Formula feeding potentiates docosahexaenoic and arachidonic acid biosynthesis in term and preterm baboon neonates

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Abstract **Infant formulas supplemented with docosahexaenoic acid (DHA) and arachidonic acid (ARA) are now available in the United States; however, little is known about the factors that affect biosynthesis. Baboon neonates were assigned to one of four treatments: term, breast-fed; term, formula-fed; preterm (155 of 182 days gestation), formula-fed; and preterm, formula+DHA/ARA-fed. Standard formula had no DHA/ARA; supplemented formula had 0.61%wt DHA (0.3% of calories) and 1.21%wt ARA (0.6% of calories), and baboon breast milk contained 0.68 ± 0.22%wt DHA and 0.62 ± 0.12%wt ARA. At 14 days adjusted age, neonates received a combined oral dose of [U-^{13}C]α-linolenic acid (LNA*) and [U-^{13}C]linoleic acid (LA*), and tissues were analyzed 14 days after dose. Brain accretion of linolenic acid-derived DHA was ~3-fold greater for the formula groups than for the breast-fed group, and dietary DHA partially attenuated excess DHA synthesis among preterms. A similar, significant pattern was found in other organs. Brain linoleic acid-derived ARA accretion was significantly greater in the unsupplemented term group but not in the preterm groups compared with the breast-fed group. These data show that formula potentiates the biosynthesis/accretion of DHA/ARA in term and preterm neonates compared with breast-fed neonates and that the inclusion of DHA/ARA in preterm formula partially restores DHA/ARA biosynthesis to lower, breast-fed levels. Current formula DHA concentrations are inadequate to normalize long-chain polyunsaturated fatty acids synthesis to that of breast-fed levels.**—Sarkadi-Nagy, E., V. Wijendran, G. Y. Diau, A. C. Chao, A. T. Hsieh, A. Turpeinen, P. Lawrence, P. W. Nathanielsz, and J. T. Brenna. **Formula feeding potentiates docosahexaenoic and arachidonic acid biosynthesis in term and preterm baboon neonates.** ***J. Lipid Res.*** **2004.** 45: **71–80.**

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It is well established that the long-chain polyunsaturated fatty acids (LCPs) docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (ARA, 20:4n-6) are required for proper neural development. Mammals can synthesize DHA from linolenic acid (LNA) and ARA from linoleic acid (LA) by a series of elongation and desaturation reactions (1). LNA and LA are considered to be dietary essential fatty acids (FAs) because mammals are incapable of synthesizing these FAs. The n-3 and n-6 FAs are not interconvertible, and it has long been assumed that they compete for the same elongation and desaturation enzymes; it was shown only recently that the Δ6-desaturase, the first step in DHA and ARA biosynthesis from LNA and LA, respectively, operates on both LNA and LA (2). Tracer studies show that preterm and term human infants (3–5) as well as term and fetal baboons (6–8) convert LNA to DHA and LA to ARA.

The most intense period of human brain growth is from ~28 weeks gestation to 18 months after birth, during which time brain DHA accretion peaks (9). Premature infants born at 28 weeks now routinely survive but have very little adipose tissue stores compared with infants born at term and must obtain all FAs from their diets rather than via placental transfer. Those consuming unsupplemented formula must synthesize all LCPs from precursors, and ample

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Abbreviations: ARA or 20:4n-6, arachidonic acid; CNS, central nervous system; DHA or 22:6n-3, docosahexaenoic acid; FA, fatty acid; FAME, fatty acid methyl ester; LA, linoleic acid; LA*, [U-^{13}C]linoleic acid; LCP, long-chain polyunsaturated fatty acid; LNA, α-linolenic acid; LNA*, [U-^{13}C]α-linolenic acid; RBC, red blood cell; RPE, retinal pigment epithelium.

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evidence demonstrates that unsupplemented formula-fed term and preterm human infants have lower DHA in the central nervous system (CNS), plasma, and red blood cells (RBCs) (10–14). Autopsy data suggest that term formula-fed infants deplete adipose tissue DHA stores by 6 months of age compared with breast-fed infants, who generally maintain DHA levels (15, 16); thus, factors concerning LCP biosynthesis are relevant for all human infants. Results are mixed for ARA, as some studies report lower ARA values in plasma and RBC lipids in formula-fed infants than in breast-fed infants (17, 18) but others do not (19).

The functional consequences of LCP supplementation are controversial. Consistent functional deficits are found for premature infants in visual function (20, 21) and in other CNS functions (22). Improvement in function is not found uniformly in studies of term infants, for whom the effects of a deficiency may resolve with later development (23, 24).

Human breast milks around the world all contain LCP (25), and commercial infant formulas containing DHA and ARA, introduced in the United States only in early 2002, have been in use in many countries for several years. Dietary LCPs support greater LCP concentrations in tissues of experimental animals and in plasma and RBCs of human infants, although there is evidence that dietary LCP and LA suppress Δ6-desaturase activity and mRNA in rats (2, 26). There are no data to indicate whether dietary LCPs decrease the biosynthesis of DHA and ARA in human infants or primates.

