stored at −80°C under N_2 until analysis.

Compositional analysis

Compositional analyses of liver, brain, and plasma fatty acids were performed by standard methods. Briefly, total lipids were extracted from an aliquot of tissue homogenate by the method of Bligh and Dyer (36). Total lipids were saponified by adding 1.0 ml 0.5 N methanolic NaOH and heating at 60°C for 10 min. FAME derivatives were prepared by adding 1.0 ml 14% BF_3 in methanol and heating at 100°C for 2 min. Hexane (1.0 ml) was then added, tubes were vortexed, and placed in the boiling water bath for an additional minute. After 1.0 ml of a saturated NaCl solution was added, tubes were centrifuged at 3000 rpm, and the top hexane layer containing the FAME was transferred to another tube. Methyl heptadecanoate (Me17:0; 99%+, Sigma Chemicals) was added as an internal standard, hexane was evaporated by a stream of N_2, and samples were resuspended in a metered amount of hexane for analysis. Butylated hydroxytoluene (BHT) was added to solvents as an antioxidant. FAME were analyzed using a Carlo Erba 5160 Mega series GC with a J&W DB23 fused silica capillary column (30 m, 0.32 mm i.d., 0.25 mm film) with H_2 carrier gas. Response factors for each FAME were determined using a standard mixture, and final values are expressed as % total fatty acids by weight.

Tracer analysis

Sample preparation for isotopic analysis was identical to that for standard GC analysis outlined above. Tracer analysis was performed using a technique combining highly enriched uniformly labeled tracers and analysis by GC-combustion isotope ratio mass spectrometry (GCC-IRMS), which has been discussed in detail elsewhere (30). Briefly, the effluent of a Varian 3400 capillary GC was swept by He carrier into a furnace held at 850°C and loaded with a solid source of O_2. Organic compounds entering the furnace were quantitatively converted to CO_2 and H_2O, and swept through a water trap that maintains chromatographic resolution. Dried CO_2 was then admitted to the ion source of a Finnigan 252 high sensitivity, high precision IRMS instrument, with dedicated detectors/electronics for each of the major masses of CO_2^+, m/z = 44, 45, and 46, and an absolute sensitivity of about 10^3 molecules/ion detected. Chromatograms from the three channels were acquired continuously during the GC run. Peaks were identified in the m/z = 44 channel, extrapolated to the other channels, and isotope ratios corresponding to R = [^{13}C]/[^{12}C] were calculated using the m/z = 46 signal to adjust for the contribution of the ^{17}O-substituted CO_2 appearing at m/z = 45. The average analytical precision of these measurements was about CV = 0.05% (= 500 ppm).

High precision IRMS data is usually expressed as the relative deviation from the international standard Pee Dee Belemnite (PDB), a carbonate relatively rich in ^{13}C with an isotope ratio defined as R = 0.0112372, using

$$\delta^{13}C_{PDB} = \left(\frac{R_{SPL} - R_{PDB}}{R_{PDB}}\right) \times 10^3 = \left(\frac{R_{SPL} - 0.0112372}{0.0112372}\right) \times 10^3 \quad \textit{Eq. 1)}$$

where $\delta^{13}C_{PDB}$ is referred to as "permil" (o/oo), and R is defined as above. This notation is less useful for tracer studies where atom fraction, or more commonly, atom percent (AP), are more convenient than ratio-based ex-

pressions. Here, we present AP or atom percent enrichments (APE) calculated by subtracting baseline isotope levels, as the index of enrichment level. This procedure also has the effect of correcting for the contribution to isotope ratio made by the methyl C added in derivatization, so no separate correction was necessary.

APE is analogous to specific activity in radiotracer experiments in that the APE responds to both the levels of tracer and tracee in the pool. Two pools in equilibrium for a particular tracee will have identical APEs.

The overall concentration of tracer per unit weight of tissue can be found by multiplying the APE by the concentration of tracee per unit tissue. This quantity, designated "total label", defines the overall conversion of labeled tracer to a particular metabolite in a tissue, and is the quantity required to test the hypothesis that overall conversion levels depend on diet. For 20 or 22 C n-3 fatty acids, only the 18 C atoms derived from the 18:3* are labeled. Therefore, a correction is required to adjust for the endogenous C added during chain elongation to permit direct comparison of conversion levels between fatty acid metabolites. This correction has been applied to the data presented here.

