Altered essential fatty acid metabolism and composition in rat liver, plasma, heart and brain after microalgal DHA addition to the diet☆

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Abstract

To investigate the effect of docosahexaenoic acid (DHA) without other highly unsaturated fatty acids (HUFA) on n-3 and n-6 essential fatty acid (EFA) metabolism and fatty acid composition in mammals, a stable isotope tracer technique was used in adult rats fed diets with or without 1.3% of algal DHA in a base diet containing 15% of linoleic acid and 3% of alpha-linolenic acid over 8 weeks. The rats were administered orally a mixed oil containing 48 mg/kg body weight of deuterated linoleic and alpha-linolenic acids and euthanized at 4, 8, 24, 96, 168, 240, 360 and 600 h after administration of the isotopes. Fatty acid compositions and the concentrations of deuterated precursors and their respective metabolites were determined in rat liver, plasma, heart and brain as a function of time. DHA, docosapentaenoic acid and eicosapentaenoic acid in the n-3 EFA family were significantly increased in all organs tested in the DHA-fed group, ranging from 5% to 200% greater in comparison with the control group. The accumulation of the metabolites, deuterated-DHA and deuterated-docosapentaenoic acid n-6 was greatly decreased by 1.5- to 2.5-fold in the dietary DHA group. In summary, feeding preformed DHA led to a marked increase in n-3 HUFA content of rat organs at the expense of n-6 HUFA and also prevented the accumulation of newly synthesized deuterated end products. This is the first study which has isolated the effects of DHA on the *de novo* metabolism on both the n-6 and n-3 EFA pathways.
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1. Introduction

Docosahexaenoic acid (DHA, 22:6n-3) may be synthesized from plant alpha-linolenic acid (18:3n-3) in mammals [1], although 4–5% of a single dose of deuterated 18:3n-3 was converted to DHA in the rat body over a 25-day period with only about 0.1% of that in the nervous system [2]. The majority of the dietary C18 essential fatty acids (EFAs) are β-oxidized [2–4]. In isotopic tracer studies, labeled DHA was incorporated into liver and brain in baboon neonates and in rats much more efficiently than when derived from labeled-18:3n-3 [5,6]. Studies of newborn rats supplied with preformed DHA *via* artificial rearing during early development indicated that 90% of the brain DHA was derived from preformed DHA intake [7,8]. Infant formula supplemented with DHA and arachidonic acid (20:4n-6) has been shown to be beneficial for optimal nervous system development in both preterm [9–12] and term infants [13,14].

What is unclear is whether the addition of preformed long chain polyunsaturated fatty acids (LC-PUFA) to the diet results in alteration of C18 metabolism or whether the LC-PUFAs are simply incorporated into tissues. In the latter case, incorporation of the LC-PUFA can lead to a decrease in other tissue LC-PUFAs since they compete for the same set of liver microsomal acyltransferases [15]. A well-known example of this is the increase in eicosapentaenoic acid (20:5n-3) and DHA content of tissues concomitant with a decrease in 20:4n-6 with dietary fish oil supplementation [16]. A well-defined dietary experiment with a single essential fatty acid variable, together with the analysis of the *in vivo* metabolism of stable isotope-labeled EFAs, can thus help to delineate these two possibilities and to determine to what extent preformed DHA may lead to a decline in C18 essential fatty acid (EFA) metabolism.

This animal study was aimed at investigating how addition of preformed DHA alters essential fatty acid composition and metabolism. A basal diet containing 15% 18:2n-6 and 3% 18:3n-3 of total fatty acids was used as the control diet to which 1.3 wt.% DHA was substituted for saturated fat in the experimental diet. The basal diet was chosen so as to have a substantial level of 18:3n-3 and not be n-3 fatty acid deficient so as to have greater relevance to physiological diets. The stable isotope tracer technique coupled with analysis by negative chemical ionization gas chromatography/mass spectrometry (GC/MS) was applied to this

investigation. A single oral dose of both deuterated 18:3n-3 and deuterated 18:2n-6 was administered to adult rats after equilibrating them to their respective diets for 8 weeks. The deuterated precursors and their metabolites in rat liver, plasma, heart and brain at various times from 4 h to 25 days after administration of isotopes were quantified as well as tissue fatty acid distributions. This is the first study which has isolated the effects of DHA on the *de novo* metabolism on both the n-6 and n-3 EFA pathways.

2. Materials and methods

2.1. Animal study

All animal procedures were carried out in accordance with the NIH guidelines concerning animal welfare, and the protocol was approved by the NIAAA Animal Care and Use Committee. Three-week-old weanling, male and female Long Evans hooded rats (Charles River Laboratories, Portage, MI, USA) were randomly divided into two groups and supplied custom diets (Dyets, Bethlehem, PA, USA), which included a basal diet (control diet) containing 15% of 18:2n-6 and 3.1% of 18:3n-3; modified from the AIN-93G standard with 10% fat [17], as specified in Table 1. The experimental diet was identical except that docosahexaenoate (DHA diet, 1.3% of 22:6n-3) was substituted for saturated fat [18]. After consuming the diets for 8 weeks, 16 male rats with a body weight (BW) of 419±31 g (mean±S.D.) and 16 female rats with BW of 309±31 g in each diet group were divided into eight groups ($n=4$) and simultaneously administered a single oral dose of ~48 mg/kg BW each of $^2H_5$-18:3n-3 and $^2H_5$-18:2n-6 ethyl ester using olive oil as the vehicle. Animals were fasted overnight prior to the administration of isotopic tracers in the morning. Animals ($n=4$ per diet group) were euthanized at 4, 8 and 24 h and at 4, 7, 10, 15 and 25 days after administration of the isotopic tracers. At sacrifice, liver, plasma, heart and brain were collected and stored at −80°C until analysis. Baseline values for GC/MS analyses were obtained from control animals gavaged with vehicle oil only.

