Low liver conversion rate of α-linolenic to docosahexaenoic acid in awake rats on a high-docosahexaenoate-containing diet

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Abstract **We quantified the rates of incorporation of α-linolenic acid (α-LNA; 18:3n-3) into "stable" lipids (triacylglycerol, phospholipid, cholesteryl ester) and the rate of conversion of α-LNA to docosahexaenoic acid (DHA; 22:6n-3) in the liver of awake male rats on a high-DHA-containing diet after a 5-min intravenous infusion of [1-^{14}C]α-LNA. At 5 min, 72.7% of liver radioactivity (excluding unesterified fatty acid radioactivity) was in stable lipids, with the remainder in the aqueous compartment. Using our measured specific activity of liver α-LNA-CoA, in the form of the dilution coefficient $\lambda_{\alpha\text{-LNA-CoA}}$, we calculated incorporation rates of unesterified α-LNA into liver triacylglycerol, phospholipid, and cholesteryl ester as 2,401, 749, and 9.6 nmol/s/g $\times$ 10^{-4}, respectively, corresponding to turnover rates of 3.2, 8.7, and 2.9%/min and half-lives of 8–24 min. A lower limit for the DHA synthesis rate from α-LNA equaled 15.8 nmol/s/g $\times$ 10^{-4} (0.5% of the net incorporation rate). Thus, in rats on a high-DHA-containing diet, rates of β-oxidation and esterification of α-LNA into stable liver lipids are high, whereas its conversion to DHA is comparatively low and insufficient to supply significant DHA to the brain. High incorporation and turnover rates likely reflect a high secretion rate by liver of stable lipids within very low density lipoproteins.** —Igarashi, M., K. Ma, L. Chang, J. M. Bell, S. I. Rapoport, and J. C. DeMar, Jr. **Low liver conversion rate of α-linolenic to docosahexaenoic acid in awake rats on a high-docosahexaenoate-containing diet.** ***J. Lipid Res.*** **2006.** 47: **1812–1822.**

Supplementary key words incorporation • turnover • synthesis • pulse labeling • infusion • diet

Docosahexaenoic acid (DHA; 22:6n-3) is a nutritionally essential PUFA that must be obtained directly through the diet or be synthesized from its dietary essential precursor, α-linolenic acid (α-LNA; 18:3n-3). Mammalian tissues can convert α-LNA to DHA through serial steps of desaturation and elongation with final peroxisomal chain shortening (1–3). Both Δ5 and Δ6 desaturases participate in this conversion, but the Δ6 desaturase is considered to be rate-limiting (1, 4). Human and rat Δ5 and Δ6 desaturases are expressed abundantly in brain, liver, and heart (5, 6).

Controversy remains regarding the extent of DHA synthesis in brain from α-LNA, compared with its delivery by blood to brain as DHA synthesized from α-LNA in the liver. In immature rats, Scott and Bazan (7) concluded that the brain does not synthesize its own DHA to a significant extent but that DHA converted from α-LNA in the liver can contribute to brain DHA via the blood stream. In adult rats fed a high-DHA-containing diet [2.3% (w/w) of total fatty acid], we recently used an in vivo kinetic pulse-labeling model that confirmed a low synthesis rate of DHA from α-LNA in brain (2), as proposed for immature rats (see above), and also showed that α-LNA was largely β-oxidized or esterified unchanged into brain phospholipid. We did not examine the rate of DHA synthesis from α-LNA in the liver of these rats, nor did we explore the kinetics of other pathways of liver α-LNA metabolism. We thought it important to try to do so here.

A number of experimental procedures have been used to examine hepatic α-LNA metabolism, including studying isolated hepatocytes (8), infusing the liver in situ (9), and injecting labeled α-LNA intravenously in an animal and measuring its distribution in different liver compartments (10). None of these studies measured the specific activity of liver α-LNA-CoA, the precursor pool for α-LNA esterification into stable lipids and for steps of DHA synthesis. This prevented calculating exact incorporation and syn-

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Abbreviations: DHA, docosahexaenoic acid (22:6n-3); di-17:0 PC, di-heptadecanoate phosphatidylcholine; EPA, eicosapentaenoic acid (20:5n-3); FAME, fatty acid methyl ester; LA, linoleic acid (18:2n-6); α-LNA, α-linolenic acid (18:3n-3).

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thesis rates as well as turnover rates and half-lives of α-LNA in stable liver lipids.

In this study, we used controlled intravenous infusion of [1-^{14}C]α-LNA in unanesthetized adult male rats to produce steady-state plasma and liver α-LNA-CoA specific activities, and measured the radioactive plasma exposure of the liver (input function) to calculate rate parameters as we did for the brain (2, 11, 12). We also microwaved the liver to stop enzymatic activity and to prevent ischemia-related changes in liver unesterified fatty acid and acyl-CoA concentrations (13–15). The rats were fed a diet containing a high DHA content [2.3% (w/w) of total fatty acid weight, contributing 0.23% of total food energy intake] compared with much lower fractional DHA weights and percentage energy intakes of current and recommended human diets in the United States (16–19). They had been studied previously by us when we determined α-LNA kinetic parameters for their brains (2). In this study, we used the labeled and unlabeled plasma α-LNA concentrations, plasma input functions, and specific activities that we had measured in that brain study (2). An abstract of part of this work has been presented (20).

MATERIALS AND METHODS

Materials

[1-^{14}C]α-LNA in 100% ethanol was purchased from Perkin-Elmer Life Sciences, NEN Life Science Products (Boston, MA). Its specific activity was 54 mCi/mmol and its purity was 98% (determined by HPLC and scintillation counting). Di-heptadecanoate phosphatidylcholine (di-17:0 PC), free heptadecanoic acid (17:0), heptadecanoyl-CoA (17:0-CoA), and acyl-CoA standards for HPLC, as well as TLC standards for cholesterol, triglycerides, and cholesteryl esters, were purchased from Sigma-Aldrich (St. Louis, MO). Standards for general fatty acid methyl esters (FAMEs) for GC and HPLC were from NuChek Prep (Elysian, MN). FAMEs for unique n-3 PUFAs (20:4n-3, 22:5n-3, 24:5n-3, and 24:6n-3) were from Larodan Fine Chemicals (Malmö, Sweden). 6-*p*-Toluidine-2-naphthalene sulfonic acid was from Acros Organics (Fair Lawn, NJ). Liquid scintillation cocktail (Ready Safe™) was purchased from Beckman Coulter (Fullerton, CA). Solvents were HPLC-grade and were purchased from Fisher Scientific (Fair Lawn, NJ) or EMD Chemicals (Gibbstown, NJ). Other chemicals and reagents, unless noted otherwise, were purchased from Sigma-Aldrich or Fisher Scientific.

