

Long Chain Polyunsaturated Fatty Acid Formation in Neonates: Effect of Gestational Age and Intrauterine Growth

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ABSTRACT

The present study was designed to evaluate the effect of gestational age and intrauterine growth on the long chain polyunsaturated fatty acid (LCP) synthesis from dietary precursors in neonates as reflected by plasma pools. These have been considered conditionally essential nutrients for normal growth, sensory maturation, and neurodevelopment. *In vivo* elongation/desaturation of deuterated d5-linoleic acid (d5-LA) to form arachidonic acid (AA), and d5- α -linolenic acid (d5-LNA) to form docosahexaenoic acid (DHA), was studied in 19 preterm appropriate-for-gestational-age (AGA) infants, 11 AGA term, and 11 intrauterine growth-retarded (IUGR) infants. They received a dose of 50 mg/kg body weight of d5-LA and d5-LNA enterally during the first days of life; d5-labeled derivatized fatty acids were determined in blood samples obtained at 24, 48, and 96 h after dosing. Lipids were extracted and fatty acids analyzed by gas chromatography and negative ion mass spectrometry. Mean concentrations, $\mu\text{g/mL}$, and d5/d0 for n-3 and n-6 precursor and products were computed at various times and were also integrated over the complete study period. Significantly higher time-integrated concentration of d5-AA and d5-DHA were observed in preterm infants relative to the other two groups. Time-integrated enrichment of DHA relative to LNA was 100-fold lower in preterms, 410-fold lower in term, and 27-fold lower in IUGR infants. Similar significant declines in product to precursor enrichments were noted for the n-6 series. A significant

negative correlation of AA and DHA formation based on time-integrated d5/d0 ratios with gestational age was noted; product/precursor enrichment *versus* gas chromatography for the n-6 series had an r of -0.5 , $p = 0.001$, and for the n-3 series had an r of -0.6 , $p = 0.0001$. Birth weight or weight adequacy did not add further strength to the relationship. We conclude that LCP formation from deuterated precursors occurs as early as 26 wk gestation, and in fact is more active at earlier gestational ages; growth retardation appears to slow down or diminish LCP formation. No quantitative estimates of LCP synthesis or nutritional sufficiency can be derived from these data. (*Pediatr Res* 47: 127-135, 2000)

Abbreviations

AA, arachidonic acid
d, deuterated
DHA, docosahexaenoic acid
GA, gestational age
AGA, adequate for gestational age
GC, gas chromatography
IUGR, intrauterine growth retardation
LCP, long chain polyunsaturated fatty acid(s)
LA, linoleic acid
LNA, α -linolenic acid

LCP are fatty acids with 20 or more-carbon atoms with two or more unsaturated bonds. They are necessary for normal development as structural components of lipid membranes and serve as precursors for the synthesis of bioactive eicosanoids. They also play an important role in determining lipid-protein

interactions in key membrane domains, which affect activity of receptors, ion channels, enzymes, and the uptake and release of compounds to and from the cell. During the last trimester of gestation there is a significant accumulation of LCP in the fetus and an increase in the relative content of both n-6 and n-3 LCP in the human brain and retina. The intrauterine accretion and the supply of LCP by the early diet are critical in determining their concentration in plasma and tissue pools. Human milk provides preformed LCP as AA, a 20-carbon 4 double bond n-6 fatty acid, and docosahexaenoic acid (DHA), a 22-carbon, 6 double bond, n-3 fatty acid; these are well absorbed and

Received March 18, 1999; accepted August 14, 1999.

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This work was supported by Fondo Nacional de Ciencia y Tecnología (Fondecyt) Chile grant 1960001. R.U. is the recipient of a Presidential Award in Science given by the Chilean Government.

readily used. Formulas presently available in North America containing mixed vegetable oils include sources of 18-carbon essential precursors for LCP biosynthesis, LA for the n-6 series, and LNA for the n-3 series, but provide no preformed LCP. Several recent reviews provide additional background on LCP metabolism and function (1–3).

Research over the past decade provides ample evidence that the supply of LCP affects the composition of phospholipids in primate brain and retina. In addition, the function of developing photoreceptors as measured by rod electroretinography, the maturation of the visual cortex as measured by behavioral tests and electrophysiologic measurements, and other CNS functions are affected in human preterm as well as term infants (4–6). The long-term significance of these changes is a topic of present research efforts and some controversy. We, and others, in 1996 established that human infants could form essential LCP from 18 carbon precursors using stable isotope-labeled LA and LNA as *in vivo* tracers (7–10).

The present study was designed to evaluate the effect of gestational age and intrauterine growth on the LCP synthesis from dietary precursors in neonates as reflected by plasma pools. This is of particular relevance in defining nutritional needs of infants, because LCP have been considered by some international technical bodies as conditionally essential nutrients; that is, they are not formed in appropriate amounts by endogenous biosynthesis from dietary precursors (11–13). The recent USA/FDA-sponsored, Life Sciences Research Office report of a state-of-the-art scientific review of the nutritional needs of healthy term infants did not find sufficient supportive evidence to include LCP among the list of required nutrients for term infant artificial formula at this time. The expert panel enthusiastically endorsed present efforts to assess the effects of supplementing infant formula with LCP and recommended that this question be reassessed within 5 y (14). However, two concurrent scientific studies published in 1998 evaluating the effect of LCP supplementation on sensory maturation and neurodevelopment concluded that term infants benefit from receiving formula containing DHA and AA (15, 16).