Fisher's least significant difference (LSD) test using the Bonferroni correction (37) were used for tests of differences, with $P < 0.01$ considered significant unless indicated otherwise.

RESULTS

Compositional

Weights. Body, liver, and brain weights for the three groups are presented in **Table 2.** The mean weight of the EFAD pups was significantly lower ($P < 0.05$) than that of the replete pups. The liver weights were indistinguishable while the brain weights were all significantly different (Hi-18:2 > EFAD > Lo-18:2). We attribute this latter, unexpected, observation to statistical fluctuation. In any case, it does not influence conclusions about conversion as all comparisons are made on a per mg tissue dry weight basis.

Fatty acids. Quantitative fatty acid profiles for liver, brain, and plasma for the three groups are presented in **Table 3, Table 4,** and **Table 5.** In all tissues the overall level of unsaturates remained approximately constant as fatty acid composition changed in response to diet. In the liver (Table 3) and plasma (Table 5), the Hi-18:2 diet caused a significant decrease in the total n-3 fatty acids compared to the Lo-18:2. The significantly lower level of 22:6n-3 in the Hi-18:2 group was matched by a reciprocal increase in 22:5n-6 as reported previously (20, 38, 39). Total n-6 fatty acids were significantly higher in the Hi-18:2 livers than the Lo-18:2 livers, with significant increases in 18:2n-6, 20:4n-6, and 22:5n-6. The total monounsaturated fatty acid in the Hi-18:2 group was

TABLE 2. Weight of animals, livers, and brains

Diet Group	Body Weight	Liver	Brain
		g	
Lo-18:2	125.43 ± 18.81^{ab}	6.69 ± 1.35	1.65 ± 0.07^{a}
Hi-18:2	122.05 ± 9.97^{a}	5.65 ± 0.76	1.94 ± 0.08^{b}
EFAD	106.75 ± 8.56^{b}	6.12 ± 0.58	1.79 ± 0.09^{c}

Values are given as means ± 95% confidence interval. Values with different superscripts are significantly different ($P < 0.01$) from the others in the column.

significantly lower than the Lo-18:2 liver, as might have been expected based on the predominance of dietary 18:2n-6 in the Hi-18:2 group. The EFAD diet had significantly lower total n-3 and n-6 fatty acids compared to the replete groups, as expected. However, total unsaturated fatty acid level was maintained by the significant increase in 20:3n-9 (known as Mead acid) and total monounsaturated fatty acids. The triene/tetraene (T/T) ratio in the EFAD liver was 1.76 indicating biochemical essential fatty acid deficiency (40). Essential fatty acids in the EFAD pups presumably arise from residual maternal stores mobilized during the suckling period.

The brain was more resilient to the dietary treatments (Table 4). Total brain saturates, monounsaturates, and n-6 fatty acids were not different among the groups. The total unsaturated fatty acids, and sum (22:6n-3 + 22:5n-6) were similar between the two replete groups, while the total n-3 fatty acids were significantly lower in the Hi-18:2 group compared to the Lo-18:2 group. This decrease, as in the liver, was primarily due to a significant decrease in 22:6n-3. The decrease in 22:6n-3 in the Hi-18:2 brain was matched by a significant increase in 22:5n-6. In the EFAD brain, the total n-3 was significantly lower than the two replete groups, as expected. The sole n-3 fatty acid detected was 22:6n-3, suggesting quantitative conversion of any n-3 fatty acid reaching the brain. Although the total n-6 level in the EFAD brain was not significantly different from the two replete groups, 18:2n-6 was significantly lower and 22:5n-6 was significantly higher, suggesting enhanced conversion of n-6 fatty acids to 22:5n-6 to maintain adequate LC-PUFA levels in the brain or greater transfer of 22:5n-6 to the pup during suckling. Measurements in progress of milk fatty acid composition indicate very low levels of 22:5n-6 and suggest the former explanation (H-M. Su, L. A. Keswick, and J. T. Brenna, unpublished results). Levels of 20:3n-9 again indicated deficiency for the EFAD brain, with T/T = 0.76, which is lower than that observed for the liver. These data confirm that 22:6n-3 is the preferred LC-PUFA in the brain, but if unavailable, 22:5n-6 will be synthesized in a reciprocal manner.