Animals

The protocol was approved by the Animal Care and Use Committee of the National Institute of Child Health and Human Development and followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Publication 80-23). Adult (2 months old) Fischer-344 (CDF) male rats were purchased from Charles River Laboratories (Portage, MI) and were housed for 4 weeks before study in an animal facility with regulated temperature, humidity, and 12 h light/12 h dark cycle. They had free access to water and to rodent chow formulation NIH-31 which contains 4% (w/w) crude fat (Zeigler Bros., Gardners, PA). The fatty acid composition of this chow is described in a prior report (2). Saturated and monounsaturated fatty acids contributed 20.1% and 22.5%, respectively, to its total fatty acid content. The n-3 PUFAs α-LNA, eicosapentaenoic acid (EPA; 20:5n-3), and DHA contributed 5.1%, 2.0%, and 2.3%, respectively, whereas the n-6 PUFAs linoleic acid (LA; 18:2n-6) and arachidonic acid contributed 47.9% and 0.02%, respectively. The 9:1 ratio of LA to α-LNA is close to the recommended dietary balance, but the energy contribution of DHA (0.23% of energy consumed) and of EPA plus DHA (0.45% of energy) in the diet were high compared with the EPA plus DHA contributions in the average diet in the United States (<0.1%) as well as in the recommended diet (0.25%) (16–19).

Surgery

A rat weighing 300 ± 39 g (SD) was anesthetized with 1–3% halothane. Polyethylene catheters filled with heparinized saline (100 IU/ml) were surgically implanted into the right femoral artery and vein, after which the skin was closed and treated with 1% lidocaine for pain control, as described previously (2). The rat was loosely wrapped in a fast-setting plaster cast that was taped to a wooden block, then allowed to recover from anesthesia for 3–4 h. Body temperature was maintained at 36–38°C using a feedback-heating device. Surgery, which took ~20 min, was performed between 10:00 AM and noon. Animals were provided with food the night before surgery.

Tracer infusion

Each rat was infused via the femoral vein catheter with 500 μCi/kg [1-^{14}C]α-LNA (2). An aliquot of [1-^{14}C]α-LNA in ethanol was dried under nitrogen gas, and the residue was dissolved in 5 mM HEPES buffer (pH 7.4) containing 50 mg/ml fatty acid-free BSA to a final volume of 1.3 ml. The mixture was sonicated at 40°C for 20 min and mixed by vortexing. A computer-controlled variable-speed pump (No. 22; Harvard Apparatus, South Natick, MA) was used to infuse the 1.3 ml solution at a rate of 0.223 $(1 - e^{-1.92t})$ ml/min (t in min), which was designed to rapidly establish a steady-state plasma radioactivity (21). Arterial blood was collected in centrifuge tubes at 0, 0.25, 0.5, 0.75, 1.5, 3, 4, and 5 min after starting the infusion. At 5 min, the rat was euthanized by an overdose of sodium pentobarbital (100 mg/kg i.v), and its head and torso were immediately subjected to high-energy focused beam microwave irradiation (5.5 kW, 3.4 s) (model S6F; Cober Electronics; Stamford, CT). Liver weight was recorded, and tissue samples that were confirmed visually to be browned or "cooked" were removed and stored at −80°C until assay. The arterial blood samples were centrifuged at 13,000 rpm for 5 min, and plasma was collected and frozen at −80°C.

Extraction and isolation of liver lipid

Total lipids from liver and plasma were extracted by the procedure of Folch, Lees, and Sloane Stanley (22). Total lipid extracts were separated into neutral lipid subclasses by TLC on silica gel 60 plates (EM Separation Technologies; Gibbstown, NJ), as described previously (3). The bands were scraped, and the silica gel was used directly to quantify radioactivity by scintillation counting and to prepare FAMEs (described below).

In addition to measuring unlabeled total phospholipid concentrations, an aliquot of total lipid extract was added to a tube and dried using a SpeedVac. To measure individual phospholipids, total lipid extracts were separated into phospholipid classes by TLC on silica gel 60 plates (23). The bands were scraped and added to the tube. The silica gel was used directly to analyze phospholipid concentrations. The phosphorous assay followed the method of Rouser, Fkeischer, and Yamamoto (24). To quantify total and free cholesterol and triacylglycerol concentrations, lipid extracts were dried using a SpeedVac, and the residue was dissolved in isopropanol. Total cholesterol and free cholesterol

concentrations were determined with a commercial kit (BioVision Research Products, Mountain View, CA), as was the triacylglycerol concentration (Sigma-Aldrich).

FAME preparation

The FAMEs were used for the GC and HPLC analyses. Lipid and aqueous extracts were methylated with 1% H_2SO_4-methanol for 3 h at 70°C (3, 25). Before methylation, an appropriate quantity of di-17:0 PC (for triacylglycerol, phospholipid, and cholesteryl ester) or of 17:0 fatty acid (for unesterified fatty acid) was added to the sample as an internal standard.

Quantification of radioactivity

Samples for measuring radioactivity were placed in scintillation vials and dissolved with liquid scintillation cocktail (Ready Safe™ plus 1% glacial acetic acid), and their radioactivity was determined using a liquid scintillation analyzer (2200CA, TRI-CARB®; Packard Instruments, Meriden, CT).

GC analysis

Fatty acid concentrations of liver lipids and plasma unesterified fatty acids were determined by GC. GC separation and analysis were performed as described (3), with fatty acid concentrations (nmol/g liver) calculated by proportional comparison of GC peak areas with the area of the 17:0 internal standard. The concentration of unesterified α-LNA in plasma also was determined this way as 41 ± 13 nmol/ml (2).

HPLC analysis

FAMEs from liver lipids were analyzed by HPLC by the method of Aveldano, VanRollins, and Horrocks (26) with modifications. The FAMEs were dissolved in acetonitrile, and the solution was fractionated by reverse-phase column HPLC using a pump (System GOLD 126; Beckman Coulter) outfitted with an ultraviolet light detector (UV/VIS-151; Gilson, Middleton, WI) and an on-line continuous scintillation counter β-RAM detector (β-RAM model 2; IN/US Systems). The reverse-phase column, Luna 5 μ C18 (2) (5 μM particle size, 4.6 × 250 mm), was from Phonomenex (Torrance, CA). Chromatography was performed using a linear gradient system of water and acetonitrile. The acetonitrile was held at 85% for 30 min, increased to 100% over 10 min, and held again at 100% for 20 min. The flow rate was 1.0 ml/min. The ultraviolet light detector was set at 205 nm.