METHODS

Subjects included in this study were clinically stable, preterm and full-term infants admitted to the neonatal unit at Hospital Sótero del Río in Santiago, Chile, between March 1996 and September 1998. They were AGA according to the Lubchenco standard, or were considered IUGR, based on fetal ultrasound with postnatal confirmation of small-for-gestational-age, defined as weight below the 10 th percentile for GA and clinical evidence of IUGR (17, 18). Gestational age was assessed by last menstrual period or with early ultrasound, and confirmed by the modified Ballard evaluation conducted soon after birth (19). Entry criteria were: infants admitted to the nursery for non-life-threatening conditions, clinically stable, and parental informed approval of infant participation. Infants with apparent malformations, unable to receive the enteral dose of tracer fatty acids for any reason or with major malformations were excluded. Most preterm infants studied presented with mild to moderate forms of respiratory distress,

whereas term infants often had mild forms of hypoxic insult or transient respiratory problems. Most subjects entered the study at 48 h and all by 96 h after birth. Decision on when to start enteral feedings was made by the attending neonatologist based on nursery routine, ranging from 3 to 96 h after birth. The type of feeding varied depending on mother's willingness to provide breast milk; if breast milk was not available, infants received formula devoid of long chain essential fatty acids. Our laboratory has analyzed breast milk from Chilean mothers after 15 d of lactation, finding that DHA ranges from 0.4–0.7% and AA from 0.5–0.8%; the ratio of AA to DHA is close to 1. Preterm formula used in the nursery contained LA 14–19%, LNA 1.3–2.5% total lipids, whereas term formula contained LA 13–33% and LNA 1.1–4.2% total lipids. Most patients received minimal amounts of formula over the 96 h of study, reaching not more than 30–50 mL/kg at the end of the study. Patients that could not be fed enterally received parenteral glucose from soon after birth; crystalline amino acids were added by d 3 of life. None of the patients included in this study received i.v. lipids, before or during the study period. Written informed consent was obtained shortly after birth, by a trained research nurse supervised by one of us (P.M.) either before or soon after feedings were begun. The study was approved by INTA's ethics committee and by the research committee at Hospital Sótero del Río, responsible for supervising research involving human subjects. The protocol was also approved by the NIAAA Institutional Review Board and received a Single Project Assurance from the Office for the Protection from Research Risks, DHHS, for international collaboration.

By d 4 of life, all subjects received 50 mg/kg of d5-LA and the same amount of d5-LNA as ethyl esters provided by Cambridge Isotope Labs (Andover, MA) by the nasogastric route. Blood was drawn (0.5 mL) from a peripheral vein or umbilical catheter, into a tube containing EDTA as anticoagulant, 24, 48, and 96 h after dosing. Plasma was separated by centrifugation shortly after sampling, divided into aliquots, and kept frozen until lipids were analyzed. Methodology for fatty acid analysis has been published but will be described briefly (7). We extracted lipids from 100 μ L plasma by using the Folch and Lees method (20), adding 50 μ g of BHT to prevent oxidation. Fatty acids were methylated with boron trifluoride-methanol according to the method of Morrison and Smith (21). Quantification was achieved by capillary gas chromatography using flame ionization detection; peaks were identified based on retention times of known standards; concentrations were calculated using C 23:0 as internal standard. The detector peak area responses to an equal weight mixture of fatty acid methyl esters from 10- to 24-carbon atoms and up to 6 double bonds were determined by using Nu-Check Prep preparation 462 (Elysian, MN). Detector responses were within 10% of the expected value. Part of the plasma lipid extract was stored at -70°C for mass spectrometric analysis of deuterated fatty acid pentafluorobenzyl esters. Samples were air-shipped on dry ice to the NIAAA Laboratory of Membrane Biochemistry and Biophysics to measure the concentration of d5-fatty acids as previously described (7). A model 5989 mass spectrometer with model 5890 gas chromatograph (Hewlett-Packard, Wilmington, DE) using a polyethylene glycol nitroterephthalic

acid ester (DB-FFAP) bonded-phase capillary column (60 m × 0.25 mm i.d., film thickness 0.25 mm) (Quadrex Corp. New Haven, CT) served to fully separate the d-5 derivatized fatty acids from the unlabeled products of LA and LNA desaturation/elongation. A model 7673 autosampler (Hewlett-Packard, Wilmington, DE) injected samples, and single-ion chromatograms at each desired M-pentafluorobenzyl ion were collected as previously described. The d-5 concentrations derived from these mass spectral measurements and the d-0 concentrations measured by flame ionization detector gas chromatography were used to compute a tracer/tracee ratio, a measure of isotopic enrichment of the metabolic conversion products. Results are expressed in terms of d-5 concentrations in $\mu\text{g/mL}$ of plasma and also as d-5/d-0 ratios at 24, 48, and 96 h. The baseline d-5 enrichment was found to be negligible in several infants and thereafter was assumed to be nil to avoid removing additional blood from the infant. The timing of blood sampling was defined based on our initial observations on kinetics of appearance of labeled metabolic products of interest (7). All data were coded and managed using computerized spreadsheets. The change, appearance, or disappearance over time for the labeled products of interest was measured at given times and also integrated over the 96 h of study. An appearance parameter, area under the curve, was created for each labeled fatty acid by application of a time-integrated algorithm (22). For each time series, trapezoidal areas are calculated by a formula, where the area equals one-half of the product of concentration multiplied by the time interval in hours. The sum of trapezoids from 0 to 96 h is the appearance parameter. We chose not to conduct further kinetic analysis of these data, given the intrinsic limitations in testing or validating the assumptions required for appropriate use of such modeling (see "Discussion").