2.2. Isotopes and chemicals

Deuterated alpha-linolenate ($^2H_5$-17, 17, 18, 18, 18-18:3n-3 ethyl ester, $^2H>95\%$) and deuterated linoleate ($^2H_5$-17, 17, 18, 18, 18-18:2n-6 ethyl ester, $^2H>95\%$) were obtained from Cambridge Isotope Laboratories (Andover, MA, USA). A dual-labeled

Table 1
Nutrient composition of diets[a]

Ingredients[b]	Amount (g/kg of diet)	
Protein (20%)		
Casein (vitamin free)	200	
Carbohydrate (60%)		
Cornstarch	150	
Dextrose	199.5	
Maltose-dextrin	150	
Sucrose	100	
Others (10%)		
Cellulose	50	
Mineral and salt mix	35	
Vitamin mix	10	
L-Cystine	3	
Choline bitartrate	2.5	
TBHQ	0.02	
Fat (10%)	100	
	Control diet	DHA diet
HCO	77.5	74.5
Safflower oil	17.7	17.7
Flaxseed oil	4.8	4.8
DHASCO	0	3
Fatty acid composition[c] (wt% of total fatty acids)		
Saturates	77.2	75.6
Monounsaturates	4.3	4.8
18:2n-6	15.3	15.8
18:3n-3	3.12	2.55
22:6n-3	ND[d]	1.28

[a] Diets were modifications of the AIN-93G.
[b] TBHQ, *tert*-Butylhydroquinone; HCO, hydrogenated coconut oil; DHASCO, docosahexaenoic acid single cell oil (Martek Biosciences Corp., Columbia, MD).
[c] C20 and C22 polyunsaturates (except 22:6n-3) were present at only trace levels.
[d] ND indicates signals were at trace levels or below.

isotope, deuterated and carbon-13-labeled gamma-linolenate (7,8-$^{13}C_2$, 99%; 2,2,3,3,4,4,5,5,6-$^2H_9$, 98%-18:3n-6 ethyl ester), was used as an internal standard for the determination of labeled fatty acids and their *in vivo* metabolites. The anion of M+11 (isotope abundance was 63.7%) for the labeled-18:3n-6 internal standard was monitored in the negative chemical ionization mode in the gas chromatography/mass spectrometric analyses. This internal standard was not detectable in the rat tissues analyzed in this report.

2.3. Chemical analysis

The right hemisphere of the brain, the whole heart and 1 g of liver were dissected and homogenized in 8 ml of methanol containing butylated hydroxytoluene (BHT, 50 mg/L) using a power homogenizer (Omni International, Marietta, GA, USA). An aliquot of the homogenate was used to extract total tissue lipids using the Folch et al. [19] method. Known amounts of the internal standard 22:3n-3 ethyl ester were added to each sample prior to lipid extraction, 0.6 μmol for liver and brain and 0.3 μmol for heart for the unlabeled fatty acid analysis by GC/flame ionization detection; 16 nmol of $^{13}C_2,^2H_9$-18:3n-6 ethyl ester was used as an internal standard for GC/MS analysis. Similarly, 200 μl of plasma spiked with 25 nmol of 23:0 ethyl ester and 0.5 nmol of $^{13}C_2,^2H_9$-18:3n-6 ethyl ester was mixed with 1 ml of BHT-methanol during total lipid extraction.

Non-labeled fatty acids in each organ were transmethylated and determined by gas chromatography/flame ionization [20,21]. A DB-FFAP capillary column (30 m×0.25 mm i.d.×0.25 μm, J&W Scientific, Folsom, CA, USA) installed in a GC 6890 Plus LAN system (Agilent Technologies, Inc., Santa Clara, CA, USA) was used to resolve various methylated fatty acids. A quantitative methyl ester reference standard of 28 fatty acids (GLC-462, Nu-Chek Prep, Elysian, MN, USA) was used to identify the retention time of methyl ester peaks.

Isotope-labeled fatty acids in each organ were measured by gas chromatography/ mass spectrometry assay [22] on a GC 6890/MSD 5973 LAN system (Agilent Technologies) with the same capillary column as above [23]. Non-labeled and labeled C24 fatty acids in all four tissues as well as 18:3n-3 and 20:5n-3 in the brain were quantified by GC/MS.

2.4. Calculation and statistics

The amounts of the fatty acids were determined by comparing the integrated areas of the fatty acid peaks on the gas chromatograms with those of the corresponding internal standards. Data were expressed as either the concentrations of fatty acid as mole per milliliter of plasma and per gram of wet tissue weight, or as the percentage of isotope initial doses (%dose) or the percentage of total fatty acid weight (wt%), as indicated in the text. Values were expressed as mean±S.E.M. ($n=4$) unless otherwise indicated. Area under the curves (AUC) was obtained by integrating the time-course curves of enrichment, from 0 to 600 h, for various labeled fatty acids using the trapezoid estimation approach [24].

Repeated measures ANOVA was applied to assess the effects of diet over the time course of labeled fatty acids using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). The significance level was set at a P value of .05 or lower. A two-tailed, pairwise t test was applied to determine significant differences in endogenous non-labeled fatty acid composition between the two groups at a P value of .05 or lower unless otherwise indicated.

3. Results

3.1. Fatty acid composition in rat plasma, liver, heart and brain

After an 8-week period on the experimental diets, there was a significant difference in the fatty acid compositions of plasma, liver (Table 2), heart and brain (Table 3) between the group fed basal diet (control group) and those fed the DHA-supplemented diet (DHA group). The DHA content in the DHA group was significantly greater than in the control group in all four tissues examined: plasma and heart were 50% greater and liver and brain were 33% and 4.6% greater, respectively. Accompanying the higher DHA content was an approximately twofold greater level of 20:5n-3 in plasma, liver and heart. Similarly, docosapentaenoic acid (22:5n-3) in the liver, brain and plasma was 30% greater in the DHA group ($P<.001$) and there were higher levels ($P<.05$) of the more minor n-3 fatty acids, 24:5n-3 and 24:6n-3 in liver and brain. There was no difference in 18:3n-3 between the two groups. There was a significantly greater total n-3 highly unsaturated fatty acid (HUFA) level in the DHA group relative to the control group in all four tissues.