Dietary manipulation resulted in significant changes in 18:2n-6 content for liver and brain, with a consistent but

TABLE 3. Fatty acid composition of livers from animals on different diets

Fatty Acid	Lo-18:2	Hi-18:2	EFAD
		weight %	
14:0	7.63 ± 0.4[a]	0.66 ± 0.09[b]	6.05 ± 0.41[a]
16:0	18.44 ± 1.80	21.65 ± 0.34	19.71 ± 0.71
18:0	15.74 ± 0.90	17.13 ± 0.62	14.35 ± 0.18
Σ Saturated	41.80 ± 2.35	39.44 ± 0.77	40.10 ± 1.18
14:1	0.32 ± 0.14[a]	0.03 ± 0.04[b]	0.49 ± 0.10[a]
16:1n-7	1.89 ± 0.45[a]	0.61 ± 0.12[b]	7.31 ± 0.58[c]
18:1n-9	8.37 ± 0.70[a]	5.01 ± 0.93[b]	13.8 ± 0.63[c]
18:1n-7	2.34 ± 0.25[a]	2.14 ± 0.74[a]	5.61 ± 0.44[c]
Σ Monounsaturated	12.93 ± 1.24[a]	7.80 ± 0.99[b]	27.21 ± 1.25[c]
20:3n-9	0.51 ± 0.12[a]	0.83 ± 0.12[a]	12.92 ± 0.48[c]
18:2n-6	12.6 ± 0.64[a]	18.16 ± 0.8[b]	4.76 ± 0.75[c]
20:3n-6	1.32 ± 0.23	0.35 ± 0.05	1.2 ± 0.24
20:4n-6	16.11 ± 1.14[a]	19.16 ± 0.77[b]	7.34 ± 0.83[c]
22:4n-6	0.61 ± 0.13[a]	1.24 ± 0.16[b]	ND
22:5n-6	1.46 ± 0.34[a]	3.52 ± 0.35[b]	1.8 ± 0.18[a]
Σ n-6	32.1 ± 1.6[a]	42.42 ± 0.48[b]	15.10 ± 1.72[c]
18:3n-3	0.31 ± 0.03	0.23 ± 0.06	ND
20:5n-3	0.37 ± 0.06	ND	0.32 ± 0.05
22:5n-3	1.24 ± 0.31[a]	0.75 ± 0.14[b]	ND
22:6n-3	10.01 ± 1.24[a]	7.4 ± 0.46[b]	2.45 ± 0.52[c]
Σ n-3	11.94 ± 1.57[a]	8.37 ± 0.55[b]	2.78 ± 0.56[c]
20:3n-9/20:4n-6	0.03	0.04	1.76
18:2n-6/18:3n-3	41	79	
n-6/n-3	2.69	5.07	5.43
22:5n-6 + 22:6n-3	11.47	10.92	4.25

Values are expressed as weight % (means ± 95% confidence interval). Values with different superscripts are significantly different from others in the row ($P < 0.01$).

nonsignificant trend in plasma values. The liver 18:2/18:3 ratio was double in the Hi-18:2 group compared to Lo-18:2, while brain levels of 18:3n-3 were too low for reliable quantification. The plasma trend was again nonsignificant but consistent with that of the liver.

Tracer analysis

The sum of ^{13}C-labeled fatty acids in liver, plasma, and brain are presented graphically in **Fig. 2.** Over the 48-h period, the liver of the EFAD pups had incorporated 4-fold more labeled fatty acids than the two replete groups. This suggests a decreased oxidation, or an increased uptake/acylation, of the 18:3* and its n-3 derivatives in the deficient state. The Hi-18:2 diet had no effect on the overall incorporation of label in the liver compared to the Lo-18:2 control.

The results were similar in the brain. The Hi-18:2 and Lo-18:2 groups incorporated similar amounts of labeled fatty acids, while the EFAD brain had incorporated about 2-fold more than the two replete groups over the same time period. Again, this higher level implies more efficient utilization of n-3 fatty acids in the EFAD animals compared to the replete groups. The sum of labeled fatty acids detected in the plasma was not significantly different between the two replete diets, as seen in the other organs. Trace levels of label were detected in the EFA of plasma of the EFAD group, suggesting that the n-3 fatty acids in this group were quickly incorporated into deficient tissues, as observed previously (41).