Statistical analysis of data included measures of central tendency and distribution. Mean and standard deviations for the relevant variables according to defined gestational age and fetal growth groupings (preterm AGA, term AGA, and IUGR groups) were computed. Comparison among the three groups was performed using the SAS General Linear Model ANOVA correcting for multiple comparisons to contrast group differences. Simple and multiple correlations were used to test the effect of gestational age and fetal growth grouping. Specific data points identified as outliers from the group data or in correlations were first verified for potential errors in data management or chemical analysis. If data points were considered valid, the results were computed with and without the outlier observations. A sample size of 10–12 infants per group was defined based on our initial observations of variability in d5-AA and d5-DHA concentration (7). For power analysis, detecting a difference of 1 SD in these variables between preterm and term infants, with an $\alpha \leq 0.05$ and a power of 0.80, was sought. The IUGR were unpredictable in distribution by gestational age (preterm and term); thus our goal was to achieve a group of at least a similar sample size over the 30-month study period. Because of the difficulty in achieving full standardization for the timing of initial enteral feeds, a *post hoc* analysis was conducted for infants who were fed before isotope dosing, compared with those who were not fed enter-

ally at the time of tracer administration. If, after controlling for major effects, the relevant measures were not different in the fed and fasted group, these infants' data would be included in the pooled data.

RESULTS

The biodemographic characteristics for the three study groups are summarized in Table 1. The large variability in gestational age for the IUGR group is evident, this is inevitable, because most (seven of 11) were preterm, whereas some (four of 11) were term. Differences in anthropometric indices between IUGR and AGA term and preterm infants are evident, ponderal index (weight divided by the square of length) was used to characterize proportionality of fetal growth.

The nature of the kinetic response for d5-labeled fatty acids for the three study groups is illustrated in Figure 1. Concentration of deuterated fatty acids measured at 24, 48, and 96 h are shown; LA and LNA are in the top panels and the respective desaturated and elongated products of interest, AA and DHA, are shown in the lower panels. Note, the major change in concentration scales for the products shown in the lower panel, namely, a 100-fold decline for d5-AA concentration relative to LA and a 10-fold drop for d5-DHA compared with LNA concentration. These results indicate that only a small fraction of the labeled precursor given orally is found in plasma LCP. Higher concentrations of precursor d5 fatty acids are found for term infants at 24 h, but because of the large interindividual variability in the time course of these responses, the comparison of integrated data by study group does not reach statistical significance; overall ANOVA comparison of the three groups has a *p* value of 0.18 for d5-LA and 0.13 for d5-LNA. The concentration of labeled products in preterm infants is significantly higher at 96 h and, also, when the d5 fatty acid concentration curve is integrated over the complete study period. Overall ANOVA in the latter case has a probability value of 0.04 for AA and 0.009 for DHA. The differences between preterm and term or IUGR groups are significant, whereas the term infants compared with the IUGR are not different.

Data on absolute concentrations of unlabeled fatty acids 24 h and 96 h after tracer administration are presented as Table 2. As noted, there are significant differences across study groups for most fatty acids and great variability in these concentrations over time; preterm have lower values relative to term infants. The trend is that precursors tend to rise and more unsaturated products tend to fall over the 96 h of study. We chose to

Table 1. *Biodemographic and anthropometric indices of infants included in three study groups*

	Pre-term AGA (<i>n</i> = 19)	Term AGA (<i>n</i> = 11)	IUGR (<i>n</i> = 11)
Gestational age (wk)	30.9 ± 2.4*	39.6 ± 0.9	35.6 ± 3.1
Weight (g)	1712 ± 491	3549 ± 320	1834 ± 437
Length (cm)	41.2 ± 4.2	50.6 ± 1.2	43.7 ± 3.6
Head circumference (cm)	29.5 ± 3.4	35.3 ± 1.8	30.3 ± 3.0
Ponderal Index (kg/cm ²)	2.4 ± 0.22	2.75 ± 0.29	2.17 ± 0.11
Gender (M/F)	11/8	7/4	5/6

* Mean ± SD.

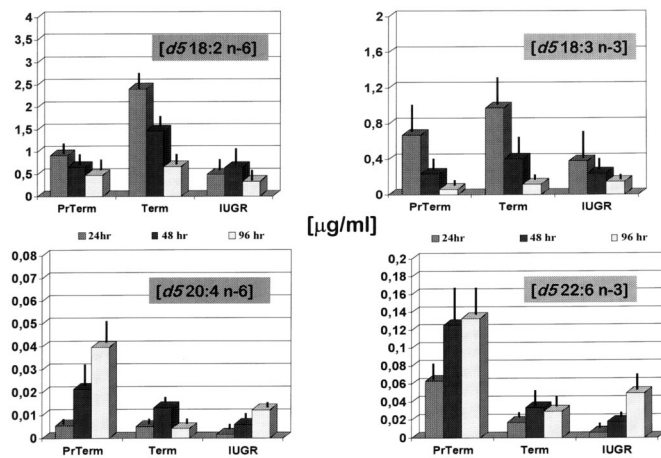


Figure 1. Deuterated pentafluorobenzyl fatty acid plasma concentrations expressed in $\mu\text{g/mL}$ after 24, 48, and 96 h of administration of 50 mg of deuterated linoleic acid (d5 18:2 n-6) and 50 mg of deuterated α -linolenic acid (d5 18:3 n-3) per kg body weight in a group of 19 preterm appropriate for gestational age (*PrTerm*), 11 term gestation infants (*Term*), and 11 Intrauterine growth-retarded infants (*IUGR*). Precursors (d5 18:2 n-6) and α -linolenic acid (d5 18:3 n-3) are in the *top panels*, and the respective desaturated and elongated products of interest arachidonic acid (d5 20:4 n-6) and docosahexaenoic acid (d5 22:6 n-3) are shown in the *lower panels*. Bars in figure are mean and SEM for each study group at 24, 48, and 96 h. Overall ANOVA comparison of d5 concentrations in the precursors integrated over time for the three infant groups has a p value of 0.18 for d5-18:2 n-6 and 0.13 for d5-18:3 n-3. The time-integrated concentration of labeled products is significantly higher in the preterm group than in either of the two other groups; overall ANOVA has a probability value of 0.04 for d5 20:4 n-6 and 0.009 for d5 22:6 n-3. The differences between the preterm and the two other groups are significant, whereas term infants compared with the IUGR are not different.