Dihomo-gamma-linolenic acid (20:3n-6) was a greater proportion of total fatty acid composition in the DHA group in all four tissues examined ($P<.001$). As seen in Tables 2 and 3, 20:3n-6 was 70%

Table 2
Non-labeled fatty acid compositions in plasma and liver (wt% of total fatty acids)

Fatty acids	Plasma		Liver	
	Control diet	DHA diet	Control diet	DHA diet
10:0	0.005±0.002	0.021±0.02	0.009±0.001	0.011±0.001
12:0	0.46±0.05	0.45±0.05	0.34±0.03	0.38±0.04
14:0	1.27±0.08	1.28±0.06	1.27±0.07	1.17±0.06
16:0	17.56±0.37	17.83±0.50	21.25±0.41	22.17±0.59
18:0	12.94±0.58	13.71±0.53	14.86±0.48	15.14±0.53
20:0	0.08±0.01	0.08±0.004	0.04±0.002	0.05±0.02
22:0	0.09±0.01	0.10±0.004	0.05±0.003	0.06±0.003
24:0	0.16±0.01	0.13±0.02	0.16±0.01	0.17±0.01
12:1	0.01±0.00	ND	0.01±0.001	0.01±0.001
14:1	0.09±0.01	0.09±0.004	0.09±0.01	0.10±0.01
16:1n-7	2.65±0.13	2.27±0.16[a]*	3.07±0.16	2.90±0.19
18:1n-9	16.16±1.12	13.26±0.95[a]**	18.07±0.78	16.66±0.87[a]***
18:1n-7	2.51±0.10	1.99±0.09[a]***	3.54±0.13	2.61±0.11[a]**
20:1n-9	0.13±0.01	0.09±0.005[a]***	0.15±0.01	0.11±0.01[a]***
22:1n-9	0.02±0.00	0.01±0.001[a]*	0.013±0.00	0.007±0.001
24:1n-9	0.58±0.05	0.68±0.04	0.10±0.02	0.08±0.005
18:3n-3	0.53±0.03	0.56±0.03	0.53±0.04	0.61±0.05
20:5n-3	0.57±0.04	1.74±0.11[b]***	0.34±0.03	1.07±0.07[b]***
22:5n-3	0.48±0.03	0.63±0.04[b]***	0.55±0.03	0.71±0.03[b]***
22:6n-3	4.47±0.15	6.72±0.18[b]***	7.52±0.27	9.99±0.33[b]***
24:5n-3	ND	ND	0.009±0.001	0.01±0.001[b]
24:6n-3	ND	ND	0.02±0.001	0.02±0.002[b]
18:2n-6	10.66±0.41	12.71±0.43[b]***	8.84±0.39	9.89±0.41
18:3n-6	0.18±0.01	0.17±0.01	0.16±0.01	0.19±0.03
20:2n-6	0.08±0.01	0.10±0.01	0.11±0.01	0.11±0.01
20:3n-6	0.53±0.03	0.90±0.05[b]***	0.53±0.04	0.77±0.05[b]***
20:4n-6	23.32±0.85	20.88±0.86[a]*	15.28±0.69	12.32±0.52[a]***
22:2n-6	ND	ND	0.02±0.002	0.02±0.001
22:4n-6	0.31±0.02	0.13±0.01[a]***	0.30±0.02	0.15±0.01[a]***
22:5n-6	0.25±0.02	0.13±0.02[a]***	0.31±0.02	0.10±0.01[a]***
24:4n-6	ND	ND	0.01±0.001	0.005±0.000[a]***
24:5n-6	ND	ND	0.007±0.001	0.002±0.000[a]***
Σ SFA	32.57±0.51	33.59±0.38[b]*	37.97±0.33	39.16±0.38[b]*
Σ MUFA	22.16±1.12	18.38±1.00[a]***	25.03±0.91	22.47±1.01
Σ PUFA	41.59±0.92	44.95±0.88[b]**	34.48±0.97	35.94±1.10
HUFA n-3	5.61±0.16	9.34±0.29[b]***	8.43±0.29	11.81±0.38[b]***
HUFA n-6	24.53±0.85	22.08±0.86[a]*	16.46±0.71	13.37±0.54[a]***
HUFA n6/n3	4.43±0.15	2.45±0.12[a]***	1.95±0.05	1.14±0.03[a]***
Total fatty acids (μmol/g wet weight tissue)	9.5±0.6	6.9±0.3[b]***	171.1±11.4	160.9±8.1

Values are presented as mean±S.E.M. (n=32) of endogenous fatty acids from the animals over the entire experimental period.

SFA, Saturated fatty acid; MUFA, monounsaturated fatty acid.

Significant differences were determined by two-tailed, paired t test with P values of .05*, .01**, .001 or lower ***.

[a] Indicates decrement of fatty acids in DHA diet group.

[b] Indicates accretion of fatty acids in DHA diet group.

greater in plasma, heart and brain and 45% greater in liver in the DHA group compared with the control group. The accretion of 18:2n-6 was also somewhat increased in the DHA group in brain, plasma and heart (P<.05). In contrast to the n-3 HUFA, the n-6 HUFA was generally lower in all tissues examined in the DHA group. For all four compartments, 20:4n-6, 22:4n-6 and 22:5n-6 showed significantly lower proportions in the DHA group (P<.05 or .001). In the DHA group, 20:4n-6 (5%), 22:4n-6 (8%) and 22:5n-6 (42%) in the brain were all lower relative to the control group and a greater decrement was observed in the other tissues. In plasma, liver and heart, 22:4n-6 and 22:5n-6 were about 50% lower in the DHA group, while 22:5n-6 was 83% lower in heart. About a 10% to 20% decrement in 20:4n-6 was observed in plasma, liver and heart. The 24:4n-6 and 24:5n-6 were significantly lower in liver but not in brain in the DHA group. Increases in some of the n-6 PUFA precursors in plasma and liver obscured changes in the total n-6 HUFA content between the two diets, but a lower level in the DHA group was observed in heart and brain (P<.001).