The atom percent excess (APE) for the n-3 fatty acids is presented in **Table 6.** In the liver, the fatty acid of highest enrichment was the dose, 18:3n-3, followed by 22:5n-3, 22:6n-3 and 20:5n-3. The pattern and absolute levels of plasma enrichment are remarkably similar to that in liver, with the exception of the lower APE for 18:3n-3. This is expected as most of the labeled fatty acids appearing in the plasma at 48 h post-dose will have been secreted directly from the liver. In the brain, no label was detected in 18:3n-3 or 20:5n-3, although substantial enrichment was observed in 22:6n-3 and 22:5n-3 in replete groups.

The extent of conversion of 18:3* to long chain n-3 derivatives was determined by calculating the total label, defined above as the mass of 18:3* found as 18:3* and as

TABLE 4. Fatty acid composition of brain from animals on different diets

Fatty Acid	Lo-18:2	Hi-18:2	EFAD
		weight %	
14:0	1.35 ± 0.07	0.65 ± 0.06	1.82 ± 0.20
16:0	20.16 ± 0.99	21.01 ± 1.29	18.47 ± 0.85
18:0	15.75 ± 0.34	15.37 ± 0.77	14.53 ± 2.41
Σ Saturated	37.27 ± 1.30	37.03 ± 0.78	34.82 ± 3.33
16:1n-7	0.91 ± 0.11[a]	0.83 ± 0.05[a]	1.47 ± 0.14[c]
18:1n-9	13.77 ± 0.53	13.36 ± 0.45	12.56 ± 0.66
18:1n-7	5.75 ± 0.35	5.73 ± 0.22	5.91 ± 0.31
Σ Monounsaturated	23.07 ± 0.51	21.69 ± 0.55	22.59 ± 0.84
20:3n-9	0.54 ± 0.04[a]	0.84 ± 0.08[a]	7.55 ± 0.78[c]
18:2n-6	1.84 ± 0.14[a]	3.13 ± 0.09[b]	0.49 ± 0.09[c]
20:3n-6	0.95 ± 0.12	0.93 ± 0.02	0.70 ± 0.17
20:4n-6	12.32 ± 0.3[a]	12.08 ± 0.14[a]	9.90 ± 0.44[c]
22:4n-6	6.03 ± 0.84[a]	5.6 ± 0.19[a]	3.79 ± 0.54[c]
22:5n-6	2.64 ± 0.4[a]	6.64 ± 0.77[b]	10.10 ± 1.03[c]
Σ n-6	23.79 ± 1.15	28.38 ± 0.91	24.96 ± 1.4
18:3n-3	ND	ND	ND
20:5n-3	ND	ND	ND
22:5n-3	0.53 ± 0.11	0.36 ± 0.14	ND
22:6n-3	14.23 ± 0.68[a]	11.24 ± 0.35[b]	5.73 ± 1.09[c]
Σ n-3	14.76 ± 0.68[a]	11.6 ± 0.36[b]	5.73 ± 1.09[c]
20:3n-9/20:4n-6	0.04	0.07	0.76
18:2n-6/18:3n-3			
n-6/n-3	1.61	2.45	4.36
22:5n-6 + 22:6n-3	16.87	17.88	15.83

Values are expressed as weight % (means ± 95% confidence interval). Values with different superscripts are significantly different ($P < 0.01$) from others in the row.

labeled elongation products per mg tissue. Because the dose to each animal is equivalent, comparison of the masses of label found in each fatty acid reflects relative conversion/incorporation. Results for the major n-3 fatty acids are presented in **Fig. 3.** About 80% of all label detected in fatty acids was found in 22:6n-3.

In the liver, 22:6n-3* as a percent of total label detected in the Hi-18:2 group is greater than that detected in the Lo-18:2 group. This unexpected result indicates that the higher dietary and tissue 18:2n-6 level of the Hi-18:2 group did not depress conversion/incorporation of 18:3* to the long chain n-3 derivatives by 48 h. Levels of 20:5n-3* were below quantifiable levels in the Hi-18:2 group but were present in the Lo-18:2n-6 group. The label in the EFAD liver was distributed between 20:5n-3 and 22:6n-3 at 29 and 71%, respectively, with no detectable 18:3n-3*. This indicates that the EFAD diet induced quantitative conversion of 18:3n-3 to long chain products.

The profile of label detection in the plasma was similar to that of the liver. The Hi-18:2 group had a greater % of label as 22:6n-3 versus the Lo-18:2 liver, although the result was not statistically significant, and again no labeled 20:5n-3 was present in the Hi-18:2 group. Although the levels were too low for reliable quantification, 18:3n-3*, 20:5n-3*, 22:5n-3*, and 22:6n-3* were all detected in EFAD plasma.