present detailed numeric results and statistical analysis of the data expressed as tracer (d5) to tracee (d0) ratio for each specific fatty acid, inasmuch as the unlabeled fatty acid concentration varies greatly within and between groups, thus potentially affecting metabolic activity. These ratios, representing isotopic enrichment, were determined at each time and also integrated over the entire study period. Time-integrated d5/d0 ratios for fatty acids of interest are summarized in Table 3. For the n-6 series, we start with the precursor 18:2 n-6, linoleic acid, progressing along the metabolic path to the elongated product 20:2 n-6, the desaturated product 20:3 n-6, and the delta 5 desaturated product of interest, 20:4 n-6 (AA). Although preterm infants relative to term and IUGR, have more than twice the d5 enrichment of 20-carbon triene and tetraene n-6 fatty acids, overall ANOVA comparing AA enrichment across groups does not reach statistical significance, $p = 0.09$. For the n-3 series, overall ANOVA of time-integrated d5 product enrichment is significantly higher in the preterm group for all C20 and C22 n-3 fatty acids, and most significant for DHA, $p = 0.012$. For both the n-6 and n-3 series, the time-integrated d5 precursor enrichment across study groups is not significantly different, suggesting that absorption and metabolic handling of LA and LNA before incorporation into plasma pools are relatively similar. Note the significant drop for all study groups in product enrichment as we move along the metabolic pathway. For example, time-integrated mean d5 enrichment of DHA relative to LNA in preterm infants is 1%, whereas for term infants it is 0.25% and for IUGR, 0.37%. The

corresponding mean figures for AA d5 enrichment relative to LA are 3% for preterm and close to 2% for both the term and IUGR groups. The comparison of time-integrated d5 fatty acid enrichment values for term and IUGR groups demonstrates that they are generally similar.

In an effort to evaluate on a relative basis metabolic conversion across study groups, we explored selected product/precursor relationships using the time-integrated d5 enrichment ratio data from each individual subject. These results are presented in Table 4. There are inherent limitations of this analysis: namely, that sampling the plasma compartment may not reflect metabolic activity across other tissues and that oxidation rates were not measured. However, if we assume that known and unknown confounders are constant across study groups, the comparison may be revealing. For example, these data suggest that for the n-6 series, the delta-6 desaturation and elongation of LA is the most restrictive step. Once 20:3 n-6 is formed, nearly half is converted to AA across all study groups. Overall metabolic conversion from LA to AA is 5-fold higher in preterm relative to term infants. In the case of the n-3 series for virtually all metabolic activity along the pathway, the preterm group is significantly more active than the term or IUGR groups. Overall conversion of LNA to DHA, on a relative basis, is 6-fold higher in preterm than in term infants, and 4-fold higher than in the IUGR group. This pattern is basically similar in all other product/precursor ratios of the n-3 series. Higher values in the preterm group remain highly significant for all product-precursor ratios except for the final 22:5 to 22:6 conversion, where the term group also has a lower value but p is only 0.08. No precise quantification can be derived from this analysis except to indicate that on a relative basis, the preterm group appears to be more active than the term or the IUGR groups. There are no significant differences between the term and IUGR group data.

To evaluate the effect of gestational age on formation of AA and DHA over the study period, we performed simple and multiple correlation analysis. The complete data set combining study groups ($n = 41$) was used, considering gestational age, birth weight, and weight adequacy as independent continuous variables and d5 fatty acid enrichment of LCP as the dependent variable. The time-integrated d5 enrichment for AA and DHA is significantly correlated with gestational age, $r = -0.42$, $p < 0.009$; for AA; d5 enrichment plotted by GA has a nonsignificant relationship. We also explored correlations of enrichment at the 96-h time point, demonstrating significant correlations for both AA and DHA; $r = -0.43$, $p = 0.01$ for DHA, and an $r = -0.46$, $p = 0.006$ for AA. Simple correlations of product enrichment with birth weight are weaker but significant ($p < 0.05$ for DHA and AA). Stepwise multiple correlation analysis demonstrated that once GA is entered, birth weight or birth weight adequacy does not add further strength to the relationship. Figure 2 (A and B) shows the correlations of time-integrated product/precursor relationships for n-6 and n-3 acids, respectively, with gestational age. The d5-integrated enrichment of AA over LA and of DHA over LNA reflects metabolic conversion over the study period. As shown in Figure 2A, for the n-6 series product/precursor relationship, AA/LA ratios were negatively correlated with gestational age;

Table 2. Plasma fatty acid concentrations ($\mu\text{g/ml}$) at 24 h and 96 h according to gestational age and fetal growth adequacy