In general, the summation of n-3 and n-6 PUFA was not significantly different in the liver, heart or brain between the two groups but was higher in the plasma of the DHA diet group (P<.01). Some monounsaturated fatty acids in the liver, plasma and heart such as 18:1n-7, 20:1n-9 and 22:1n-9 comprised a lower proportion of total fatty acids in the DHA diet group (P<.05), but there was a higher level of 18:1n-9 in the brain.

3.2. Alteration of essential fatty acid metabolism by dietary DHA

3.2.1. Dietary DHA decreases $^2H_5$-22:6n-3 accumulation

DHA synthesized from its precursor $^2H_5$-18:3n-3 was greatly decreased in rats fed with the DHA diet compared to rats fed the control diet over the entire experimental period, as seen in Fig. 1 for rat liver (P<.05) and plasma (P<.01), and Fig. 2 for heart (P<.05) and brain (P<.01). The maximal amount of $^2H_5$-22:6n-3 in the control group and DHA group, expressed as a percentage of the dosed $^2H_5$-18:3n-3, was as follows: plasma, 0.14% vs. 0.04%, at 24 and 96 h; liver, 2.2% vs. 0.6%, at 24 and 8 h; heart, 0.08% vs. 0.03%, at 240 h; brain, 0.10% vs. 0.03%, at 360 and 600 h, respectively. The $^2H_5$-22:6n-3 in the two dietary groups reached their maximal values at similar time points except in liver where the DHA diet group reached its maxima at 8 h. The generally higher maximal amounts of $^2H_5$-22:6n-3 in the control group engendered a greater slope in both the appearance and decay phases of the time courses.

Statistical analyses of the time-course curves for $^2H_5$-22:5n-3 indicated no significant differences in brain, liver or heart, although significance (P<.05) was detected in the plasma compartment (Fig. 1).

Table 3
Non-labeled fatty acid compositions in heart and brain (wt% of total fatty acids)

Fatty acids	Heart		Brain	
	Control diet	DHA diet	Control diet	DHA diet
10:0	0.017±0.003	0.017±0.003	ND	ND
12:0	1.07±0.11	1.08±0.13	0.01±0.00	0.01±0.00[a]**
14:0	1.67±0.15	1.65±0.16	0.20±0.01	0.21±0.02
16:0	15.04±0.57	15.61±0.58	18.94±0.16	18.76±0.12
18:0	18.76±0.52	18.72±0.56	16.84±0.16	16.70±0.08
20:0	0.11±0.003	0.12±0.00	0.42±0.01	0.41±0.01
22:0	0.06±0.003	0.07±0.004[b]*	0.34±0.01	0.34±0.01
24:0	0.08±0.005	0.07±0.004	0.58±0.03	0.57±0.03
12:1	0.02±0.002	0.02±0.002	ND	ND
14:1	0.12±0.01	0.11±0.02	0.03±0.01	0.04±0.01
16:1n-7	2.16±0.24	1.99±0.26	0.35±0.01	0.36±0.004
18:1n-9	11.47±0.92	10.51±0.98	17.86±0.10	18.36±0.09[b]***
18:1n-7	3.77±0.07	3.26±0.07[a]***	4.05±0.04	4.02±0.02
20:1n-9	0.12±0.01	0.11±0.01[a]*	2.13±0.05	2.20±0.03
22:1n-9	0.02±0.001	0.01±0.000	0.20±0.01	0.21±0.003
24:1n-9	0.08±0.01	0.03±0.01[a]***	1.77±0.12	1.81±0.11
18:3n-3	0.27±0.01	0.27±0.02	0.005±0.000	0.007±0.000[b]***
20:5n-3	0.11±0.01	0.30±0.01[b]***	0.02±0.001	0.05±0.001[b]***
22:5n-3	1.08±0.05	1.01±0.04	0.16±0.01	0.21±0.003[b]***
22:6n-3	9.64±0.44	14.59±0.65[b]***	14.00±0.12	14.64±0.10[b]***
24:5n-3	ND	ND	0.03±0.002	0.05±0.001[b]***
24:6n-3	ND	ND	0.10±0.006	0.14±0.002[b]***
18:2n-6	11.74±0.39	12.70±0.35[b]*	0.48±0.01	0.59±0.01[b]***
18:3n-6	0.03±0.001	0.02±0.001[a]***	ND	ND
20:2n-6	0.09±0.01	0.09±0.002	0.13±0.004	0.15±0.004[b]***
20:3n-6	0.35±0.02	0.43±0.01[b]***	0.37±0.01	0.44±0.01[b]***
20:4n-6	17.82±0.67	13.90±0.58[a]***	8.87±0.06	8.40±0.04[a]***
22:2n-6	0.03±0.00	0.03±0.003	0.06±0.01	0.06±0.00
22:4n-6	0.70±0.03	0.31±0.01[a]***	3.08±0.02	2.82±0.02[a]***
22:5n-6	0.78±0.05	0.13±0.01[a]***	0.38±0.01	0.22±0.01[a]***
24:4n-6	ND	ND	0.42±0.02	0.41±0.007
24:5n-6	ND	ND	0.10±0.005	0.09±0.002
Σ SFA	36.80±0.33	37.32±0.32	37.33±0.27	37.01±0.12
Σ MUFA	17.74±1.14	16.04±1.26	26.39±0.20	27.00±0.14b*
Σ PUFA	42.63±1.35	43.79±1.42	27.56±0.17	27.73±0.15
HUFA n-3	10.83±0.49	15.91±0.69[b]***	14.32±0.12	15.08±0.11[b]***
HUFA n-6	19.68±0.72	14.81±0.61[a]***	13.27±0.09	12.44±0.06[a]***
HUFA n6/n3	1.85±0.04	0.94±0.02[a]***	0.93±0.01	0.83±0.01[a]***
Total fatty acids (μmol/g wet weight tissue)	62.2±5.1	59.2±3.5	97.8±1.4	95.1±1.1

Footnotes are the same as those for Table 2.