Quantification of total LCP biosynthesis in target organs is not possible in human infants because tissue sampling is required. Accessible tissues, blood or buccal mucosal cells (27, 28), must be sampled and used as proxies for organ status (29). Data on the relationship between tracers detected in plasma/RBC and organs are sparse. Animal studies are required for direct sampling of target organs; because of the unique size and structure of the human CNS, it is best modeled by nonhuman primates. Much important early data on the relationship of dietary n-3 FAs to visual function were generated in term neonatal rhesus monkeys (30). The baboon is a common model for prematurity (31) and is omnivorous in the wild (32), making it an excellent model for preterm human LCP nutrition.

In a recent paper, we reported on the influence of prematurity and LCP supplementation on total FAs of brain and related tissues of preterm baboon neonates at 4 weeks adjusted age (33). Here, we report the results of isotopic tracers administered to these same animals to determine the influence of LCP supplementation and prematurity on ARA and DHA biosynthesis. We also assess relative quantitative LCP biosynthesis and relate brain and retina precursor-derived ARA and DHA accretion to blood-borne precursor-derived ARA and DHA accretion to assist in the interpretation of human studies.

MATERIALS AND METHODS

The design of this study was described in detail in a previous publication (33). Important details will be outlined here.

Animals and diets

The animal protocol was approved by the Cornell Institutional Animal Care and Use Committee; the facility is approved by the Association for the Assessment and Accreditation of Laboratory Animal Care. Nineteen pregnant baboons (*Papio cynocephalus*) were transported in the first half of gestation from the Southwest Foundation for Biomedical Research in San Antonio, TX, to Cornell University. Animals were housed in separate cages within sight of at least one other animal and were fed a commercial primate diet containing fishmeal, which served as an abundant source of n-3 LCP. Pregnant females/neonate pairs were randomly assigned to one of four categories: term, breast-fed (B); term, formula-fed (T−); preterm, formula-fed (P−); and preterm, formula-fed supplemented with LCP (P+). Nine animals delivered spontaneously at approximately 182 days of gestational age. From these nine, four neonates were assigned to the B group and nursed for 4 weeks. Samples of breast milk were obtained on the day of neonate necropsy. The remaining five animals, the T− group, were transferred to the primate nursery within 12 h of birth and fed a standard formula [commercially available preterm infant formula without LCP (Enfacare; Mead-Johnson Nutritionals, Evansville, IN)] for 4 weeks. Cesarean sections were performed on 10 animals at 155 ± 5 days of gestational age, as estimated by ultrasound measurement of fetal head circumference and biparietal diameter (34). The P− group consisted of five premature animals fed the standard formula, Enfacare, identical to the T− group. The remaining five preterm neonates, the P+ group, were fed a formula of powdered Enfacare blended with an encapsulated oil providing 151 mg of DHA and 302 mg of ARA per 100 g of Enfacare formula powder (all formulas and encapsulated oils were kindly provided by Mead-Johnson Nutritionals). Neonates were fed ad libitum.

The three animals that were 6 weeks of age at euthanasia and were included in our previous report (33) on total FA compositions were excluded from this analysis. Thus, all animals reported in this study were 4 weeks adjusted age at the time of euthanasia.

Isotopically labeled tracer doses

All animals received a single combined oral dose of 20.9 ± 1.5 mg of [U-^{13}C]LNA (LNA*) and 21.0 ± 2.3 mg of [U-^{13}C]LA (LA*) (Spectra Stable Isotopes, Columbia, MD). LNA* and LA* were 98% uniformly ^{13}C-labeled and were in FFA form. The dose was administered to each of the 19 neonates at 14 days before euthanasia; this corresponded to 14 days postnatal age for term neonates and 14 days adjusted age for preterm neonates (39 ± 5 days postnatal age).

The dose was prepared as follows. LNA* and LA* were pipetted and weighed into a sterilized 20 ml vial containing 4 ml of freshly prepared warm formula. The mixture was vortexed thoroughly, and 100 μl was withdrawn and saved for isotopic analysis. The remaining vial contents were drawn into a 5 ml syringe. The vial was washed twice with 1 ml of warm fresh formula, which was then drawn into a second syringe. The contents of both syringes were administered orally within 1 h of preparation to the neonates, immediately before a regular feeding.

Sampling

Neonates were euthanized by exsanguination under halothane general anesthesia. Organs were collected and individually placed in freezer bags. Blood was collected in EDTA-containing Vacutainer tubes, and RBCs and plasma were rapidly separated by centrifugation. The eyes were immediately dissected, and retinas and retinal pigment epithelium (RPE) were placed in sterile saline solution. All samples, including retinas and RPE in saline,

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were flash-frozen in liquid nitrogen and transferred to a −80°C freezer, where they were kept until analysis.

Analyses

Total lipids were extracted from tissue homogenates using the Bligh and Dyer method (35) modified to ensure that the solvent mixture for each tissue type was a single phase (36). FAs were derivatized to fatty acid methyl esters (FAMEs) with BF_3 in methanol (Sigma, St. Louis, MO), and the FAMEs were dissolved in heptane containing butylated hydroxytoluene (Sigma) as an antioxidant. Freshly prepared diheptadecanoyl-phosphatidylcholine was added as an internal standard to each tissue sample before homogenization. Freshly prepared triheptadecanoin (Matreya, Inc., Pleasant Gap, PA) was added to the formula and breast milk extraction mixtures as an internal standard.

Total FA concentrations were determined by gas chromatography (HP 5890 gas chromatograph with a flame ionization detector) using a BPX-70 capillary column (60 m × 0.32 mm × 0.25 μm film thickness; SGE, Austin, TX) and H_2 carrier gas. Response factors for each FA were obtained using an equal-weight FAME mixture, and data were calibrated with methyl heptadecanoate as an internal standard.