	Pre-term AGA (n = 19)		Pre vs. term (p value)	Term AGA (n = 12)		Pre vs. IUGR (p value)	IUGR (n = 11)		Overall ANOVA (p value)
	Mean	SE		Mean	SE		Mean	SE	
18:2 _{n-6} 24h	118.99	30.17	0.04	227.92	34.08	0.70	83.80	18.24	0.001
18:2 _{n-6} 96h	217.94	32.81	0.44	317.46	89.64	0.94	195.99	51.69	0.33
p value*	0.08			0.52			0.69		
20:2 _{n-6} 24h	4.91	0.98	0.14	8.00	1.56	0.04	1.20	0.20	0.001
20:2 _{n-6} 96h	7.86	1.46	0.10	8.03	1.54	0.14	3.40	1.30	0.12
p value	0.26			0.1			0.12		
20:3 _{n-6} 24h	23.67	2.03	0.10	32.30	2.59	0.46	18.77	4.09	0.01
20:3 _{n-6} 96h	23.89	2.19	0.97	25.06	4.69	0.51	19.19	3.46	0.43
p value	0.1			0.31			0.99		
20:4 _{n-6} 24h	85.46	8.01	0.06	129.42	11.78	0.10	84.23	20.51	0.04
20:4 _{n-6} 96h	87.72	6.44	0.58	107.07	22.52	0.72	75.19	14.96	0.31
p value	0.98			0.57			0.93		
18:3 _{n-3} 24h	1.85	0.64	0.02	6.47	2.12	0.95	1.32	0.67	0.01
18:3 _{n-3} 96h	3.77	0.68	0.05	9.54	3.70	0.85	4.85	1.36	0.05
p value	0.14			0.76			0.06		
20:5 _{n-3} 24h	3.34	0.40	0.001	8.44	1.18	0.60	4.86	1.79	0.001
20:5 _{n-3} 96h	3.91	0.53	0.01	9.35	2.53	0.87	4.65	1.19	0.01
p value	0.82			0.92			0.99		
22:5 _{n-3} 24h	1.93	0.35	0.001	5.42	0.75	0.26	3.71	1.28	0.001
22:5 _{n-3} 96h	2.65	0.43	0.001	6.94	1.71	0.94	3.01	0.80	0.001
p value	0.58			0.65			0.92		
22:6 _{n-3} 24h	27.83	2.81	0.001	65.05	7.53	0.78	34.19	10.02	0.001
22:6 _{n-3} 96h	25.83	2.10	0.001	57.17	14.18	0.73	31.90	7.92	0.001
p value	0.81			0.85			0.99		

* p value corresponds to probability that mean values at 24 h are different from the 96 h value based on ANOVA.

Table 3. Integrated d5/d0 fatty acid ratio (r) over complete study period according to gestational age and fetal growth adequacy

	Pre-term AGA (n = 19)		Pre vs. term (p value)	Term AGA (n = 11)		Pre vs. IUGR (p value)	IUGR (n = 11)		Overall ANOVA (p value)
	Mean	SE		Mean	SE		Mean	SE	
r18:2 _{n-6}	0.859*	0.232	0.36	0.434	0.142	0.50	0.507	0.129	0.29
r20:2 _{n-6}	0.557	0.262	0.42	0.114	0.045	0.84	0.360	0.077	0.41
r20:3 _{n-6}	0.197	0.037	0.13	0.086	0.041	0.20	0.099	0.027	0.07
r20:4 _{n-6}	0.026	0.007	0.15	0.008	0.004	0.23	0.010	0.003	0.09
r18:3 _{n-3}	42.37	10.91	0.59	25.87	13.96	0.58	25.64	6.27	0.45
r20:5 _{n-3}	5.513	1.163	0.07	1.819	0.804	0.16	2.502	0.750	0.04
r22:5 _{n-3}	2.141	0.511	0.03	0.377	0.164	0.18	0.950	0.250	0.02
r22:6 _{n-3}	0.423	0.110	0.04	0.064	0.029	0.06	0.097	0.033	0.01

* d5/d0 ratios for each fatty acid are integrated over 96 h of study using sum of trapezoidal areas (arbitrary units).

$r = -0.36$, p is 0.02. If the two outliers are removed from the analysis, the corresponding r is -0.5 , and p is 0.001. For the n-3 series, DHA/LNA ratios were also negatively correlated with gestational age ($r = -0.47$, $p = 0.0018$), and they rise to an r of -0.6 with a p of 0.0001 when the three outliers are removed from the correlation analysis.

Based on the mixed composition of the IUGR group in terms of GA and the significant impact this variable had on d5 enrichment of LCP, we conducted a *post hoc* analysis matching IUGR infants with one or more infants of comparable GA who had normal fetal growth. The control group with adequate birth weight for gestational age and similar duration of gestation had a mean GA of 35 wk, with a range from 31 to 39 wk. Mean birth weight for the control AGA group was 2401 g (range

1620–3970 g), whereas for the IUGR the mean was 1834 g, ranging from 1220 to 2520 g. This *post hoc* analysis is presented as Table 5, correcting for differences in gestational age found in the initial groups shown in Table 3. As observed from these data, group differences in time-integrated d5 enrichment of various fatty acids disappear when groups are matched by GA. However, these data should be interpreted with caution, inasmuch as sample size is small and variability high. In addition, for virtually all metabolic products of both the n-6 and n-3 series, mean d5 enrichment values of AGA controls are around twice those of the IUGR group. The IUGR infants (as shown in Fig. 1) demonstrate a slower decline in d5 concentrations of LA and LNA and a slower rate of rise in AA and DHA. Comparison of gestational age-matched AGA and IUGR

Table 4. Product/precursor relationship of integrated d5/d0 fatty acid ratios (r) according to gestational age and fetal growth adequacy