In plasma, there was a markedly higher level of ²H₅-22:5n-3 accretion and what appeared to be a much faster appearance rate in the control diet group.

The absorption of precursor ²H₅-18:3n-3 into the plasma was decreased in the DHA group and exhibited a much higher maximal value in the control group. In heart and brain, similar tendencies were observed although no significant changes were observed. The time point of the maximal concentration for ²H₅-18:3n-3 was 4 h later in the control group in plasma and heart, but similar in the liver and brain.

No significant differences were observed in ²H₅-20:5n-3 accretion between the dietary groups, although there was a tendency for a greater content in liver, heart and brain in the DHA group.

Few differences were observed for the accretion of the more minor n-3 and n-6 metabolites including ²H₅-labeled -20:3n-3, -20:4n-3, -24:5n-3, -24:6n-3 in liver (Supplemental Figure 1). However, the accumulation of ²H₅-24:5n-3 was significantly lower in brain. ²H₅-24:6n-3 in the DHA diet group also had a similar tendency, albeit not significant.

3.2.2. Dietary DHA decreases ²H₅-22:4n-6, ²H₅-22:5n-6 and ²H₅-20:4n-6 accumulation

Accumulation of ²H₅-22:4n-6 and ²H₅-22:5n-6 was greatly decreased in rat plasma, liver and heart in the DHA diet group ($P<.05$), as presented in Figs. 3 and 4. The ²H₅-22:5n-6 was a weak

signal on the GC/MS ion chromatograms at this dose of ²H₅-18:2n-6 in the control group and difficult to detect in the DHA group. ²H₅-20:4n-6 was lower in plasma ($P<.05$) and exhibited a similar tendency in liver, heart and brain in the DHA group. No difference was detected for ²H₅-20:2n-6. Deuterated C24 n-6 fatty acids could not be quantified except in the brain (Supplemental Figure 1). ²H₅-24:4n-6 was detected in brain in both dietary groups with a tendency of a greater maximal value of 7.8 vs. 5.7 ppm of ²H₅-18:2n-6 dose for the control group and the DHA group, respectively. A similar situation was observed for ²H₅-24:5n-6.

3.2.3. Dietary DHA increased accumulation of ²H₅-20:3n-6 in rat liver and brain

There was a significant increase in ²H₅-20:3n-6 in liver and brain in the DHA diet group ($P<.05$). In plasma, ²H₅-18:2n-6 and its first metabolite ²H₅-18:3n-6 had similar accretion in the two dietary groups. The ²H₅-18:3n-6 could not be quantified in the rat heart and brain due to trace level signals.

3.3. Deuterium enrichment comparisons between diets

The concentrations of the stable isotope precursors and their metabolites were combined with measurements of the respective endogenous fatty acids and expressed as enrichment, i.e., the tracer divided by the tracee. A summary of the comparison of the main fatty
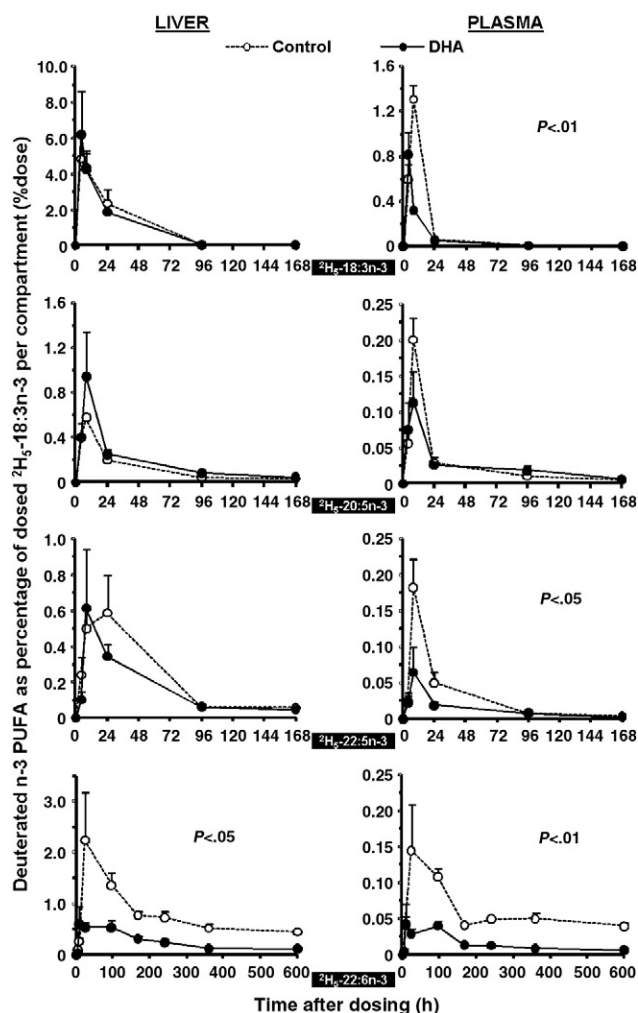
Fig. 1. Time-course curves of the labeled n-3 fatty acids in rat liver (left) and plasma (right) after a single foral dosage of $^2H_5$-18:3n-3 in the control diet group (○) and DHA diet group (●). Values were expressed as % dose per compartment as a function of time over 600 h (mean±S.E.M., n=4). Repeated measures ANOVA with diet as a between-group factor was performed to determine the significant difference with a P value of .05 or lower.

acid enrichment between the two dietary groups is presented in Table 4. For $^2H_5$-22:6n-3, the tracee was decreased in the DHA group and coupled with an increase in the endogenous DHA pool leading to a very significant decline in the enrichment in $^2H_5$-22:6n-3 in all tissues examined. Both $^2H_5$-20:5n-3 and $^2H_5$-22:5n-3 enrichments were also significantly decreased in the DHA group. This was in part due to the greater denominator, since the non-labeled 20:5n-3 and 22:5n-3 pools were generally increased in the tissues. The effect on enrichment using this measure, the area under the time-course curves, was somewhat greater than that resulting from the increased pool sizes of these two n-3 fatty acids. In brain, there was a significant decline in the $^2H_5$-20:4n-6 and $^2H_5$-22:5n-6 enrichments in the DHA group and $^2H_5$-22:5n-6 enrichment also declined in the plasma.