Analysis of long-chain acyl-CoAs

Long-chain acyl-CoAs were extracted from microwaved liver using an affinity chromatography method with slight modifications (27). After 5 nmol of heptadecanoyl-CoA (17:0-CoA) was added as an internal standard to ~1 g of liver, the sample was homogenized in 25 mM KH_2PO_4 (Tissuemizer; Tekmar, Cincinnati, OH). The homogenate was adjusted with isopropanol and acetonitrile to isopropanol /25 mM KH_2PO_4/acetonitrile (1:1:2, v/v/v), then sonicated using a probe sonicator (model W-225; Misonix, Farmingdale, NY). A small volume (~3% of total) of saturated $(NH_4)_2SO_4$ solution was added to the homogenate to precipitate proteins, after which the sample was mixed vigorously for 5 min and centrifuged. The supernatant was washed with hexane (equal volume) to remove nonpolar lipids and then diluted with a 1.25-fold volume of 25 mM KH_2PO_4. Extracting nonpolar lipids with hexane was important, as it markedly improved HPLC separation of acyl-CoAs compared with samples that were not extracted (data not shown). The solution was passed three times through an oligonucleotide purification cartridge (ABI Masterpiece™, OPC®; Applied Biosystems, Foster City, CA), and then the cartridge was washed with 25 mM KH_2PO_4. Acyl-CoA species were eluted with a small volume of isopropanol-1 mM glacial acetic acid (75:25, v/v).

HPLC separation and analysis for acyl-CoA were performed as described (2). Under the HPLC system, 14:0-CoA, EPA-CoA, and α-LNA-CoA coeluted as a single peak (2). This peak was collected and saponified with 2% (w/v) KOH/ethanol at 100°C for 45 min and acidified with HCl, and then fatty acids were extracted with hexane. The unesterified fatty acids were converted to FAMEs and separated by HPLC to measure radioactivity. The concentrations of the FAMEs that came from the acyl-CoA species also were determined by GC. Thus, the concentrations of 14:0, EPA, and α-LNA in the original acyl-CoA peak were determined by proportional comparison of their GC peak areas with each other.

Calculations

The general pulse-labeling equations for determining the in vivo kinetics of a fatty acid in any organ, after the intravenous infusion of a radiolabeled fatty acid to produce a steady-state plasma radioactivity, have been described elsewhere (2, 11, 12, 21, 28). These equations were applied to a model for liver α-LNA metabolism (see Fig. 3 below). In this model, unesterified plasma α-LNA enters the liver unesterified α-LNA pool (not shown in Fig. 3 below), from where it is delivered to the α-LNA-CoA pool through the action of an acyl-CoA synthetase. From there, it can be converted to DHA-CoA by elongation and desaturation enzymes. Both α-LNA and DHA (as well as n-3 PUFA conversion intermediates) can be transacylated from their acyl-CoA forms into phospholipid, triacylglycerol, or cholesteryl ester (called "stable" lipids). The esterified fatty acids can be released from these stable lipids back to the unesterified liver fatty acid pool (data not shown) and then activated again to acyl-CoA by an acyl-CoA synthetase, or they can be secreted into blood while esterified within the stable lipids as packaged in VLDLs. A fatty acid in the liver acyl-CoA pool also can be transferred by carnitine *O*-palmitoyl transferase to mitochondria for β-oxidation (29). Aqueous radiolabeled β-oxidation fragments that are formed (predominantly acetyl-CoA) then can be recycled into cholesterol, saturated long-chain fatty acids, or any number of other products.

Incorporation coefficients $k^*_{i(\alpha\text{-LNA})}$ (ml/s/g liver), representing the transfer of unesterified [1-^{14}C]α-LNA from plasma into stable liver lipid *i*, were calculated as follows:

$$k^*_{i(\alpha-\mathrm{LNA})} = \frac{c^*_{\mathrm{liver},i(\alpha-\mathrm{LNA})}(T)}{\int_0^T c^*_{\mathrm{plasma}(\alpha-\mathrm{LNA})}\,dt} \qquad (Eq.1)$$

where $C^*_{\mathrm{liver},i(\alpha\text{-LNA})}(T)$ (nCi/g liver) is α-LNA radioactivity in *i* at time *T* (5 min) after starting tracer infusion, *t* is time after starting infusion, and $C^*_{\mathrm{plasma}(\alpha\text{-LNA})}$ (nCi/ml plasma) is plasma radioactivity of unesterified α-LNA (2). The coefficient $k^*_{i(\alpha\text{-LNA}\rightarrow\mathrm{DHA})}$ (ml/s/g liver), representing the synthesis of DHA from α-LNA and subsequent DHA incorporation into stable lipid *i*, was calculated as follows:

$$k^*_{i(\alpha-\mathrm{LNA}\rightarrow\mathrm{DHA})} = \frac{c^*_{\mathrm{liver},i(\mathrm{DHA})}(T)}{\int_0^T c^*_{\mathrm{plasma}(\alpha-\mathrm{LNA})}\,dt} \qquad (Eq.2)$$

where $C^*_{\mathrm{liver},i(\mathrm{DHA})}(T)$ (nCi/g liver) is DHA radioactivity in *i* at *T* (5 min).

The rate of incorporation of unlabeled unesterified α-LNA from plasma into liver lipid *i*, $J_{in,i(\alpha\text{-LNA})}$, and the rate of DHA

synthesis from α-LNA followed by DHA incorporation into *i*, $J_{in,i(\alpha\text{-LNA}\rightarrow\text{DHA})}$, were calculated as follows (nmol/s/g liver):

$$J_{in,i(\alpha-LNA)} = k^*_{i(\alpha-LNA)} C_{plasma(\alpha-LNA)} \quad (Eq.3)$$

$$J_{in,i(\alpha-LNA\rightarrow DHA)} = k^*_{i(\alpha-LNA\rightarrow DHA)} C_{plasma(\alpha-LNA)} \quad (Eq.4)$$

where $C_{plasma(\alpha\text{-LNA})}$ is the concentration (nmol/ml) of unlabeled unesterified α-LNA in plasma.

A dilution factor, $\lambda_{\alpha\text{-LNA-CoA}}$, represents the steady-state ratio of liver α-LNA-CoA specific activity to the specific activity of unesterified α-LNA in plasma:

$$\lambda_{\alpha-LNA-CoA} = \frac{C^*_{liver(\alpha-LNA-CoA)}/C_{liver(\alpha-LNA-CoA)}}{C^*_{plasma(\alpha-LNA)}/C_{plasma(\alpha-LNA)}} \quad (Eq.5)$$

The rate of incorporation, $J_{FA,i(\alpha\text{-LNA})}$, of unlabeled α-LNA from the liver α-LNA-CoA pool into lipid *i* equals (nmol/g/s):

$$J_{FA,i(\alpha-LNA)} = J_{in,i(\alpha-LNA)}/\lambda_{\alpha-LNA-CoA} \quad (Eq.6)$$

As seen in Fig. 3 below, this rate equals the rate of deacylation (recycling) of α-LNA from stable lipid *i* plus the rate of α-LNA secretion into blood as part of lipid *i* packaged within VLDLs. Thus, $J_{FA,i(\alpha\text{-LNA})}$ is an upper limit for the rate of secretion of α-LNA within VLDLs.