Tracer analysis for ^{13}C was performed using a high-precision gas chromatography-combustion isotope ratio mass spectrometer (Finnigan MAT 252, Bremen, Germany) described in detail previously (37).

Isotope calculations

Calibrated isotopic data are acquired as delta values expressing the relative deviation from an international standard Pee Dee Belemnite (PDB) in parts per thousand. ($^{13}C/^{12}C = R_{PDB} = 0.0112372$):

$$\delta^{13}C_{PDB} = \frac{R_f - R_{PDB}}{R_{PDB}} \times 1{,}000 \qquad \textit{(Eq. 1)}$$

R_f is the ratio of the heavy to light isotope for the sample. Extracting R_f from equation 1, atom percent (AP) of the heavier isotope in the analyte peak can be calculated as follows:

$$AP_f = \frac{R_f}{1 + R_f} \times 100 \qquad \textit{(Eq. 2)}$$

Atom percent excess (APE) is calculated by subtracting the baseline sample atom percent from the postdose sample atom percent. Baseline values were derived from analysis of samples of a single undosed fetus euthanized at cesarean section as described previously (6). The total amount of tracer that accumulates in a particular tissue can be calculated by multiplying the APE by the tracee concentration (Q_f) in tissues. If the dose FA is elongated from endogenous nonlabeled sources (i.e., LNA to DHA and LA to ARA), this value must be corrected by the number of moles of C in the analyte divided by the number of moles of C in the dose FA, to be expressed in terms of the moles of precursor that have entered the product pool. This value is termed the molar dose equivalent (D*) (38) and is calculated as follows:

$$D^* = \frac{APE_f}{100} \times Q_f \times \frac{[C]_f}{[C]_d} \qquad \textit{(Eq. 3)}$$

To eliminate artifactual differences resulting from different oral amounts, the results are normalized by the amount of labeled FA consumed by the animal (d):

$$\%Dose = \frac{D^*}{d} \qquad \textit{(Eq. 4)}$$

The %Dose value reflects the transformation of labeled FA into products and incorporated into organs in terms of molar equivalents of the dosed FAs, LNA* and LA*, for labeled DHA (DHA*) and ARA (ARA*), respectively.

The labeled FAs are tracers for the unlabeled FA tracees consumed in the feed. The relevant pool sizes that must be taken into account are the differing tracee concentrations, which in this case are LNA and LA in the diets. Normalizing the data to the relative tracee levels permits conclusions to be drawn about the fate of dietary FAs in the meal immediately subsequent to dosing. Changes in FA pools, such as plasma FAs, induced by differing experimental diets are characteristic of the metabolism under study and therefore need not be considered separately. In our treatments, the LNA and LA concentrations were essentially identical for the T−, P−, and P+ groups but different for the B group. The breast milk %Dose values were adjusted by dividing them by the ratio $M_{FA,F}/M_{FA,B}$, where M represents molarity, FA represents either LA or LNA, B represents breast milk, and F represents formula. In the present context, the result can be interpreted as the %Dose that would have been found if the concentrations of breast milk LA and LNA were equivalent to those of the other treatments. For simplicity and consistency with previous reports (8, 39), we refer to this as %Dose.

Correlations among tracer concentrations in plasma, RBC, and tissue pools were calculated from individual data from each animal without regard to treatment group to determine the degree to which pools accessible in human infants correlate with tissues. Each animal was represented by a single data point for DHA* and ARA* for each pool.

Statistics

Data are expressed as means ± SD. Within each organ, means were compared with the SAS System for Windows version 8.02 (SAS Institute, Inc., Cary, NC) using the Mixed Procedure by writing specific contrasts for the main effects of group (B, T−, P+, and P−) and of labeled FAs (DHA* and ARA*). Significance was declared at $P < 0.05$. Regression equations were calculated in Excel 2000 for Windows98 (Microsoft, Seattle, WA).

RESULTS

Body and organ weight data are summarized in **Table 1**. Premature baboons at cesarean section averaged 73% of the birth weight of term neonates ($P < 0.05$). At the time of dosing, 2 weeks adjusted age, differences in body weight were no longer significant; at 4 weeks adjusted age, there were no significant differences in body, brain, or liver weights between the groups.

Diets and total FA

The FA compositions of breast milk, unsupplemented formula (P− and T− groups), supplemented formula (P+ group), and the adult female's feed are shown in **Table 2**. The feed LNA-LA ratio of 10.8 is similar to that of the Western human diet and supported a breast milk LNA-LA ratio of 12.8. Formula ratios were similar to one another, at 9.6 and 9.8 for the unsupplemented and supplemented groups, respectively. The unsupplemented formula was nearly devoid of LCP, whereas the supplemented formula contained twice as much ARA and a similar amount of DHA as did breast milk. Breast milk contained significant amounts (0.85%) of 22:6n-3 LCP precursors,

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TABLE 1. Characteristics of the animal groups

Group	Breast-fed (B)	Term Formula (T−)	Preterm Formula (P−)	Preterm Supplemented (P+)
Gender	One female, three males	Three females, two males	Three females, two males	Three females, two males
Gestational age at cesarean section (days)	Spontaneous birth	Spontaneous birth	156 ± 5	154 ± 2
Age at euthanasia (days)[a]	28	28	28 ± 2	28 ± 2
Birth weight (g)[b]	880 ± 74[A]	872 ± 78[A]	632 ± 61[B]	608 ± 71[B]
Body weight at dosing (g)	1,071 ± 82	931 ± 84	824 ± 185	869 ± 206
Body weight at euthanasia (g)	1,221 ± 103	1,138 ± 131	1,071 ± 187	1,035 ± 220
Brain weight (g)	104 ± 5	96 ± 7	93 ± 8	94 ± 9
Liver weight (g)	31 ± 6	34 ± 5	31 ± 6	29 ± 6

Data are expressed as means ± SD.