4. Discussion

There are few studies of EFA metabolism wherein the diet is well controlled and adequately described. It is critical that dietary studies entail a single variable such as in the present study where DHA was added to a diet that contained adequate levels of both 18:2n-6 and 18:3n-3. Since competition between n-3 and n-6 fatty

acids, for example, is well known, fatty acid metabolic studies that do not carefully prescribe the levels of these fatty acids in the diet may be irreproducible and difficult to interpret.

When 1.3% of the dietary fatty acid weight is provided as DHA, there is a marked increase in DHA content in all tissues studied. This increase was quite sizeable in peripheral tissues like plasma, liver and heart but was modest in the brain. This is consistent with previous reports that the adult mammalian brain is fairly resistant to EFA compositional changes after the PUFAs are laid down [25]. The 20:5n-3 also increased in the plasma and liver of the order of about threefold but still comprised only 1.1–1.7% of the total fatty acids. The levels of 20:5n-3 in the heart and brain were very small with respect to the DHA content, the principal n-3 fatty acid in these organs. Accretion of 20:5n-3 has been observed in human studies after a DHA supplement [26–28]. This has been ascribed to retroconversion from partial β-oxidation of DHA [29,30]. Similar retroconversion to 20:5n-3 is reported in rats after a 22:5n-3 supplement [31]. This result indicates that dietary DHA may also serve as a source of 20:5n-3, as previously suggested in other studies [26,32], suggesting that a supplement of both 20:5n-3 and DHA may not be necessary to increase levels of n-3 HUFAs. As has often been observed [33,34], this increased level of n-3 HUFAs was accompanied by a decrease in 20:4n-6 and other n-6 HUFAs, including 22:4n-6 and 22:5n-6.

Accompanying this increase in organ DHA was a marked decrease in the accumulation of the newly synthesized HUFA in liver and some
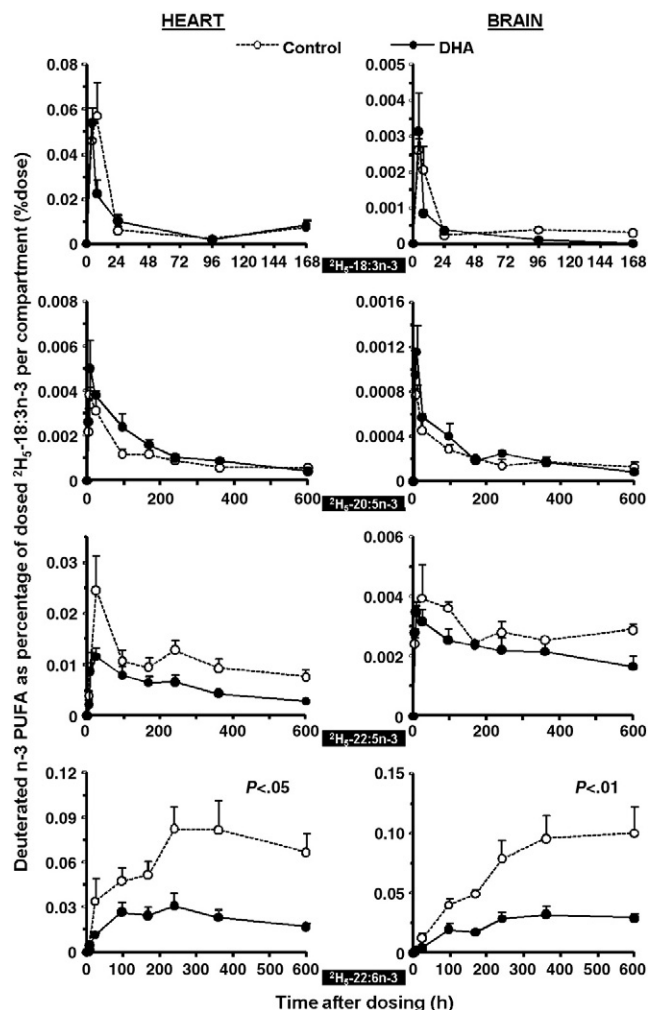