In the brain, label in the Lo-18:2 and Hi-18:2 groups was distributed exclusively between 22:5n-3 and 22:6n-3, at 27 and 73%, respectively. As in the liver, the predominance of brain 18:2n-6 in the Hi-18:2 group did not depress conversion at 48 h. In the EFAD brain, all the label was detected in 22:6n-3.

The total labeled fatty acid found in whole organ liver and brain was estimated using measured mean organ wet weights and a literature value for the ratio of dry weight to wet weight for brain of 0.21 (15) and assuming this value for liver. The results expressed as a fraction of the dose are presented in **Table 7.** The values for liver are more than 10-fold greater than those for brain across all diet groups. Values for the replete groups are comparable, while the EFAD group retains 4-fold more label in the liver and 2-fold more in the brain. These results are in

TABLE 5. Fatty acid composition of plasma from animals on different diets

Fatty Acid	Lo-18:2	Hi-18:2	EFAD
		weight %	
14:0	7.40 ± 0.84[a]	0.70 ± 0.25[b]	0.55 ± 0.07[b]
16:0	20.25 ± 1.50	24.25 ± 0.96	15.96 ± 2.63
18:0	16.36 ± 0.39	15.57 ± 1.26	21.53 ± 0.94
Σ Saturated	44.01 ± 2.51	40.52 ± 0.59	38.04 ± 2.45
14:1	0.08 ± 0.1	ND	ND
16:1n-7	1.04 ± 0.38	0.7 ± 0.56	ND
18:1n-9	7.52 ± 1.18[a]	4.46 ± 1.29[b]	22.66 ± 1.93[c]
18:1n-7	1.48 ± 0.22[a]	0.82 ± 0.25[a]	5.41 ± 0.59[c]
Σ Monounsaturated	10.22 ± 1.53[a]	6.01 ± 0.73[b]	28.24 ± 1.72[c]
20:3n-9	0.47 ± 0.06[a]	0.45 ± 0.16[a]	18.04 ± 2.05[c]
18:2n-6	15.92 ± 1.34	20.0 ± 2.42[b]	7.37 ± 1.76[c]
20:3n-6	1.36 ± 0.25[a]	0.22 ± 0.35[b]	1.10 ± 0.86[a]
20:4n-6	16.15 ± 0.65[a]	24.98 ± 3.48[a]	3.41 ± 1.24[c]
22:4n-6	0.60 ± 0.16	1.05 ± 0.38	ND
22:5n-6	1.34 ± 0.51	1.99 ± 0.96	0.77 ± 0.24
Σ n-6	35.39 ± 0.63[a]	48.24 ± 3.81[a]	12.65 ± 7.23[c]
18:3n-3	0.34 ± 0.12	0.22 ± 0.21	ND
20:5n-3	1.36 ± 0.25[a]	0.22 ± 0.36[b]	ND
22:5n-3	1.14 ± 0.19	0.70 ± 0.43	ND
22:6n-3	6.33 ± 1.47[a]	3.86 ± 2.28[a]	0.55 ± 0.24[c]
Σ n-3	8.21 ± 1.83[a]	4.84 ± 3.13[a]	0.55 ± 0.24[c]
20:3n-9/20:4n-6	0.03	0.02	5.29
18:2n-6/18:3n-3	47	91	
n-6/n-3	4.31	9.97	23
Σ 22:5n-6 + 22:6n-3	7.67	5.85	1.32

Values are expressed as weight % (means ± 95% confidence interval). Values with different superscripts are significantly different ($P < 0.01$) from others in the row.

remarkably good agreement with published radiotracer experiments in rats of similar ages and on different dietary regimens (42, 43).

DISCUSSION

The Lo-18:2 diet can be considered a control because it is in the range typical of the western diet and commercial infant formula (44). The Hi-18:2 diet is near the extreme attainable with commonly available natural oils while maintaining 36 en% as fat and 18:3n-3 levels at 2 g/kg diet (0.36 en%), which is sufficient for requirements in growing rats (33). In vitro, 18:2n-6 and 18:3n-3 are observed to compete for Δ^6-desaturation, the first step in the biosynthesis of LC-PUFA (45, 46). By simple analogy, the increased tissue 18:2n-6 concentration in the Hi-18:2 group would have been expected to slow the rate of 18:3* conversion. In vivo tissue compositional studies of dietary 18:2n-6 and 18:3n-3 are consistent with this idea. For instance, early work showed that increasing dietary 18:2n-6 levels inhibited accretion of 18:3n-3-derived LC-PUFA