[a] The breast-fed and term, formula-fed group ages are chronological, counted from birth (postnatal age). The ages of preterm, formula-fed and preterm, supplemented groups are adjusted ages. The normal gestational period for baboons is 182 days.

[b] Values that do not share the same uppercase superscript are significantly different at $P < 0.05$.

primarily 20:5n-3 and 22:5n-3. In adult humans, the rate-limiting step of 22:6n-3 synthesis appears to be the Δ6-desaturation of 18:3n-3 (40), and n-3 LCPs are assumed to serve as more efficient precursors for 22:6n-3 than for 18:3n-3, although there is no direct evidence to support this point. Also present in breast milk and in the supplemented formula was adrenic acid (22:4n-6), the elongation product of 20:4n-6. Eicosadienoic acid (20:2n-6), an n-6 LCP that is not an intermediate in the pathway of 20:4n-6 synthesis (41), was present in all diets. Fat constitutes ~47% of calories in formula. In early 2003, three US manufacturers' consumer information services reported ARA-DHA ratios in their supplemented formulas (%wt of FAs) of 0.64:0.32, 0.45:0.25, and 0.40:0.15 (C. Stark, personal communication). Thus, the ARA and DHA concentrations in our supplemented formula were higher than those found in commercial formulas; however, the ratio of ARA to DHA was equivalent to that in commercial formula. Importantly, the DHA concentration in the supplemented formula was similar to that in the present breast-fed group.

Table 3 shows abstracted results of total FA concentrations for reference; details are presented elsewhere (33). DHA was dramatically lower in most tissues of the unsupplemented groups, with liver values of ~36% of the breast-fed and supplemented groups. DHA supplementation normalized DHA concentrations in retina, RPE, and liver, but brain, plasma, and RBC DHA concentrations were less than those of the breast-fed group. In contrast, ARA concentrations in the unsupplemented groups were in no cases lower than those in the breast-fed group and were increased significantly in the brain and liver of the unsupplemented preterm group. Supplementation with ARA did not significantly alter brain, retina, RPE, or RBC levels but did increase liver and plasma ARA compared with breast-feeding.

Tracer results

Figure 1 presents tracer results for brain, retina, and RPE. DHA* in brain (on a whole-brain basis), retina, and RPE was significantly lower for the B group than for the formula groups (T−, P+, and P−). P+ group values were significantly lower than P− group values in all tissues, indicating that the LCP supplementation partially attenuated DHA synthesis to be more in line with synthesis in the gold standard breast-fed state. Whole-brain DHA* was ~0.25 ± 0.06%Dose in the B group and 2- to 3-fold greater in the formula groups (P−, 0.80 ± 0.15; T−, 0.69 ± 0.16; P+, 0.63 ± 0.18). Retina and RPE DHA* were 4- to 5-fold greater in the formula groups than in the B group.

Although whole-brain conversion/accretion of ARA* was approximately twice that of the B group compared

TABLE 2. FA composition of breast milk, a standard LCP-free formula, LCP-supplemented formula, and maternal diet (wt% of total FAs) expressed as means ± SD

FAs	Breast Milk	Standard (T−, P−) Formula	Supplemented (P+) Formula	Female's Feed
Σ SFA	27.87 ± 8.49	30.22 ± 2.66	32.36 ± 0.23	30.37 ± 0.66
Σ MUFA	38.95 ± 8.37	44.72 ± 2.18	43.01 ± 0.35	35.27 ± 0.99
18:2n-6	27.23 ± 3.99	22.35 ± 0.42	20.11 ± 0.14	27.93 ± 1.36
18:3n-6	0.27 ± 0.09	0.38 ± 0.04	0.18 ± 0.04	0.28 ± 0.07
20:2n-6	0.78 ± 0.14	ND	0.08 ± 0.11	0.25 ± 0.16
20:3n-6	0.42 ± 0.11	ND	0.19 ± 0.06	1.31 ± 0.06
20:4n-6	0.62 ± 0.12	ND	1.21 ± 0.09	0.24 ± 0.04
22:4n-6	0.22 ± 0.06	ND	ND	ND
18:3n-3	2.12 ± 0.53	2.33 ± 0.02	2.06 ± 0.05	2.58 ± 0.22
20:5n-3	0.34 ± 0.13	ND	0.12 ± 0.02	0.85 ± 0.11
22:5n-3	0.51 ± 0.15	ND	0.06 ± 0.09	0.20 ± 0.03
22:6n-3	0.68 ± 0.22	ND	0.61 ± 0.03	0.73 ± 0.03
18:2n-6/18:3n-3	12.84	9.59	9.76	10.83

FA, fatty acid; LCP, long-chain polyunsaturated fatty acid; MUFA, monounsaturated fatty acid; ND, not detectable; SFA, saturated fatty acid.