Fig. 2. Time-course curves of the labeled n-3 fatty acids in rat heart (left) and brain (right) after a single oral dosage of $^2H_5$-18:3n-3. See legend to Fig. 1 for details.
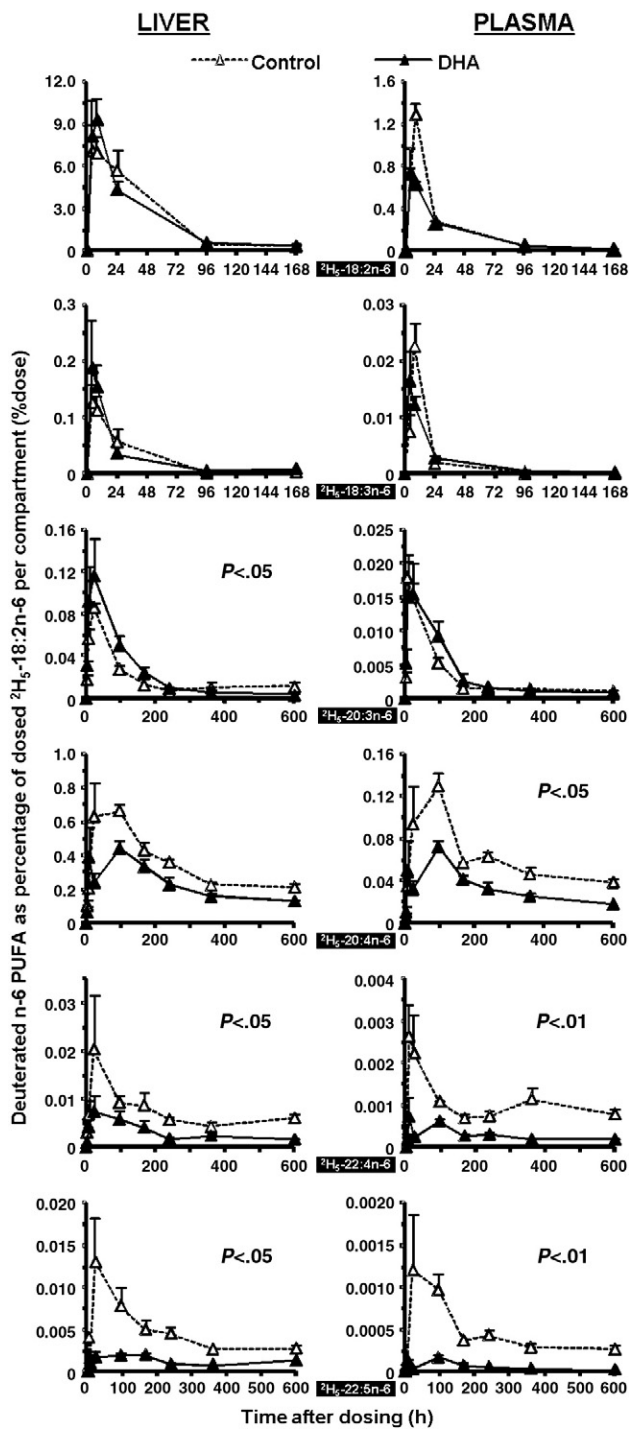
Fig. 3. Time-course curves of the labeled n-6 fatty acids in rat liver (left) and plasma (right) after a single oral dosage of $^2H_5$-18:2n-6. See legend to Fig. 1 for details.

in 20:3n-6 in all tissues studied. In liver and brain, this difference in content was accompanied by an increase in the deuterated intermediate in the DHA diet condition (Figs. 3 and 4). There was no evidence of an increase in the Δ5 desaturase product, $^2H_5$-20:4n-6, in the DHA diet; in fact, the $^2H_5$-20:4n-6 had increased in plasma and the tissue content of the unlabeled compound had decreased in all tissues.

One of the most notable observations in this study is the very marked decline in $^2H_5$-18:3n-3 incorporation into $^2H_5$-DHA in the DHA-treated animals in all tissues studied, including the brain. It is of
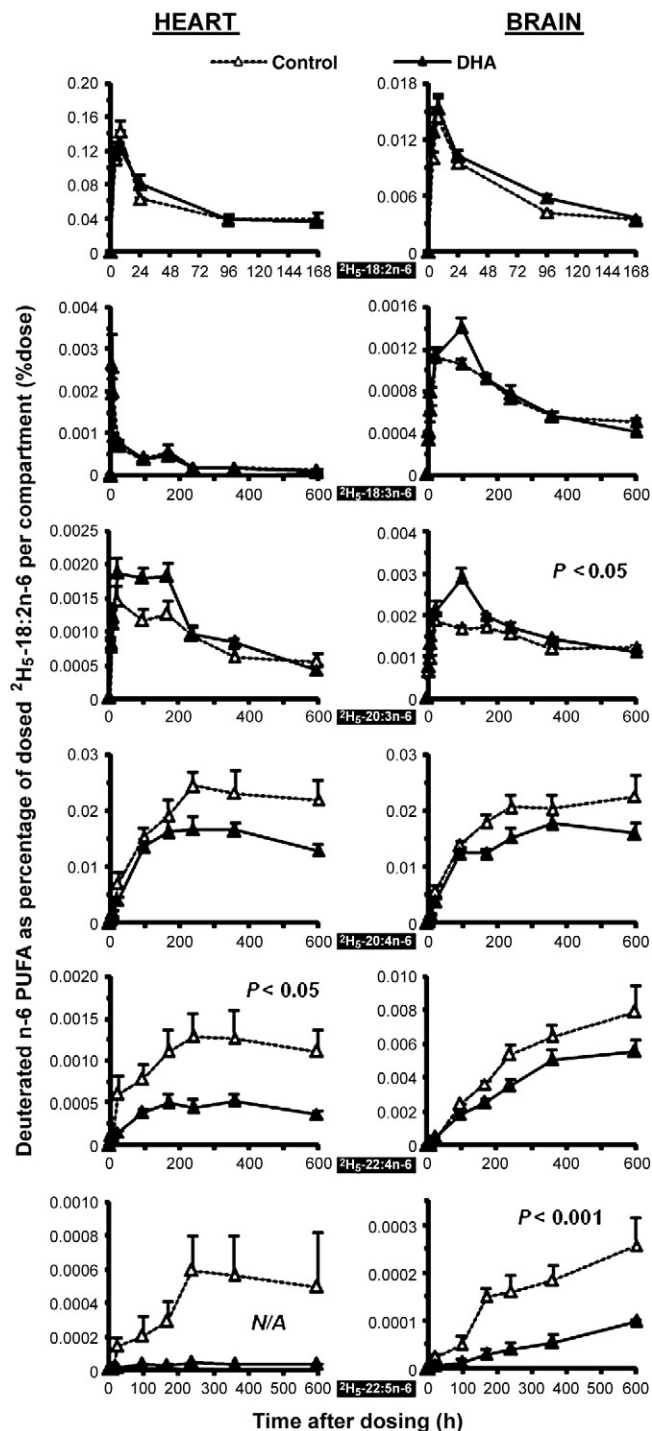
Fig. 4. Time-course curves of the labeled n-6 fatty acids in rat heart (left) and brain (right) after a single oral dosage of $^2H_5$-18:2n-6. N/A indicates P value is not available. See legend to Fig. 1 for details.