The turnover rate, $F_{FA,i(\alpha\text{-LNA})}$, of α-LNA within liver lipid *i* equals:

$$F_{FA,i(\alpha-LNA)} = \frac{J_{FA,i(\alpha-LNA)}}{C_{liver,i(\alpha-LNA)}} \quad (Eq.7)$$

where $C_{liver,i(\alpha\text{-LNA})}$ is the concentration of α-LNA in *i*. The corresponding half-life of α-LNA in *i* equals:

$$t_{1/2,i(\alpha-LNA)} = 0.693/F_{FA,i(\alpha-LNA)} \quad (Eq.8)$$

With regard to DHA synthesis, if the specific activities of liver α-LNA-CoA and DHA-CoA have not reached steady states during the 5 min infusion period, the rate of synthesis of DHA from α-LNA followed by DHA incorporation will be less than the actual rate, as shown:

$$\text{Rate of DHA synthesis/esterification} \geq J_{in,i(\alpha-LNA\rightarrow DHA)}/\lambda_{\alpha-LNA-CoA} \quad (Eq.9)$$

Statistical analysis

Data are expressed as means ± SD (n = 6). HPLC profiles for FAMEs made from total lipid, triacylglycerol, phospholipid, and cholesteryl ester (profile not shown) were pooled samples from six animals. Percentage radioactivities corresponding to [1-^{14}C]α-LNA and [^{14}C]DHA in liver lipid fractions were determined from these HPLC profiles. Means ± SD are given, with significance taken as $P \leq 0.05$.

RESULTS

Concentrations of stable liver lipids

Table 1 presents the unlabeled concentrations of "stable" lipids in microwaved liver. The total phospholipid concentration is ~2.5 times the triacylglycerol concentration, which in turn is approximately twice the cholesterol concentration.

TABLE 1. Concentrations of stable lipids in microwaved rat liver

Lipid	Concentration
	μmol/g liver
Triacylglycerol	12.4 ± 5.0
Cholesterol	5.8 ± 0.4
Cholesteryl ester	0.6 ± 0.4
Total phospholipid	29.7 ± 2.1
Phosphatidylcholine	16.4 ± 1.7
Phosphatidylethanolamine	6.6 ± 1.2
Phosphatidylserine	0.8 ± 0.3
Phosphatidylinositol	2.4 ± 0.9
Sphingomyelin	2.4 ± 1.4

Values represent means ± SD (n = 6).

Liver fatty acid composition

Table 2 presents concentrations of unlabeled unesterified fatty acids and of fatty acids esterified in stable liver lipids. α-LNA concentrations in triacylglycerol, phospholipid, and cholesteryl ester were 457 ± 190, 52 ± 12, and 2.7 ± 1.9 nmol/g liver, respectively, whereas corresponding DHA concentrations were much higher: 1,657 ± 716, 5,388 ± 947, and 20 ± 13 nmol/g liver. EPA (20:5n-3) concentrations were 680 ± 141, 656 ± 169, and 15 ± 4 nmol/g liver, respectively. DPA (22:5n-3) concentrations were 1,055 ± 367, 1,122 ± 143, and 3.4 ± 2.0 nmol/g liver, respectively. The ~2-fold concentration ratio of total fatty acids in phospholipid compared with triacylglycerol is consistent with the relative concentrations of these stable lipids in liver (Table 1) and with the fact that triacylglycerol contains three rather than two esterified long-chain fatty acids.

Distribution of radioactivity

Figures 1A, B and **Table 3** provide values for radioactivity in the different liver compartments after 5 min of [1-^{14}C]α-LNA infusion. Total lipid radioactivity was calculated when excluding unesterified fatty acid radioactivity, as we could not distinguish intravascular from parenchymal contributions. Total radioactivity equaled 3,300 ± 649 nCi/g liver, of which 2,423 ± 561 nCi/g liver (72.7%) was in the lipid fraction and the remainder, 877 ± 85 nCi/g liver (27.3%), was in the aqueous fraction. Radioactivity in triacylglycerol, phospholipid, and cholesteryl ester equaled 1,737 ± 618, 532 ± 304, and 17.4 ± 6.4 nCi/g liver (53, 16, and 0.53% of total liver radioactivity), respectively (Fig. 1B).

As illustrated in Table 3, radioactivity attributable to [1-^{14}C]α-LNA in triacylglycerol, phospholipid, and cholesteryl ester equaled 1,263 ± 449, 472 ± 269, and 4.6 ± 0.9 nCi/g liver, compared with 3.9 ± 2.5, 1.6 ± 0.9, and 0.001 ± 0.001 nCi/g, respectively, for [^{14}C]DHA. Thus, the DHA/α-LNA radioactivity ratio in triacylglycerol, phospholipid, and cholesteryl ester equaled 0.3, 0.3, and 0.02%, respectively.

Radiolabeled intermediates along the expected pathways of conversion of [1-^{14}C]α-LNA to [^{14}C]DHA (1, 30) could be detected in HPLC chromatograms of total lipid, triacylglycerol, phospholipid, and cholesteryl ester (**Fig. 2**).

TABLE 2. Fatty acid composition of liver lipids

Fatty Acid	Esterified Fatty Acid			Unesterified Fatty Acid
	Triacylglycerol	Phospholipid	Cholesteryl Ester	
	nmol/g liver			
14:0	288 ± 91	93 ± 9	3.5 ± 2.5	6.0 ± 3.2
16:0	10,289 ± 3,268	11,407 ± 822	227 ± 45	275 ± 115
16:1n-7	1,166 ± 404	519 ± 108	27 ± 9	16 ± 9
18:0	653 ± 198	14,182 ± 1769	90 ± 34	70 ± 18
18:1n-7	1,319 ± 423	1,977 ± 155	18 ± 9	27 ± 12
18:1n-9	7,044 ± 2,004	1,603 ± 195	86 ± 31	79 ± 18
18:2n-6	8,308 ± 2,725	8,595 ± 776	127 ± 46	132 ± 90
18:3n-3	457 ± 190	52 ± 12	2.7 ± 1.9	6.7 ± 5.6
20:4n-6	824 ± 175	12,963 ± 1258	144 ± 47	49 ± 21
20:5n-3	680 ± 141	656 ± 169	15 ± 4	30 ± 26
22:5n-6	59 ± 12	108 ± 28	1.0 ± 0.6	4.7 ± 3.4
22:5n-3	1,055 ± 367	1,122 ± 143	3.4 ± 2.0	18 ± 16
22:6n-3	1,657 ± 716	5,388 ± 947	20 ± 13	34 ± 26
Total	33,800 ± 10,714	58,663 ± 6,391	764 ± 244	748 ± 382

Values represent means ± SD (n = 6).

Of liver lipid radioactivity excluding radiolabeled unesterified fatty acids (2,423 nCi/g liver), radioactivity of the n-3 intermediates 18:4, 20:3, 20:4, 20:5, 22:5, 24:5, and 24:6 equaled 5.0, 2.6, 3.4, 9.5, 1.8, 1.8, and 0.2% (sum = 24.3%), respectively, compared with 0.3% for 22:6 and 75.4% for α-LNA (18:3). Each of the radiolabeled intermediates was identified in triacylglycerol and phospholipid (Fig. 2). Radiolabels of 14:0, 16:0, 18:0, and 18:1, which would have come from radiolabeled carbon recycling, were not detected.