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TABLE 3. Selected polyunsaturated tissue FA composition at 4 weeks adjusted age

FA	Breast-fed (B)	Term, Formula (T−)	Preterm, Formula (P−)	Preterm, Supplemented (P+)
Brain				
18:2n-6	1.29 ± 0.12^{a}	1.20 ± 0.10^{a,b}	1.25 ± 0.15^{a,b}	1.05 ± 0.07^{b}
18:3n-3	ND	ND	ND	ND
20:4n-6	13.01 ± 0.09^{a}	13.16 ± 0.80^{a}	14.26 ± 0.36^{b}	13.50 ± 0.63^{a,b}
22:6n-3	13.49 ± 0.40^{a}	11.21 ± 0.55^{b}	8.78 ± 0.26^{c}	12.18 ± 0.27^{d}
Retina				
18:2n-6	2.77 ± 0.16^{a,b}	2.77 ± 0.33^{a,b}	2.98 ± 0.34^{a}	2.36 ± 0.18^{b}
18:3n-3	ND	ND	ND	ND
20:4n-6	10.27 ± 0.38	10.78 ± 0.30	11.11 ± 0.82	10.97 ± 0.39
22:6n-3	23.16 ± 1.06^{a}	19.00 ± 0.92^{b}	16.46 ± 1.82^{c}	22.13 ± 1.21^{a}
RPE				
18:2n-6	8.31 ± 0.22^{a}	7.03 ± 0.39^{b}	7.55 ± 0.35^{b}	6.19 ± 0.31^{c}
18:3n-3	0.12 ± 0.03	0.13 ± 0.05	0.11 ± 0.02	0.10 ± 0.01
20:4n-6	18.52 ± 0.55	18.84 ± 0.64	18.98 ± 0.65	19.74 ± 0.65
22:6n-3	7.45 ± 0.55^{a}	5.23 ± 0.66^{b}	4.16 ± 0.37^{c}	7.55 ± 0.25^{a}
Liver				
18:2n-6	18.31 ± 0.57^{a}	16.71 ± 1.1^{a}	17.18 ± 2.20^{a}	13.34 ± 0.79^{b}
18:3n-3	0.59 ± 0.06^{a}	0.37 ± 0.12^{b}	0.19 ± 0.10^{b}	0.29 ± 0.11^{b}
20:4n-6	11.58 ± 0.59^{a}	14.62 ± 1.03^{b}	15.36 ± 1.37^{b,c}	17.16 ± 1.24^{c}
22:6n-3	10.67 ± 0.90^{a}	4.13 ± 0.27^{b}	3.57 ± 0.32^{b}	10.62 ± 1.01^{a}
RBCs				
18:2n-6	13.06 ± 0.75^{a}	13.35 ± 1.81^{a}	14.81 ± 1.78^{a}	9.97 ± 0.59^{b}
18:3n-3	0.25 ± 0.01	0.27 ± 0.08	0.27 ± 0.06	0.20 ± 0.04
20:4n-6	17.14 ± 1.14^{a}	17.47 ± 1.51^{a}	18.51 ± 1.85^{a}	21.20 ± 0.54^{b}
22:6n-3	8.39 ± 1.00^{a}	4.52 ± 0.30^{b}	3.17 ± 0.59^{c}	7.15 ± 0.72^{a}
Plasma				
18:2n-6	27.94 ± 1.18^{a}	24.32 ± 2.3^{b}	24.32 ± 1.11^{b}	19.23 ± 1.18^{c}
18:3n-3	0.58 ± 0.02	0.55 ± 0.12	0.62 ± 0.11	0.46 ± 0.04
20:4n-6	8.38 ± 0.69^{a}	8.09 ± 1.04^{a}	8.29 ± 0.75^{a}	13.75 ± 1.11^{b}
22:6n-3	6.48 ± 0.76^{a}	1.96 ± 0.26^{b}	1.60 ± 0.06^{b}	5.21 ± 0.63^{c}

Values are means ± SD of percent by weight of total FAs. For B, n = 4; for P−, P+, and T, n = 5. ND, not detectable; RBCs, red blood cells; RPE, retinal pigment epithelium.

^{a,b,c} Different lowercase superscripts in a row indicate significantly different values ($P < 0.05$).

with the formula groups, the differences reached significance only in RPE and not in brain or retina. In RPE, there were no differences among formula groups.

Tracer data for liver, RBC, and plasma are presented in **Fig. 2**. Patterns for these pools were remarkably similar to those in brain, retina, and RPE, and differences were significant for all ARA* comparisons. The difference between the B and formula groups was most dramatic for DHA*, for which the B group had ~6-fold lower DHA* in all tissues. DHA* was significantly lower in liver, plasma, and RBC for the P+ compared with the P− group, consistent with results in brain. In whole liver, ARA* was <0.005%Dose in the B group and ~0.01%Dose in the formula groups. RBC and plasma maintained a smaller differential for ARA* between the B and formula groups. All comparisons between B group and formula group ARA* were significant. These results directly indicate that the accretion of precursor-derived DHA* and ARA* in brain and their appearance in related pools are potentiated in formula feeding compared with breast-feeding.