	Pre-term AGA (n = 19)		Pre vs. term (p value)	Term AGA (n = 11)		Pre vs. IUGR (p value)	IUGR (n = 11)		Overall ANOVA (p value)
	Mean	SE		Mean	SE		Mean	SE	
r20:4/r18:2 _{n-6}	0.0364*	0.010	0.1	0.0075	0.002	0.5	0.0214	0.007	0.09
r20:2/r18:2 _{n-6}	0.0215	0.003	1.0	0.0214	0.003	0.01	0.006	0.002	0.001
r20:3/r20:2 _{n-6}	3.568	0.270	0.79	3.256	0.450	0.07	2.512	0.253	0.07
r20:4/r20:3 _{n-6}	0.452	0.066	0.9	0.437	0.162	0.9	0.52	0.206	0.9
r20:3/r18:2 _{n-6}	0.075	0.010	0.01	0.051	0.008	0.24	0.022	0.007	0.002
r22:6/r18:3 _{n-3}	0.3795	0.081	0.01	0.0621	0.026	0.01	0.0884	0.021	0.0017
r20:5/r18:3 _{n-3}	0.6679	0.104	0.01	0.2629	0.064	0.03	0.3132	0.044	0.004
r22:6/r20:5 _{n-3}	0.5613	0.064	0.01	0.1796	0.058	0.01	0.2688	0.051	0.0002
r22:6/r22:5 _{n-3}	2.6268	0.262	0.08	1.5831	0.450	0.01	0.9179	0.194	0.0004

* Product/precursor relationship of d5-enrichment for each fatty acid integrated over 96 h of study.

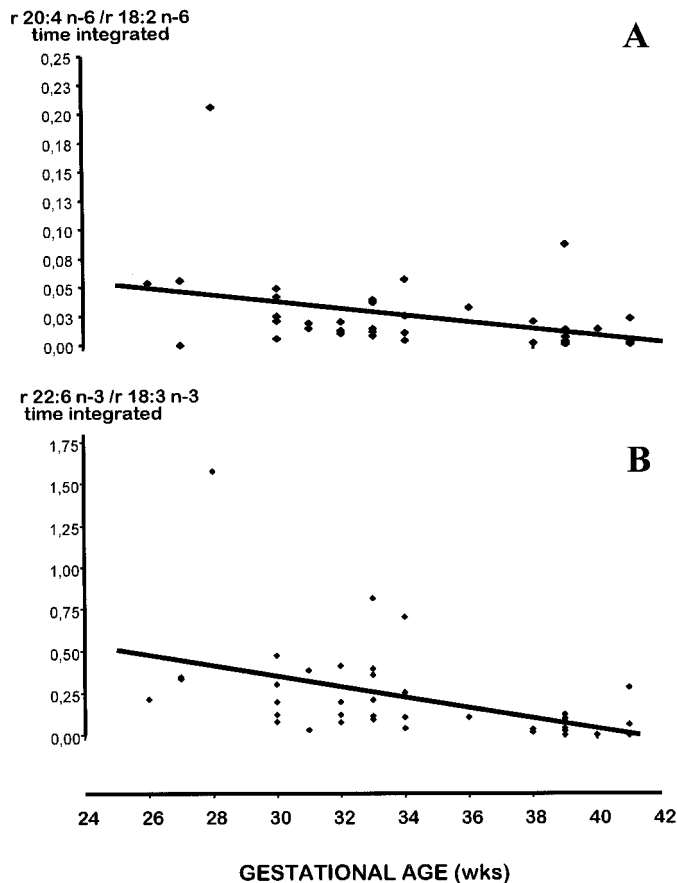


Figure 2. A (top panel) and B (lower panel) show the correlation of product/precursor relationships for n-6 and n-3, respectively, with gestational age, using the time-integrated d5 enrichment ratios. Linear regression of time-integrated d-5 enrichment in 20:4 n-6/18:2 n-6 was negatively correlated with gestational age; F value 5.8, $r = -0.36$, p is 0.02. After the two outliers are removed from the analysis, the corresponding F value is 12.4, r is -0.5 , and p is 0.001. As shown in lower panel, linear regression of time-integrated d-5 enrichment in 22:6 n-3/18:3 n-3 was also negatively correlated with gestational age; F value 11.3, $r = -0.47$, p is 0.0018, and rise to an F of 12.4, r of -0.6 with a p of 0.0001 when the three outliers are removed from the correlation analysis.

infants demonstrated significantly lower d5 DHA and d5DHA/d5LNA time-integrated areas for the IUGR group (data not shown). Thus, a 96-h study period may be insufficient to fully evaluate the capacity of this group to form LCP.

To evaluate the effect of feeding status at the time of isotope administration, we conducted a *post hoc* analysis of data from

eight subjects who had been fed before isotope administration, comparing them with a group matched by gestational age and birth weight. This is presented in Table 6. No group differences in time-integrated d5 and d0 concentrations or d5 enrichment of various fatty acids are found in this evaluation. These data should be interpreted with due consideration of the limitations imposed by the small sample size. In addition, we should observe that mean concentration of d5-AA and d5-DHA in the enterally fed group is nearly half that found in non-fed controls, whereas d0 concentrations are similar or slightly higher, suggesting the possibility of lower d5 precursor conversion to LCP when feedings are given. This *post hoc* analysis appears to be insufficient for a definitive evaluation of the effect of feeding on the capacity of newborns to form LCP from precursor EFAs. We are presently completing a more thorough evaluation of this hypothesis in a group of infants studied before feedings and after full feeds are established.