Liver acyl-CoA concentration and associated radioactivity

HPLC separation of aqueous liver acyl-CoA extract yielded unlabeled concentrations and radioactivities of individual acyl-CoA species in the aqueous liver compartment (**Table 4**). Concentrations of unlabeled α-LNA-CoA, EPA-CoA, and DHA-CoA equaled 1.2 ± 1.1, 1.6 ± 0.97, and 2.2 ± 0.8 nmol/g liver, respectively. Their respective radioactivities equaled 21.0 ± 19.3, 5.0 ± 3.4, and 8.5 ± 8.3 nCi/g liver, giving specific activities of 17.5, 3.1, and 3.9 nCi/nmol, respectively. The lower specific activities of EPA-CoA and DHA-CoA likely reflect the contributions of unlabeled unesterified plasma EPA and DHA to these respective pools and possibly the nonattainment of a steady state. Radiolabeling also was evident of 14:0-CoA, 16:0-CoA, 18:0-CoA, and 18:1-CoA, indicating recycling of radiolabeled carbon from [1-^{14}C]α-LNA.

Distribution of liver radioactivity

Figure 3 summarizes the disposition of plasma-derived unesterified [1-^{14}C]α-LNA and its metabolic products in liver after the 5 min tracer infusion. Of total liver radioactivity, 72.7% was in the lipid fraction and 27.3% was in the aqueous phase (Fig. 2). Of stable lipid radioactivity, 52.0% was in triacylglycerol, 15.9% was in phospholipid, 0.5% was in cholesteryl ester, and 0.5% was in other lipids (Fig. 1B). Of cholesteryl ester radioactivity (0.5%), the sterol body and acyl chain components contained 0.3% and 0.2%, respectively.

[1-^{14}C]α-LNA, [^{14}C]DHA, and all of the [^{14}C]n-3 acyl intermediates involved in [^{14}C]DHA synthesis constituted 51.7, 0.2, and 16.7%, respectively, of net liver lipid radioactivity, whereas radiolabeled α-LNA-CoA, EPA-CoA, and DHA-CoA contributed 0.6, 0.2, and 0.3%, respectively.

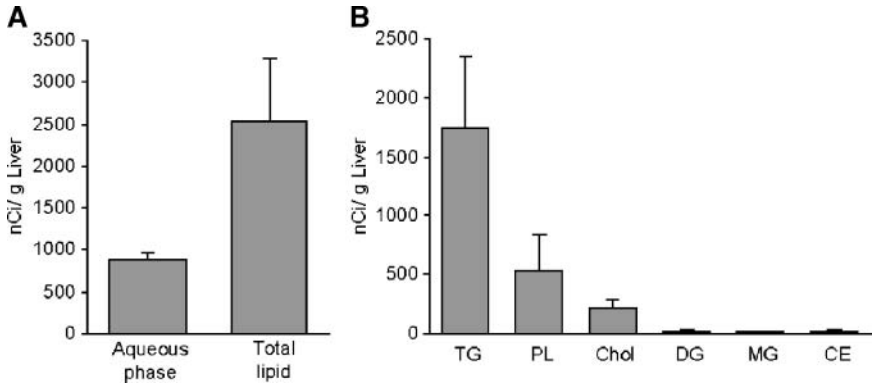

Fig. 1. Radioactivity distribution in whole liver (aqueous + total lipid fractions) (A) and liver neutral lipid classes (B) after a 5 min intravenous infusion of [1-^{14}C]α-linolenic acid (α-LNA; 18:3n-3). TG, triacylglycerol; PL, phospholipid; Chol, cholesterol; DG, diacylglycerol; MG, monoacylglycerol; CE, cholesteryl ester. Values are means ± SD (n = 6).

TABLE 3. Radioactivity, incorporation coefficients, and incorporation rates of unesterified plasma α-LNA into different liver lipid components

Lipid	Radioactivity	Incorporation Coefficients	Incorporation Rates
	nCi/g liver	*ml/s/g* $\times 10^{-4}$	*nmol/s/g* $\times 10^{-4}$
Total (aqueous + total lipids)	3,300 ± 649		
Total lipids	2,423 ± 561		
α-LNA	1,827 ± 464		
DHA	7.27 ± 1.8		
Triacylglycerol	1,737 ± 618		
α-LNA	1,263 ± 449	22.5 ± 7.7	1,000 ± 622
DHA	3.9 ± 2.5	0.1 ± 0.04	5.5 ± 3.4
Phospholipid	532 ± 304		
α-LNA	472 ± 269	7.9 ± 2.2	312 ± 94
DHA	1.6 ± 0.9	0.03 ± 0.01	1.1 ± 0.3
Cholesteryl ester	17.5 ± 6.3		
α-LNA	4.6 ± 0.9	0.1 ± 0.02	3.7 ± 1.9
DHA	0.001 ± 0.001	0.0001 ± 0.00002	0.004 ± 0.002

DHA, docosahexaenoic acid (22:6n-3); α-LNA, α-linolenic acid (18:3n-3). Values represent means ± SD (n = 6).

Radioactive saturated and monounsaturated fatty acids were not detected, although 2.2% of net radioactivity was in saturated and monounsaturated acyl-CoA species (14:0, 16:0, 18:0, and 18:1n-9).

α-LNA incorporation and turnover rates in liver lipids

We used equations 1–9 to calculate kinetic rate parameters of unlabeled α-LNA and DHA in liver using the relevant labeled and unlabeled concentrations. In this analysis, we used our individual published unesterified plasma α-LNA concentrations for these animals (mean = 41 ± 13 nmol/ml plasma) (equations 3, 4), individual values for integrated plasma α-LNA radioactivity over the 5 min infusion period (input function; mean = 571,870 ± 141,337 nCi/ml plasma/s) (equation 1), and individual values for plasma specific activity (mean = 66 ± 43 nCi/