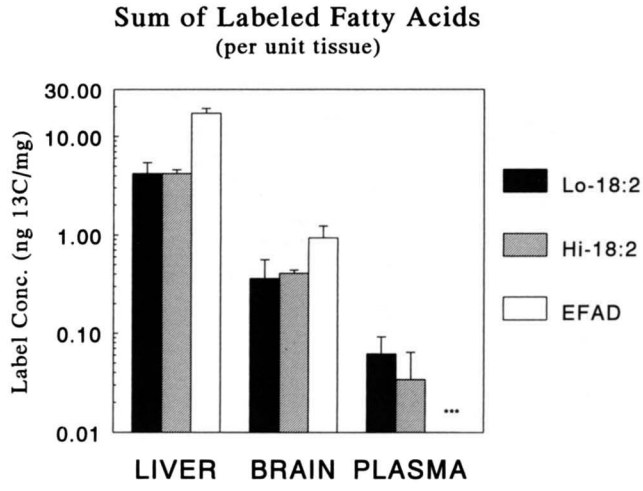

Fig. 2. Sum of labeled fatty acids found in each group for liver, brain (per mg dry weight), and plasma (per ml), plotted in semilog format. Error bars reflect ± 95% confidence interval. Lo-18:2 and Hi-18:2 diet groups are not significantly different in any of these tissues. Labeled fatty acids of EFAD liver and brain are significantly higher than those of the Lo-18:2 and Hi-18:2 groups ($P < 0.01$). Labeled fatty acids of EFAD plasma are below quantifiable limits; ***, below quantifiable levels.

TABLE 6. Atom percent excess (APE) of ^{13}C in n-3 fatty acids of liver, plasma, and brain from animals on different diets

Fatty Acid	Lo-18:2	Hi-18:2	EFAD
Liver			
18:3n-3	0.56 ± 0.18	0.491 ± 0.074	ND
20:5n-3	0.250 ± 0.014[a]	0.22 ± 0.14[a]	9.3352 ± 0.0047[b]
22:5n-3	0.326 ± 0.079	0.372 ± 0.061	ND
22:6n-3	0.26 ± 0.52[a]	0.342 ± 0.039[a]	3.70 ± 0.27[b]
Brain			
18:3n-3	ND	ND	ND
20:5n-3	ND	ND	ND
22:5n-3	0.133 ± 0.055	0.16 ± 0.34	ND
22:6n-3	0.0113 ± 0.0031[a]	0.0152 ± 0.0026[a]	0.1052 ± 0.0098[b]
Plasma			
18:3n-3	0.26 ± 0.14	0.266 ± 0.068	ND
20:5n-3	0.33 ± 0.14	0.259 ± 0.019	ND
22:5n-3	0.355 ± 0.099	0.34 ± 0.53	ND
22:6n-3	0.238 ± 0.077	0.3027 ± 0.0021	ND

Values are expressed as means ± 95% confidence intervals. Values with different superscripts are significantly different ($P < 0.01$) from others in the row; ND, not determined because the concentration of fatty acid was too small to quantify accurately.

(47), while increasing 18:3n-3 levels inhibited accretion of 18:2n-6-derived LC-PUFA (48). A very recent study in adult humans indicates that dietary 18:2n-6 levels alter plasma levels of deuterated 18:2n-6 and 18:3n-3 and their LC-PUFA products, based on area-under-the-curve (AUC) calculations (49). Overall desaturation products of deuterated 18:3n-3 (and 18:2n-6) were about 50% lower when dietary 18:2n-6 was doubled. Aside from possible