Potentiation

The synthesis of DHA* and ARA* was significantly increased in most pools of formula-fed neonates compared with breast-fed neonates. To more carefully evaluate these results, we define a parameter, potentiation, as the arithmetic difference in precursor-derived LCP (DHA* or ARA*) in each formula group minus that in the B group. For instance, for the brain,

$$Potentiation(\text{DHA*, T}-) = \text{DHA*(T}-) - \text{DHA*(B)} \quad (Eq.\ 5)$$

where DHA*(x) on the right side of the equation represents brain DHA* in group x (x = T− or B in the example shown).

In all cases, DHA* increased more than ARA* in the formula groups. To summarize the degree of increase, we averaged the potentiation within pools and present them as summary data in **Table 4**. DHA potentiation due to formula ranges from 189% (brain) to 846% (plasma), and ARA potentiation ranges from ~69% for brain to 230% for liver at these 14 day time points.

A notable trend in these data is that DHA* potentiation is greater than ARA* potentiation for every pool. To express this quantitatively, we constructed a potentiation ratio by taking the ratio of the potentiation from DHA*−ARA*, shown in the last line of Table 4. These calculations show that brain DHA* accretion increased almost 3-fold in formula feeding compared with ARA* accretion, 7-fold in plasma, and >3-fold in the RPE, retina, and liver.

Tracer correlations

Table 5 presents equations resulting from linear regression analyses of plasma and RBC DHA* and ARA* versus brain, liver, and retina DHA* and ARA*. All correlations

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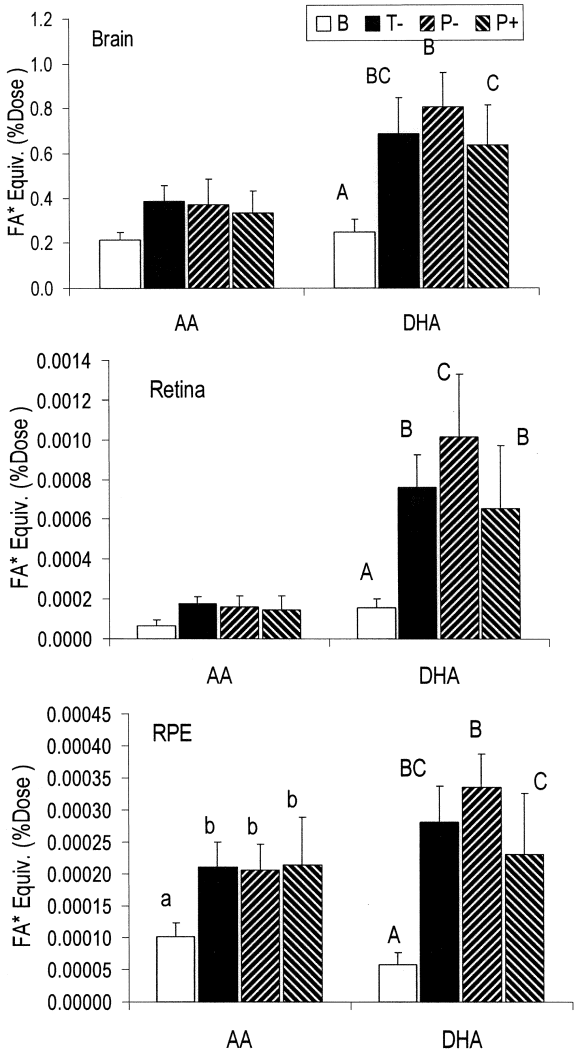

Fig. 1. [U-^{13}C]arachidonic acid (AA) and [U-^{13}C]docosahexaenoic acid (DHA) found in the whole brain, whole retina, and whole retinal pigment epithelium (RPE) at 2 weeks after dosing with [U-^{13}C]linoleic acid (LA*) and [U-^{13}C]α-linolenic acid (LNA*). Data are expressed as %Dose; breast-fed group data were adjusted for different dilutions by breast milk LA and LNA as discussed in Materials and Methods. Values are means ± SD; n = 5 in the T−, P−, and P+ groups, and n = 4 in the B group. Values are different ($P < 0.05$) if they do not share a common letter (lowercase for AA; uppercase for DHA). B, breast-fed; FA, fatty acid; P−, preterm, formula-fed; P+, preterm, formula plus long-chain polyunsaturated fatty acid-fed; T−, term, formula-fed.