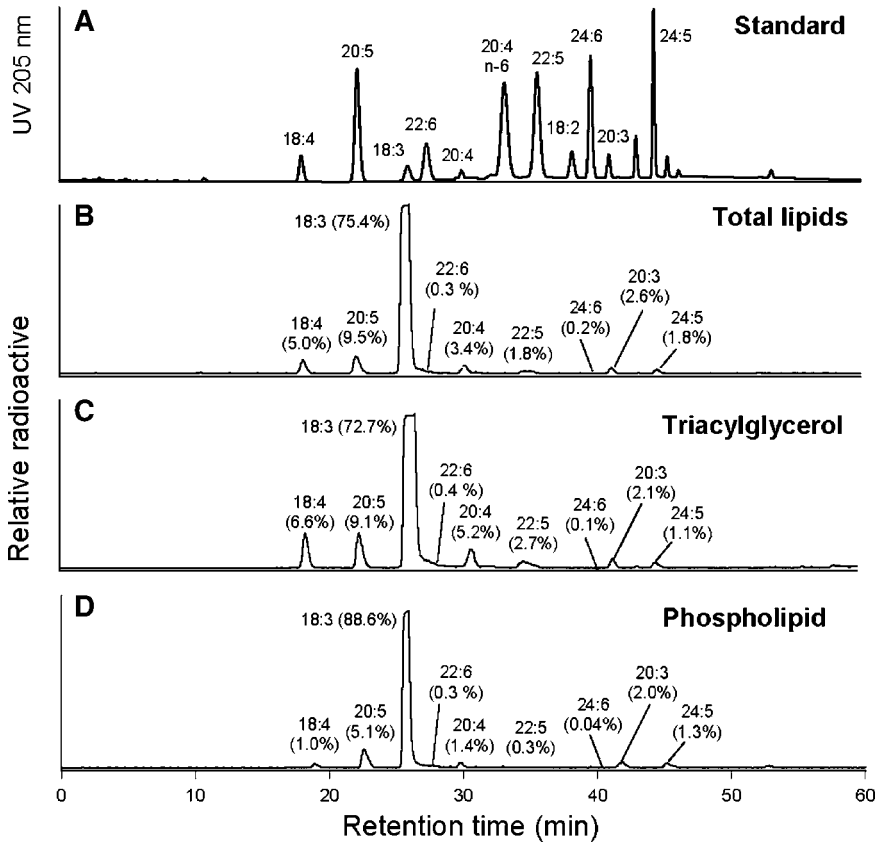

Fig. 2. HPLC chromatograms of fatty acid methyl ester (FAME) standards (A), total lipid radioactivity (B), triacylglycerol radioactivity (C), and phospholipid radioactivity (D) prepared from liver lipid extracts of rats infused intravenously with [1-^{14}C]α-LNA. Ultraviolet light absorbance was monitored at 205 nm for standards. Samples from six animals were pooled to generate each FAME profile.

TABLE 4. Acyl-CoA concentrations and their corresponding radioactivities in rat liver after 5 min of [1-^{14}C]α-LNA infusion

Acyl-CoA	Concentration	Radioactivity	Specific Activity
	nmol/g liver	*nCi/g liver*	*nCi/nmol*
18:3-CoA	1.2 ± 1.1	21.0 ± 19.3	17.5
Eicosapentaenoic acid-CoA	1.6 ± 0.97	5.0 ± 3.4	3.1
DHA-CoA	2.2 ± 0.75	8.5 ± 8.3	3.9
14:0-CoA	0.96 ± 0.94	3.4 ± 2.4	
16:0-CoA	15.1 ± 10.4	27.6 ± 4.6	
18:0-CoA	3.1 ± 1.7	24.4 ± 16.1	
18:1-CoA	3.1 ± 1.8	18.1 ± 12.9	

Values represent means ± SD (n = 6).

nmol) (2). In that study, we also reported that plasma specific activity had reached a steady state by 0.5 min into the [1-^{14}C]α-LNA infusion period and that plasma triacylglycerol, phospholipid, and cholesteryl ester contained traces (0.95, 0.52, and 0.076%, respectively) of total plasma radioactivity at 5 min, whereas the unesterified fatty acid fraction contained 97%. Additionally, ~96% of plasma radioactivity was unesterified [1-^{14}C]α-LNA, whereas ≤0.2% was [^{14}C]DHA. Radioactive plasma [^{14}C]EPA was not detected.

The second and third data columns in Table 3 provide incorporation coefficients and incorporation rates of plasma-derived α-LNA entering liver triacylglycerol, phospholipid, and cholesteryl ester. Values for $k^*_{i(\alpha\text{-LNA})}$ were >200-fold the values for $k^*_{i(\alpha\text{-LNA}\rightarrow\text{DHA})}$. Multiplying each incorporation coefficient by the unesterified unlabeled α-LNA plasma concentration for the animal [mean = 41 ± 13 nmol/ml (2)] gave respective incorporation rates $J_{in,i}$ for α-LNA and DHA in the same ratios as the incorporation coefficients. These data suggest negligible conversion of plasma-derived unesterified α-LNA to esterified DHA within stable liver lipids within the 5 min study period compared with direct esterification of unchanged α-LNA.

After the 5 min [1-^{14}C]α-LNA infusion, the mean dilution coefficient $\lambda_{\alpha\text{-LNA-CoA}}$ (equation 5) of liver α-LNA-CoA equaled 0.42 ± 0.28 (n = 6) (**Table 5**) and did not differ significantly ($P > 0.05$) from the value that we determined after 3 min of infusion, 0.28 ± 0.21 (n = 5). Thus, $\lambda_{\alpha\text{-LNA-CoA}}$ had reached a steady state by 5 min, a requirement for the exact application of our kinetic model (21, 28). Using 0.42 for $\lambda_{\alpha\text{-LNA-CoA}}$, we calculated rates $J_{FA,i(\alpha\text{-LNA})}$ of incorporation of unlabeled α-LNA into stable liver lipids *i* from the α-LNA-CoA pool (equation 6) and corresponding turnover rates $J_{FA,i(\alpha\text{-LNA})}$ (equation 7) and half-lives (equation 8) of α-LNA in *i* (Table 5). For triacylglycerol, the stable lipid into which most [1-^{14}C]α-LNA was incorporated, $J_{FA,i(\alpha\text{-LNA})}$, $F_{FA,i(\alpha\text{-LNA})}$, and half-life equaled 2,401 ± 1,363 nmol/s/g × 10^{-4}, 3.20 ± 1.66%/min, and 21.7 min, respectively; for phospholipid, the respective values equaled 749 ± 207 nmol/s/g × 10^{-4}, 8.7 ± 2.1%/min, and 8.0 min; for cholesteryl ester, the respective values equaled 9.61 ± 4.47 nmol/s/g × 10^{-4}, 2.90 ± 1.63%/min, and 23.4 min. Thus, α-LNA half-lives in the three stable lipids ranged from 8 to 24 min.

Mean net $J_{FA,i(\alpha\text{-LNA}\rightarrow\text{DHA})}$ calculated from mean net $J_{in,i(\alpha\text{-LNA}\rightarrow\text{DHA})}$ in Table 3 by equation 9 equaled (6.6 nmol/s/g × 10^{-4})/0.42 = 15.8 nmol/s/g × 10^{-4}. This rate represents a lower limit for the rate of synthesis of DHA from α-LNA in the liver. It is only 0.5% of the total α-LNA incorporation rate into the three stable lipids, 3,164 nmol/s/g × 10^{-4} (Table 5).