DISCUSSION

The most significant finding of this study is that preterm infants appear to be able not only to form AA and DHA from labeled parent essential fatty acid precursors, but also in fact their overall metabolic activity and or transport may be greater than that found in term infants. Beyond the inherent methodological limitations of this study, this finding is of interest, because it appears to contradict the notion that preterm infants are more vulnerable to LCP deficiency, inasmuch as presumably they have immature biochemical systems. However, we should first consider that the indices of metabolic conversion measured in this study are based on plasma pools and may be fully inappropriate to evaluate the LCP accretion of critical organs such as the CNS and the developing retina. In fact, work in non-human primates by Brenna's group indicate that there is a tissue-specific incorporation of DHA, favoring the brain and the retina (23, 24). Thus, despite active synthesis of LCP, nervous system accretion may still be restricted, because what is made, as reflected by plasma phospholipid pools, may not be sufficient to meet the accretion needs of preterm infants. Data from Martinez suggest that neural LCP accretion during the third trimester of human gestation is 3- to 5-fold higher, on a body mass basis, early on, compared with at the time of completion of pregnancy (25).

Table 5. Integrated d5/d0 fatty acid ratio (τ) over complete study period according to fetal growth adequacy in groups matched by gestational age

	AGA Control (n = 14)		IUGR (n = 11)		p value
	Mean	SE	Mean	SE	
r18:2 _{n-6}	0.864*	0.285	0.507	0.129	0.32
r20:2 _{n-6}	0.306	0.086	0.36	0.077	0.67
r20:3 _{n-6}	0.159	0.04	0.099	0.027	0.27
r20:4 _{n-6}	0.019	0.007	0.010	0.003	0.28
r18:3 _{n-3}	28.04	9.48	25.64	6.27	0.84
r20:5 _{n-3}	4.133	1.38	2.50	0.75	0.36
r22:5 _{n-3}	1.514	0.53	0.950	0.25	0.35
r22:6 _{n-3}	0.282	0.115	0.096	0.032	0.19

* d5/d0 ratios for each fatty acid are integrated over 96 h of study using sum of trapezoidal areas.

Table 6. Integrated fatty acid d5 and d0 concentrations, and d5/d0 ratios (τ) over complete study period according to feeding status at time of isotope dosing (groups matched by gestational age)

	Enterally fed (n = 8)		No enteral feeds (n = 10)		p value
	Mean	SE	Mean	SE	
d5 22:6 _{n-3}	4.6462*	1.69	8.826	4.97	0.4425
d5 20:4 _{n-6}	0.900	0.22	2.277	1.09	0.2439
d0 22:6 _{n-3}	3795†	927	2667	450	0.26
d0 20:4 _{n-6}	8389	1483	8150	1076	0.895
r22:6 _{n-3}	0.1687‡	0.08	0.3337	0.19	0.437
r20:4 _{n-6}	0.0100	0.03	0.0262	0.01	0.222
r20:4/r18:2	0.01677	0.07	0.0462	0.02	0.186
r22:6/r18:3	0.1604	0.06	0.2759	0.15	0.4871

* Integrated d5 concentrations over 96 h of study using sum of trapezoidal areas ($\mu\text{g/ml/96 h}$).

† Integrated d0 concentrations over 96 h of study using sum of trapezoidal areas ($\mu\text{g/ml/96 h}$).

‡ d5 enrichment and product/precursor relationship described in footnote for Figures 2 and 3.

Second, we need to closely examine the results of this study before making firm conclusions; the interpretation of the comparative data rests on the assumption that known and unknown confounders are acting similarly across all study groups. The very nature of this assumption conflicts with our extremely limited capacity to validate even minor specific components of this complex metabolic process in humans. For now, we cannot even measure isotope distribution space, nor take into account the size or enrichment of the active precursor pool for LCP biosynthesis. We further assume that the metabolic pool of interest is homogenous and that turnover rates remain stable during the study period. In the future, we may be able to test whether oxidation rates for specific fatty acids of interest change across gestational age, or are affected by metabolic demands for energy, or alternatively are commanded by the need of key organs for structural lipids (26). However, until we can evaluate the tissue-specific turnover rates, and incorporation or accretion into functionally relevant organ or even cellular pools, we will not be able to define sufficiency or insufficiency in a conclusive manner (23–26).

Third, even the most sophisticated biochemical measure cannot define nutritional adequacy unless it is used in conjunction or has been validated as a surrogate measure for functional normalcy. This should be considered the crucial step in evaluating any development in the biochemical assessment of LCP status. Function related to LCP should include, at the very least, assessment of growth, maturation of sensory systems, and development of the nervous system. In addition, the ade-

quacy of eicosanoid-related functions will surely become progressively more significant as we move beyond the basic survival of immature preterm infants (27). Further considerations of the potential use of these type of data in defining nutritional needs of preterm and term infants should take these points into account. We should not prematurely withdraw from this daunting challenge but should rather be extremely cautious in the interpretation of these results. We purposefully avoided kinetic modeling of these data based on single or multiple compartments, not because this is difficult but because we considered that the assumptions inherent to the prevalent kinetic models cannot be adequately tested, in the specific case of fatty acid metabolism studies in human infants.

Clinical studies are plagued by confounders, some of which can be standardized by the methodology used; such is the case with isotope dosing or time of sampling, but multiple other factors that affect LCP biosynthesis remain unknown. Moreover, in examining the data from this study, we find that the kinetics of metabolic conversions has a large interindividual variability; the correlation analysis of product/precursor relationships demonstrates that at best, only 25–30% of the variance can be explained by gestational age (Fig. 2). Our routine assessment of clinical variables or even the physiologic monitoring these infants receive, does not provide us with a clear idea of the relevant confounders. Overall energy balance may affect fatty acid oxidation rates and possibly modify metabolic conversions; occurrence of even mild or unidentified morbid processes, multiple routine nursery practices that induce mild

or moderate cold stress, may all affect the variables we are measuring (26, 28). The large interindividual variability in metabolic conversions observed has important implications in defining nutritional needs for this population group. We suggest that this be considered in establishing dietary standards and in assessing adequacy of essential fatty acid intakes of neonates.