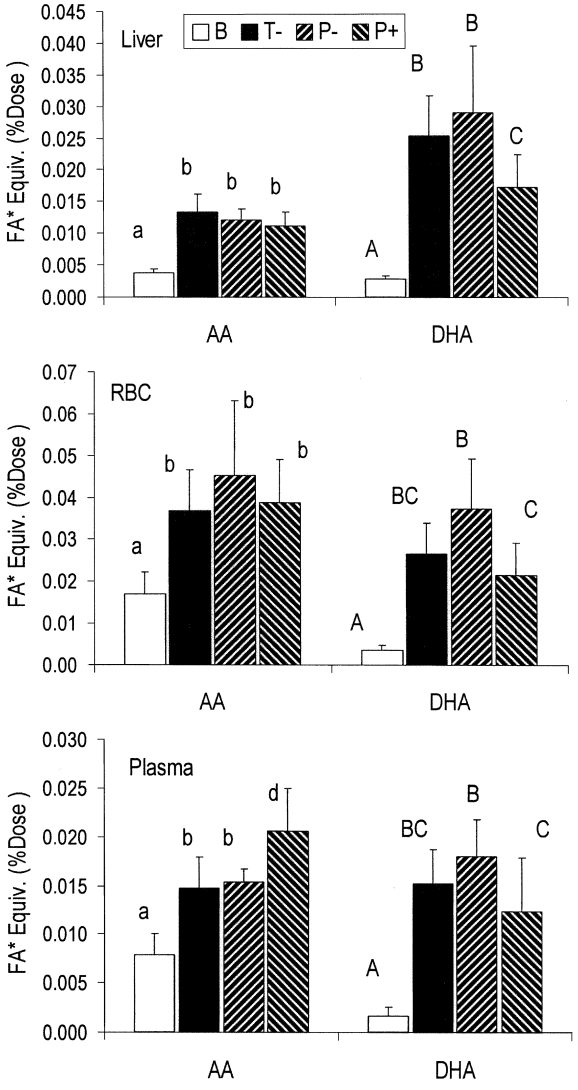

Fig. 2. AA and DHA found in whole liver, red blood cells (RBCs; per 100 ml of blood), and plasma (per 100 ml) at 2 weeks after dosing with labeled LA* and LNA*. Details as in Fig. 1.

were significant at $P < 0.01$, and the results for the most highly correlated pools, RBC and retina DHA*, are presented in **Fig. 3** to illustrate the linearity of the relationship. Values from all animals regardless of treatment are plotted, and each point represents one animal. DHA* in organs was more highly correlated with blood values than was ARA* for each comparison. One factor to explain this is the lower variability of ARA* relative to DHA*; because measurement error tends to be approximately the same for both FAs, the lower biological variability of ARA* should be a smaller fraction of the total variability than that of DHA*. The greater potentiation of DHA* accretion compared with ARA* accretion shown in Table 4 supports this interpretation. The regression slopes listed in Table 5 show that RBCs were a much better predictor of brain and retina DHA* and ARA* than was plasma, whereas plasma was a better predictor of liver levels. The slopes of the regression lines reflect the relative responses of the two pools to changes in tracer concentrations. Slopes for DHA* ranged from 2.3- to 8.8-fold greater than the slopes for ARA*.

DISCUSSION

Breast-feeding is widely regarded as the gold standard of infant nutrition and serves as a guide for dietary recommendations for infants. Micropremies (birth weight <800 g) have survived at appreciable rates only in the last two decades, so there is little reason to believe that breast milk evolved to meet the special needs of the very preterm in-

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TABLE 4. Summary of potentiation data

Variable	Pool					
	Brain	Retina	RPE	Liver	RBC	Plasma
%Potentiation						
ARA*	69	136	108	230	137	114
DHA*	189	420	394	758	666	846
Potentiation ratio (DHA–ARA*)	2.7	3.1	3.7	3.3	n.s.	7.4

%Potentiation refers to the average percentage increase in precursor-derived arachidonic acid (ARA) or docosahexaenoic acid (DHA) in formula groups (T−, P−, and P+) compared with the B group. The DHA-ARA potentiation ratio is the factor increase of DHA over ARA. These data show that α-linolenic acid-derived DHA brain accretion increases, on average, 2.7-fold more than linoleic acid-derived ARA accretion due to formula feeding, with other measured pools showing slightly larger increases. Values shown are average percentage increases of all formula groups in excess of the B ARA* or DHA* value. n.s., not significant.

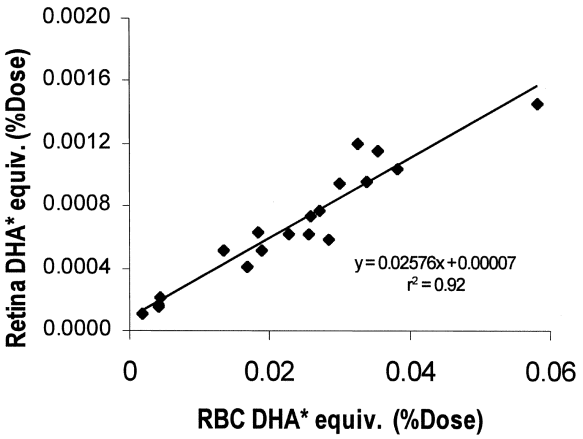

Fig. 3. Relationship between RBC DHA accretion and retina DHA accretion expressed as %Dose. Each point is derived from one animal. The regression equation and r^2 are statistically significant at $P < 0.001$.

fant (42). In addition, conventional infant formulas were developed several decades ago and were aimed at promoting the optimal growth of term infants.

Among the major difficulties facing researchers in the field of human FA requirements is the response of tissue FAs to diet. It is widely recognized that tissue and plasma FA concentrations respond to diet. Improvements in function cannot be directly inferred and must be demonstrated (43). Furthermore, the wide variation in the regulation of individual FA concentrations from tissue to tissue makes extrapolation difficult from compartments accessible in humans, primarily plasma and RBCs. Finally, dietary FAs interact metabolically by apparent competition (44) and precursor/product feedback; thus, inclusion of LCP in formula may result in the attenuation of LCP synthesis from precursors (2). Isotopic tracer data can assist in unraveling the relative contributions of various precursors to a product pool, thereby revealing direct measures of metabolic partitioning and clues to mechanism. Here, DHA* and ARA* reflect only that fraction of DHA and ARA that has been synthesized from their 18-carbon precursors consumed with a meal 2 weeks before analysis at identical adjusted ages of 4 weeks. Because brain turnover of DHA (6, 7) and ARA (39) is slow, the respective tracer concentrations plateau in brain at approximately this time and interpretation is straightforward; kinetics in other pools is more complex and should be approached with caution.