DISCUSSION

In this study, we extended our general pulse-labeling infusion model (2, 11, 28, 31) to estimate, in unanesthetized rats, rates of unesterified α-LNA incorporation into stable liver lipids and corresponding turnover rates and half-lives, as well as a lower limit for the rate of α-LNA

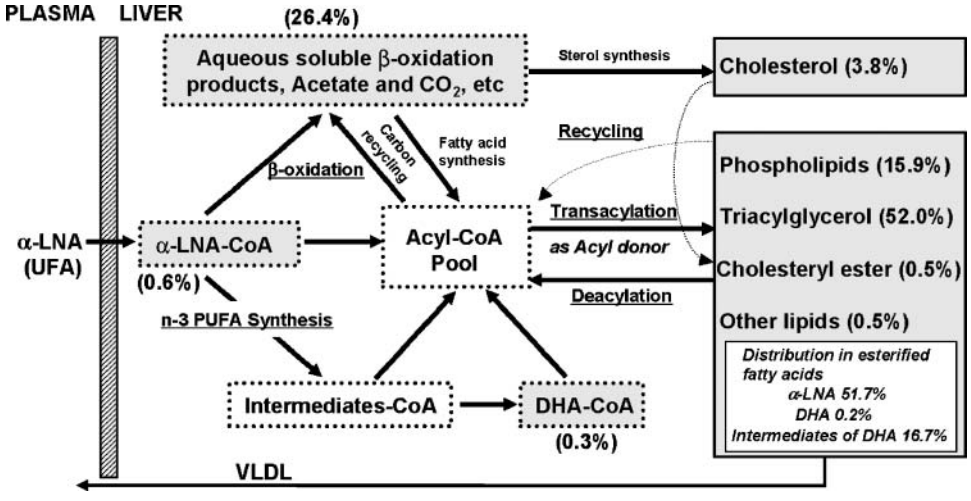

Fig. 3. Percentage distribution of radioactivity in liver compartments of rats at 5 min after intravenous infusion of [1-^{14}C]α-LNA. Percentages were calculated by dividing radioactivity in each compartment by the net liver (excluding unesterified fatty acid) radioactivity. The back arrow to blood represents secretion within VLDL. DHA, docosahexaenoic acid (22:6n-3).

TABLE 5. Rates of incorporation of α-LNA from the liver α-LNA-CoA pool, and turnover and half-life, in stable liver lipids

Lipid	$\lambda_{\alpha\text{-LNA-CoA}}$	$J_{FA,\ i\ (\alpha\text{-LNA})}$	$F_{FA,\ i\ (\alpha\text{-LNA})}$	Half-Life
		nmol/s/g × 10⁻⁴	*%/min*	*min*
Triacylglycerol	0.42 ± 0.28	2,401 ± 1,363	3.20 ± 1.66	21.7
Phospholipid		749 ± 207	8.70 ± 2.10	8.0
Cholesteryl ester		9.61 ± 4.47	2.90 ± 1.63	23.4
Total		3,160		

Values represent means ± SD (n = 6).

conversion to DHA followed by incorporation of this DHA into the stable lipids. Our rats were adult males fed a diet abundant in DHA. Our ability to calculate rates and related kinetic parameters depended on our taking into account integrated plasma radioactivity (plasma input function) during the 5 min [1-^{14}C]α-LNA infusion as well as $\lambda_{\alpha\text{-LNA-CoA}}$, the ratio of steady-state liver α-LNA-CoA specific activity to that of unesterified plasma α-LNA.

In vivo incorporation rates of unesterified plasma α-LNA have not been reported, as far as we know. Our calculated incorporation rate into liver phospholipid plus triacylglycerol, 1,312 nmol/s/g × 10^{-4} (475.2 nmol/h/g) (Table 3), is equivalent to 3,684 nmol/h/mg protein [taking liver protein content as 12.9% wet weight (32)]. This in vivo rate is 75 times the rate of 50 nmol/h/mg protein reported for α-LNA incorporation into glycerolipids in isolated hepatocytes from adult rats fed Purina Rodent Chow (33), which attests to the importance of making in vivo rather than in vitro measurements. When taking into account the steady-state value of 0.42 for the dilution coefficient $\lambda_{\alpha\text{-LNA-CoA}}$, rates of incorporation $J_{FA,i(\alpha\text{-LNA})}$ of α-LNA from the α-LNA-CoA pool equaled 2,401, 749, and 9.6 nmol/s/g × 10^{-4}, respectively (sum = 3,160 nmol/s/g × 10^{-4}).

$J_{FA,i(\alpha\text{-LNA})}$ may largely reflect the secretion into blood of α-LNA within stable lipids packaged in VLDLs (equation 6). VLDL secretion involves the translocation of apolipoprotein B across the endoplasmic reticular membrane, is facilitated by a microsomal triglyceride transfer protein, and is regulated by insulin (34). It is a slow process compared with our 5 min infusion study period, because radiolabeled VLDLs appear in plasma of unanesthetized rats only 15–20 min after the liver has been exposed to radiolabeled fatty acids through the oral route (35). The actual secretion rate might be measured in longer term in situ liver perfusion or feeding experiments or in vitro (35–38). In cultured hepatocytes, the fraction of triglyceride secreted ranges from 35% in the absence of insulin to 65% in the presence of insulin, with the remainder being recycled via biolysis (39, 40). VLDL secretion rates have been reported for the human liver (37).

The fact that 72.7% of net liver radioactivity after 5 min of [1-^{14}C]α-LNA infusion was in stable lipids (of which 52.0% was in triacylglycerol and 15.9% was in phospholipid) agrees roughly with prior longer term studies. One study reported that 90% of net radioactivity was in liver lipid (60% in triacylglycerol) 2 h after [1-^{14}C]α-LNA perfusion of the in situ rat liver (9). Another indicated that 92% of net radioactivity was in triacylglycerol at 22 h after intraperitoneal infusion of [1-^{14}C]α-LNA and that 2.4% of triacylglycerol fatty acid radioactivity was labeled DHA (10). Thirty minutes after intravenously infusing 2 week old rats with [1-^{14}C]α-LNA, ~95% of radioactivity was in stable liver lipids (36).

α-LNA is elongated and desaturated to 24:6n-3 by the following steps, 18:3→18:4→20:4→20:5→22:5→24:5→24:6, after which 24:6n-3 is shortened to DHA by one round of β-oxidation in peroxisomes (1, 30). Radiolabels of these synthesis intermediates were detected in liver (total 21.7% of net radioactivity). Radioactive 24:6n-3 was at a trace level compared with the other labeled intermediates (9- to 25-fold less) (Fig. 2). This may have been attributable to the high DHA content of the diet, as conversion of 22:5n-3, 24:5n-3, and 24:6n-3 to DHA was reduced in rat brain astrocytes cultured in a DHA-containing medium (41). An additional pathway of α-LNA metabolism has been reported, in which 20:3n-3, 22:4n-3, and 24:4n-3 intermediates are converted to 24:5n-3 and then to DHA in the usual manner (42), and we also detected radiolabeled 20:3n-3 in stable lipid (2.6% of net radioactivity) (Fig. 2). We did not detect esterified radiolabeled 14:0, 16:0, 18:0, or 18:1, which would have been derived from β-oxidation products of [1-^{14}C]α-LNA, but we did detect radiolabeled 14:0-CoA, 16:0-CoA, 18:0-CoA, and 18:1-CoA (which contributed ~2.2% of its net liver radioactivity) (Table 4, Fig. 3).