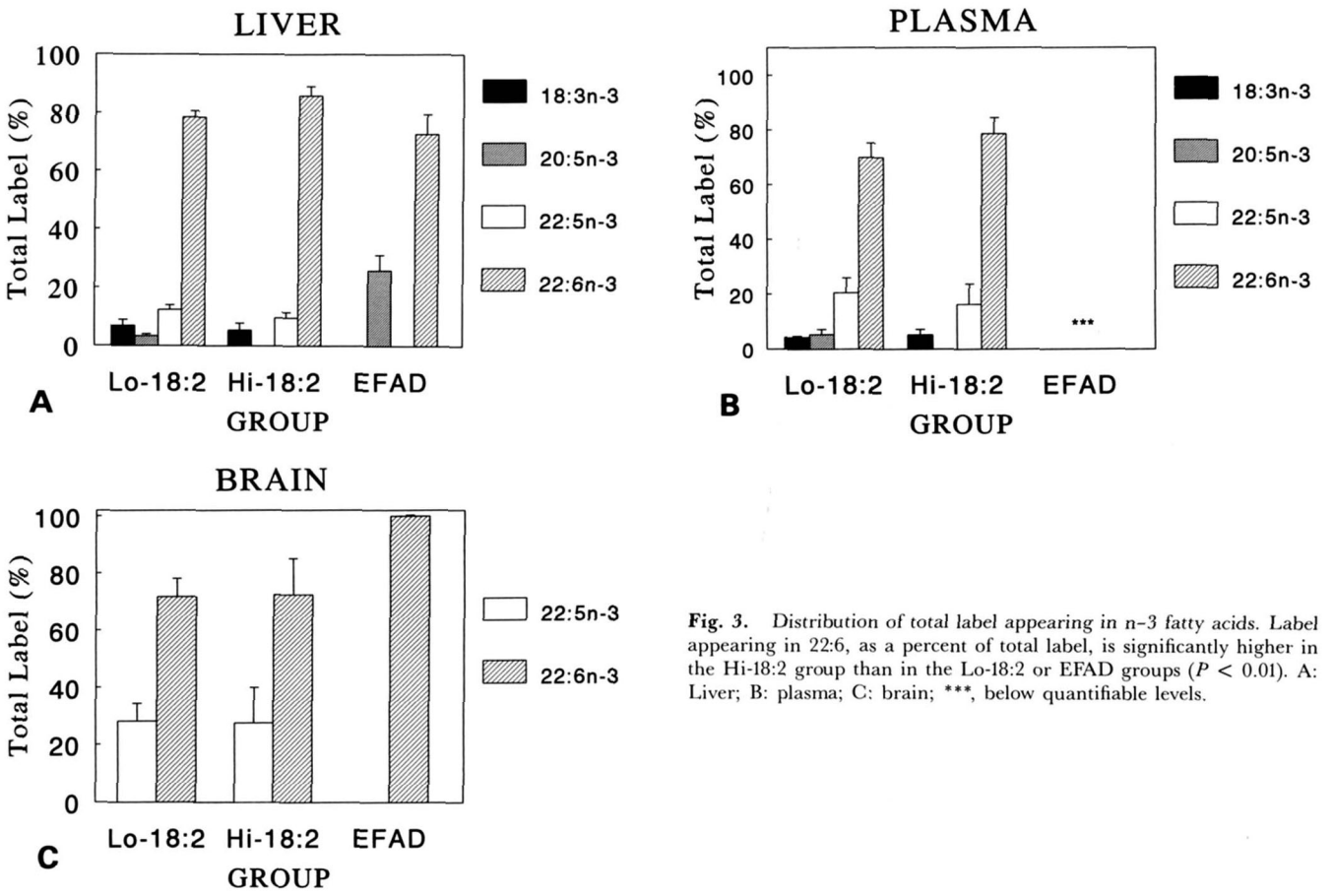

Fig. 3. Distribution of total label appearing in n-3 fatty acids. Label appearing in 22:6, as a percent of total label, is significantly higher in the Hi-18:2 group than in the Lo-18:2 or EFAD groups ($P < 0.01$). A: Liver; B: plasma; C: brain; ***, below quantifiable levels.

TABLE 7. Total label found in fatty acids in whole liver and brain from animals in each dietary group

Tissue	Lo-18:2	Hi-18:2	EFAD
		%	
Liver	0.59	0.50	2.2
Brain	0.013	0.017	0.035

Values are expressed as percentage of the dose.

differences in species, developmental state (i.e., growing rats vs. mature humans), and methodology (organ endpoint vs. plasma AUC), our failure to observe a change in 18:3* to 22:6* conversion between the replete groups may reflect metabolism in organs prior to incorporation, as is implied by differences we observe between plasma and brain 18:3*.