extrahepatic tissues in adult rats. Preformed DHA suppressed the accumulation of $^2H_5$-DHA and $^2H_5$-22:5n-3 derived from $^2H_5$-18:3n-3 and also of $^2H_5$-22:4n-6 and $^2H_5$-22:5n-6 derived from $^2H_5$-18:2n-6. However, the accumulation of the $^2H_5$-18:3n-3, $^2H_5$-18:2n-6, as well as their intermediate metabolites showed little difference with or without performed DHA in diet. These results are consistent with the hypothesis that Δ6 desaturase is unaffected by the addition of DHA to the diet, while the data does suggest a decreased activity of Δ5 desaturase. An additional observation that provides some support for this notion is the observed small but statistically significant increase

Table 4
Comparison of area under the time-course curves over 600 h for enrichment of n-3 and n-6 HUFAs

Tissues	Fatty acids	$AUC_{0 \to 600\ h}$ (mol/mol%×h)	
		Control diet	DHA diet
Plasma	20:5n-3	618.1±39.5	281.4±23.3***
	22:5n-3	735.7±28.7	325.8±21.8***
	22:6n-3	408.1±28.0	92.5±6.0***
	20:3n-6	175.5±5.7	156.8±16.6
	20:4n-6	81.2±7.9	71.1±6.9
	22:4n-6	103.3±5.5	94.7±8.3
	22:5n-6	61.2±4.4	35.2±6.7*
Liver	20:5n-3	350.9±16.9	168.7±18.8***
	22:5n-3	364.1±29.4	189.1±17.5**
	22:6n-3	234.2±12.0	60.2±5.0***
	20:3n-6	79.4±11.1	70.6±8.5
	20:4n-6	51.8±3.1	46.6±1.8
	22:4n-6	56.4±5.9	56.3±5.5
	22:5n-6	32.7±2.4	24.4±4.0
Heart	20:5n-3	538.4±64.8	265.3±4.6**
	22:5n-3	600.1±92.1	330.5±19.5*
	22:6n-3	387.8±49.3	87.9±10.6***
	20:3n-6	146.4±20.5	142.8±10.3
	20:4n-6	62.2±6.2	56.8±0.5
	22:4n-6	90.2±11.1	82.2±2.4
	22:5n-6	28.3±8.7	8.0±1.0 NA
Brain	20:5n-3	189.6±26.8	105.5±6.9*
	22:5n-3	378.6±24.7	221.9±21.2**
	22:6n-3	104.0±9.6	32.8±4.0***
	20:3n-6	75.2±2.4	75.0±4.5
	20:4n-6	38.9±1.4	31.7±1.2**
	22:4n-6	34.0±1.5	26.9±1.2**
	22:5n-6	9.6±0.7	5.2±1.1*

AUC values are presented as mean±S.E.M. ($n=4$).
Two-tailed, unpaired t test determined the significant difference between diet groups at a P value of .05*, .01**, .001 or lower***.
NA indicates P value is not available.

interest to follow the deuterium through both the n-3 and n-6 pathways to see where this phenomenon is first observed. No statistically significant changes were observed at the level of $^2H_5$-20:5n-3 and only in plasma for its elongation product, $^2H_5$-22:5n-3. In the brain, only the $^2H_5$-24:5n-3 was less in the DHA diet group and no differences could be observed (albeit with very low level signals) for $^2H_5$-24:6n-3. In the n-6 pathway, the effects of the DHA diet became apparent in three of the four tissues for both $^2H_5$-22:4n-6 and $^2H_5$-22:5n-6 and could not be observed in the C24 PUFAs of the brain. Thus there is evidence of a decrease in the net formation of the Δ5 desaturase products subsequent to their elongation that continues, for the most part, afterwards. Dietary PUFAs, except for 18:2n-6, are likely to induce fatty acid oxidation enzymes *via* the peroxisome proliferator-activated receptor α (PPARα) as a "feed-forward" mechanism [35].

Previous investigations have suggested that a variety of LC-PUFAs including DHA and 20:5n-3 [36,37] or DHA and docosapentaenoic acid (22:5n-6) [38] or DHA and 20:4n-6 [39] exert feedback regulation on the accumulation of HUFA. The mechanism of this feedback effect from fish oil may be through reduction of liver microsomal enzymatic activity [40] or through down-regulation of the hepatic abundance of Δ5 or Δ6 desaturase mRNA [41,42]. Two transcriptional factors, sterol regulatory element-binding protein-1c [43] and PPARα [44], are involved in the feedback regulation exerted by PUFAs. In these studies, the Δ5 and Δ6 desaturases may be induced by fat-free diets, addition of EPA/DHA from fish oil, then leads to lower levels of activity. However, no study has isolated the effects of DHA on the *de novo* metabolism of EFAs of both the n-6 and n-3 pathways.

It is also of interest to note that, although there was a much smaller AUC for $^2H_5$-DHA in all tissues examined in the DHA dietary group, there was no reduction in tissue content of 24:5n-3 or 24:6n-3

in those tissues where they could be measured (brain and liver). This may suggest that a decrement in Δ6 desaturase activity is not the explanation for the changes in $^2H_5$-DHA accumulation in tissues. This was supported by preliminary observations of primary hepatocytes where no decrease in Δ6 desaturase activity measured *in vitro* was observed in the DHA-fed group relative to the control diet. One possible explanation is that the rate of conversion of 24:6n-3 to DHA may be inhibited in the DHA-supplemented group.

The following are the supplementary materials related to this article. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2010.06.008.

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