Of net liver radioactivity (excluding unesterified labeled fatty acids), ≤0.5% was attributable to [^{14}C]DHA (Fig. 3), of which 0.3% was [^{14}C]DHA-CoA and 0.2% was [^{14}C]DHA esterified in triacylglycerol, phospholipid, and cholesteryl ester. In contrast, 51.7% of liver radioactivity at 5 min was esterified [1-^{14}C]α-LNA, and 0.6% was [1-^{14}C]α-LNA-CoA. Only a small fraction of 5 min liver radioactivity was esterified labeled DHA. We estimated a lower limit for the rate of synthesis of α-LNA to DHA, and DHA esterification into stable liver lipid, as 15.8 nmol/s/g × 10^{-4}. This value was only 0.5% of the rate of 3,164 nmol/s/g × 10^{-4} for the direct incorporation of α-LNA into stable lipids from the α-LNA-CoA pool. Perhaps longer term infusion studies could help to better ascertain the actual DHA synthesis rate using a serial compartmental analysis (43, 44).

In the same rats fed a high-DHA-containing diet, we recently used our in vivo kinetic model to estimate that the rate of brain DHA synthesis from α-LNA equaled 0.22 nmol/s/g × 10^{-4}, 1% of the reported rate of incorporation of plasma unesterified plasma DHA into brain phospholipids, 22 nmol/s/g × 10^{-4} (3, 45, 46). Our cur-

rent study showing that DHA synthesis from α-LNA in liver is ~15.8 nmol/s/g × 10^{-4} indicates that DHA synthesis from α-LNA in the liver does not contribute to brain DHA in these rats, because 1% or less of the DHA secreted within VLDLs, once released by hydrolysis, would be expected to be taken up by brain (28, 31).

Approximately 30% of the plasma [1-^{14}C]α-LNA that entered liver was converted to aqueous β-oxidation products (26.4%) and the cholesterol (3.8%) derived from such products. As some [^{14}C]CO_2 may have been lost by respiration, the 30% is only an approximation of the oxidation fraction. In this regard, α-LNA is reported to be β-oxidized more rapidly than other C18 fatty acids and to be transported more rapidly than DHA by carnitine *O*-palmitoyl transferase into liver mitochondria (29, 47, 48).

Plasma fatty acids can enter liver when unesterified by passive diffusion or facilitated transport (49, 50) or via hepatocyte lipoprotein receptors when esterified within lipoproteins (51–56). The dilution coefficient $\lambda_{\alpha\text{-LNA-CoA}}$ (equation 4) equals the steady-state rate of entry of unesterified plasma α-LNA into the liver α-LNA-CoA pool, divided by the sum of this rate and rates of entry via plasma lipoproteins and from deacylation of stable lipids (because α-LNA cannot be synthesized de novo, its synthesis makes no contribution to α-LNA-CoA) (11, 12, 28). Thus, the 5 min value of 0.42 for $\lambda_{\alpha\text{-LNA-CoA}}$, which we confirmed to be a steady-state value by doing a 3 min determination as well, means that 42% of liver α-LNA-CoA came from unesterified plasma α-LNA and 58% came from the other sources.

Our concentrations of stable lipids in microwaved liver agree generally with published concentrations in non-microwaved liver (57, 58). Our unlabeled liver acyl-CoA concentrations, although in the range of some reported values, differ for some acyl-CoA species by >4-fold from those values (59–62). These differences may be attributable to differences in diet, methods for measuring acyl-CoA, or to the fact that we but not others analyzed microwaved liver (59–62). Transient ischemia in the absence of microwaving can cause marked changes in tissue concentrations of unesterified fatty acids, their metabolites, and acyl-CoAs (13–15).

In our rats fed a diet containing 5.1% (w/w total fatty acids) α-LNA, α-LNA constituted 0.6% of net liver fatty acid (Table 2). In comparison, α-LNA equaled 0.2% of liver fatty acid in rats fed a diet containing 0.13% (w/w) α-LNA (63) and 0.31% in rats fed a diet containing 0.1% α-LNA (64). Clearly, the liver α-LNA concentration depends on dietary α-LNA. Additionally, the conversion of α-LNA to DHA depends on the dietary content of n-3 PUFAs (4, 65–68). One reason for this is that the transcription of genes for the Δ5 and Δ6 desaturases is influenced by n-3 PUFA levels (69). Additionally, the transcription factor sterol-regulatory element binding protein-1, which positively regulates the transcription of the Δ5 and Δ6 desaturase genes, is reduced in the liver of rats fed DHA (4–6).

Our diet had a high DHA content [2.3% (w/w) of total fatty acid] and a high energy contribution (0.23%) compared with the respective values for EPA plus DPA in the average human diet in the United States (<0.1% of energy) (16–19). Thus, it is possible that the high rate of α-LNA oxidation and its low rate of conversion to DHA in this study reflected disproportionately high dietary n-3 PUFA levels. We could test this possibility directly, using our kinetics model, by comparing our results with results from rats with no n-3 PUFAs or just α-LNA but no DHA in their diet. Additionally, we could determine α-LNA incorporation rates and half-lives and rates of β-oxidation in animals in which α-LNA conversion to DHA would be expected to be disturbed (models of type I diabetes, peroxisomal disorders, alcoholism) (52, 70, 71) as well as during fetal and postnatal development (7, 72, 73).

The α-LNA incorporation rate $J_{FA,i(\alpha\text{-LNA})}$ into liver phospholipid equaled 749 ± 207 nmol/s/g × 10^{-4} (Table 5), 52 times the published rate of 14.3 nmol/s/g × 10^{-4} for α-LNA incorporation into brain phospholipid in the same animals (2). The higher rate for the liver is consistent with the fact the liver secretes incorporated α-LNA and DHA within VLDLs, whereas esterified n-3 PUFAs in brain are largely recycled and retained (3, 45). As expected from the high incorporation rate, half-lives of α-LNA equaled 21.7, 8, and 23.4 min, respectively, in triacylglycerol, phospholipid, and cholesteryl ester (Table 3). These are short compared with those in brain, in which α-LNA half-life in phospholipid equaled 78 min (2), and reflect the high-rates of lipoprotein secretion and α-LNA metabolism by the liver.

In summary, we have estimated for the first time exact rates of incorporation of α-LNA from the plasma and the liver precursor α-LNA-CoA pool into stable liver lipids, triacylglycerol, phospholipid, and cholesteryl ester, in unanesthetized rats fed a high-DHA-containing diet. High uptake, incorporation, and turnover rates of α-LNA are consistent with the role of the liver in secreting stable lipids packaged within VLDLs. We also have estimated a lower limit for the rate of conversion of α-LNA to DHA in the liver. This value is only 0.5% of the rate of esterification of α-LNA into stable lipids, again suggesting that liver synthesis of DHA does not provide significant DHA to the brain in rats with a high dietary DHA content.

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