Recently, Lin, Connor, and Anderson (34) have shown in a chick embryo model that n-3 fatty acids are more efficiently incorporated from yolk to chick in embryos of safflower oil-fed hens (high 18:2n-6, low 18:3n-3) than in embryos with a more plentiful source of 18:3n-3. This latter finding is consistent with the present tracer results as it indicates that high 18:2n-6 levels do not inhibit overall n-3 LC-PUFA accretion from available 18:3n-3. The preference of the Δ^6-desaturase for n-3 over n-6 fatty acids observed in vitro (50) may play a key role in ensuring that the ultimate conversion level of 18:3n-3 does not depend critically on tissue 18:2n-6 content. Mechanisms involving feedback inhibition of 18:3n-3 conversion (45, 50, 51), may also slow conversion rates when competitive inhibitors, such as 18:2n-6, are low.

In the livers of all groups, 90+% of the detected dose was converted from 18:3n-3 to long chain products, with complete conversion to 22:6n-3 at the level of about 80% and little dietary influence. The EFAD liver incorporated about 4-fold more label than the replete groups. These results indicate that a decrease in 18:3n-3 oxidation or increase in uptake/acylation occurs in the EFAD animal compared to the replete animals in response to the 22:6n-3 deficiency. Label in the EFAD liver was in exclusively in 20:5n-3 and 22:6n-3 at 27% and 71%, respectively. The accumulation of label in 20:5n-3 indicates that the regulation step in the conversion of 18:3n-3 to 22:6n-3 in this tissue is at the level of elongation of 20:5n-3. Radiolabeled 20:5n-3 accumulation in liver phospholipids of EFA-deficient rats has been observed previously (52). The absence of 18:3* in the EFAD tissues suggests that n-3 fatty acids were converted more quickly to long chain derivatives compared to the replete groups, with possible up-regulation of conversion mechanisms to meet 22:6n-3 demands.

Overall, there was a lower level of label incorporation in brain compared to liver as reported previously (43, 53, 54). The replete groups incorporated similar amounts of label in the brain, distributed between 22:5n-3 and 22:6n-3 at 28 and 72%, respectively. The Hi-18:2 diet had no affect on the accretion of label despite the significant increase in brain 18:2n-6 in the Hi-18:2 group.

The EFAD brain incorporated 2-fold more label, found exclusively as 22:6n-3, than the replete groups, indicating that the brain can respond to a deficient state. In brain of the replete groups, there were no differences in label distribution or overall incorporation, indicating that high 18:2n-6 levels do not impair brain conversion of 18:3* to 22:6n-3*. However, overall endogenous 22:6n-3 were depressed and 22:5n-6 were elevated in the Hi-18:2 group. These results suggest that high levels of tissue n-6 fatty acids compete at the level of incorporation rather than as inhibitors of conversion. While low 22:6n-3 and elevated 22:5n-6 levels are associated with functional deficiencies (9, 12, 13, 16, 17), there are insufficient quantitative data available to assess whether the present condition is functionally significant.

High precision GCC-IRMS

Biomedical applications of the tracer approach used here has been reviewed recently (55). The combination of highly enriched tracers and GCC-IRMS affords greater sensitivity across all compounds using chromatography than by using conventional electron impact (EI) GC-MS, as the mass spectrometer is optimized for detection of a single species (CO_2). In this respect, the GCC-IRMS system is analogous to a chromatographic radioactivity detector for radiotracers. The sensitivity of the technique is similar to that of negative ion chemical ionization, which has recently been applied to essential fatty acid tracer studies (56).

The 1-mg isotope dose (10 mg/kg body weight) used in this study represents 10-20% of the 18:3n-3 contained in a day's feed consumption for these animals. Doses of 0.1-1 mg/kg have yielded satisfactory results in similar pilot and ongoing studies in humans and non-human primates (54). The relatively high tracer dose in the present study was used to maximize detection of trace metabolites. Enrichment levels in this study, expressed as signal-to-noise ratio (S/N = mean/sd), range up to 5,000 for small, fast-turnover pools of highly enriched metabolites such as those in the plasma.

Label detectability in this technique is limited by baseline stability and the reproducibility of peak definition algorithms. Lower limits for high precision (and accuracy) are usually quoted for a minimum sample size of 10 ng, although signal can be detected by the IRMS instrument for samples at least 5 orders of magnitudes below this level. In the present study, metabolites were detected at levels lower than those quantified and reported, such as in plasma, although techniques for quantification and accurate assignment of enrichment for these levels are not yet available. Hardware and software modifications are

under development to facilitate calibration of low analyte levels, and are expected to improve routine GCC-IRMS isotope ratio determinations for signals 1-2 orders of magnitude lower than at present (57).

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