This study expands the initial report by Salem *et al.* which documented for the first time DHA biosynthesis from d5-LNA in nine human infants and confirms unequivocally that AA can be endogenously made from LA as originally reported by Demmelmair *et al.*, in four infants (7, 8). The latter author used the rise in natural abundance of ^{13}C -AA after feeding corn oil formula as an indicator of endogenous synthesis of AA, because the corn oil formula was richer in ^{13}C -LA relative to the baseline diet (8). Work by Carnielli *et al.*, who used uniformly labeled ^{13}C -LA and ^{13}C -LNA in five infants, provided additional confirmation for endogenous synthesis of AA and DHA using a carbon label (9). These studies were followed by a larger study conducted with uniformly labeled ^{13}C -LA and ^{13}C -LNA in 37 infants fed formulas with different LNA concentrations and no LCP. Unfortunately, blood was sampled for up to 24 h after tracer dosing, possibly underestimating isotope incorporation into LCP, inasmuch as the published work has demonstrated that LCP enrichment continues to rise for 72–96 h after isotope administration. Kinetic modeling of these data attempted to define fractional turnover rates, documenting a decrease in LA to AA conversion from 1.1% to 0.4% when the high LNA (3.2%) diet was fed. In addition, they evaluated the effect of postnatal age and found a significant negative correlation between postnatal age and fractional rate of conversion (10, 29). A problem with this data set is that the authors assumed product enrichment peaked within the 24-h period of study; this assumption was not tested. In fact, the work of Carnielli and our own published work are in sharp contrast with that assumption, showing that LCP isotopic enrichment rises after 24–48 h of dosing and peaks at around 72–96 h (7, 9). Presently, no kinetic model to quantify LCP synthesis in infants from isotopic enrichment of metabolic products has been validated.

Even our simplistic area under the curve integration assumes that we are able to model concentrations over time using a linear interpolation between data points forming a trapezoid. It is evident that this will underestimate the area, when data points form a convex curve and, conversely, will overestimate the area when the curve is concave. The limited number of samples obtained in this study during the initial 24 h will predictably underestimate the precursor concentrations over time. There was also an underestimation of product formation since the study was restricted to 96 h, which for some subjects is before the downslope of the concentration curve has been completed.

Work in neonatal non-human primates by Brenna *et al.* using direct measurements of uniformly labeled ^{13}C DHA and LNA incorporation into brain, retina, and other tissues after labeled fatty acid administration has served to assess organ-specific distribution. These data indicate that over a 2-wk period, 1.7% of the labeled preformed DHA is incor-

porated into the brain, incorporation into retina is similar, whereas for retinal pigment epithelium it is very small, only 0.002%. Labeled DHA incorporation into liver is 2.5%, for red cells is 0.4%, and for plasma is 0.2%. If the labeled precursor, LNA, is administered and labeled DHA organ accretion is followed in comparison to that observed when preformed labeled DHA is given, bioequivalence can be assessed. Organ-specific bioequivalence of DHA relative to LNA in terms of labeled DHA incorporation is 7:1 for brain, 12:1 in retina, 15:1 for retinal pigment epithelium, 27:1 for liver, 29:1 for plasma, and 51:1 for red blood cells 2 wk post-dose (23, 24). These data indicate that the transport and accumulation of DHA across organs differ widely and that metabolic conversion of LNA to DHA in terms of incorporation is also tissue specific.

The data from this study suggest that d5-LCP concentrations, d5/d0 enrichment of labeled DHA and AA and labeled products relative to precursors, an index of metabolic conversion, are affected by time of sampling after tracer administration and by gestational age. Fetal growth retardation appears to slow down rates of incorporation or limit endogenous synthesis. Comparative analysis of the three study groups indicates that the drop in time-integrated mean d5 enrichment of n-3 products is greater than that observed for n-6 metabolites, suggesting that AA biosynthesis is relatively more active than that of DHA. Alternatively, DHA removal from the plasma pool by tissues may be greater; disappearance in this case may be due to oxidation or to tissue accretion. Unfortunately, the nature of human clinical studies appears to preclude at present an approach that can answer this question. Perhaps studies in infants, who subsequently die or undergo surgery, may serve to assess tissue-specific incorporation of labeled products, at least for some organs. In addition, future developments in magnetic resonance spectroscopy, using natural abundance or exogenously administered ^{13}C -stable isotope-labeled substrates, may provide a noninvasive method for the evaluation of organ-specific LCP incorporation and turnover (30, 31). A recent report in 10 breast-fed term infants using ^{13}C -labeled linoleic acid as an oral tracer determined that only 7.4% of the dose was oxidized over the initial 6 h of study based on the appearance of ^{13}C -labeled CO_2 . This study is in accordance with our results indicating that the conversion of LA to 20:3 n-6 is limited but most 20:3 n-6 is desaturated to AA. It also is in accordance with our observation of the relatively small endogenous conversion of LA to LCPs in term infants (32).

In conclusion, we have confirmed that metabolic elongation and desaturation of LA and LNA to form AA and DHA occurs during the first days of life in humans, including very immature preterm neonates. Our results suggest that the formation of LCP from linoleic and α -linolenic acid is more active at earlier gestational ages and decreases with advancing development. Alternatively, these data may indicate that removal from plasma pool due to oxidation or accretion is faster in the more immature infants. Fetal growth restriction appears to slow down or diminish the capacity of newborns to form LCP from dietary precursors. Further refinement and advancement of methodologies may provide a way to quantify endogenous LCP synthesis and organ-specific turnover rates.

Acknowledgment. The authors thank Gloria Icaza, Ph.D., who provided data management and statistical support for this work.

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