TABLE 5. Linear regression results between RBC (R), plasma (P), brain (B), retina (Ret), and liver (L) ARA* and DHA*

ARA*	DHA*
B = 6.15 R + 0.11 [0.81]	B = 16.2 R + 0.2 [0.86]
B = 11.4 P + 0.16 [0.34]	B = 26.6 P + 0.3 [0.58]
Ret = 0.0034 R + 0.00002 [0.67]	Ret = 0.03 R + 0.0007 [0.91]
Ret = 0.0077 P + 0.00002 [0.43]	Ret = 0.04 P + 0.0001 [0.65]
L = 0.1477 R + 0.0052 [0.29]	L = 0.6755 R + 0.0038 [0.67]
L = 0.4996 P + 0.0029 [0.41]	L = 1.4773 P + 0.0012 [0.79]

r^2 values are given in brackets. All regression slopes are significant at $P < 0.01$.

Comparison of DHA* and ARA* between the B and T− groups shown in Figs. 1 and 2 demonstrates that feeding of LCP-free formula upregulates accretion of LCPs from their precursors. This may be attributable to increased synthesis and/or improved transport efficiency or to lower degradation in the liver; our data cannot distinguish among these possibilities. LCP* accretion in the preterm groups compared with the B and T− groups shows that prematurity does not further upregulate this process. DHA synthesis, as shown in Figs. 1 and 2, comparing the P+ and P− groups, shows that DHA inclusion in formula partially decreases DHA* synthesis/accretion. At least at these dietary levels, supplemental DHA does not restore accretion to the lower, breast-fed levels. Moreover, ARA inclusion in formula does not measurably change ARA synthesis/accretion. It is well known that tissue FA levels are influenced by dietary FA concentrations (43). However, this observation does not equate to improvement in function, inasmuch as excess tissue FA, such as DHA, may be stored. Our previous results unambiguously demonstrate that preterm neonates fed DHA/ARA formula actively lay down amounts of LNA-derived DHA similar to those of breast-fed neonates (33). The data shown in Table 3 demonstrate that DHA total concentrations are compromised, in some cases dramatically, by formulas without DHA, whereas ARA concentrations are either not affected or, paradoxically, increase. These results imply that tissue incorporation of DHA and ARA is subject to very different control mechanisms. More specifically, the low levels of total plasma and liver DHA imply that combined liver and brain synthesis is inadequate to achieve breast-fed DHA levels. In contrast, ARA concentrations are more tightly regulated. ARA synthesis remained high in the formula-fed groups even though ARA levels in formula-fed neonates were nearly twice those of breast-fed neonates.

Figures 1 and 2 also show that LA–derived ARA* is 2- to 3-fold greater in the formula groups than in the B group for liver and RPE ($P < 0.05$), whereas for brain and retina,

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the differences are smaller and only the B and T− comparison is significant. The differential increase in LNA–derived DHA* of the formula groups over the B group is more dramatic, as shown in Table 4.

A possible explanation for the potentiation of precursor-derived LCP is that formula-fed neonates consumed less LCP on average than did breast-fed neonates; therefore, demand was greater. Breast milk consumption could not be measured in nursing neonates; however, Table 1 shows that body weights among groups were not different at necropsy, and the B group averaged the least weight gain (not significantly different) between dosing and necropsy, suggesting that caloric intake was not a decisive factor.

An alternative explanation is the presence of efficient LCP precursors in breast milk that were not in formula. Breast milk contained a lower concentration of n-6 LCP, considering the summed concentration of ARA (0.62%wt) and its precursor 20:3n-6 (dihomo-γ-linolenic acid; 0.42%wt) totaling 1.04%wt, compared with supplemented formula, with 1.21%wt ARA and 0.19%wt 20:3n-6 totaling 1.40%wt. These breast milk values are for milk sampled at necropsy; it is likely that LCP concentrations were higher in colostrum. Human colostrum/milk is highest in LCP at day 2 postpartum and decreases by 20–50% in the first weeks (25, 45). Applying these figures to our baboons as estimates suggests that the B group consumed nearly equivalent amounts of ARA and its efficient LCP precursor, compared with the P+ group. In contrast, although DHA in the supplemented formula (0.61%wt) was similar to that in breast milk (0.68%wt), the sum of 20:5n-3 and 22:5n-3, both efficient DHA precursors, was 0.85%wt in the breast milk but only 0.18% in the supplemented formula. Considering that colostrum and earlier breast milk is much higher in n-3 LCP, we conclude that breast milk DHA and its efficient n-3 precursors may have been considerably greater in breast milk than in the P+ formula. A greater increase in DHA synthesis caused by this difference in demand would account for our results. Failure to find consistently greater increases in DHA* synthesis in the P− and T− groups would fit with this scenario if this synthetic rate is nearly maximal. A compensatory increase in DHA synthesis could lead to an increase in ARA synthesis via common enzymes or other mechanisms responsible for the interactive metabolic regulation of n-3 and n-6 levels. Thus, we cannot rule out the possibility that inadequate DHA levels in the P+ diet influenced ARA biosynthesis as well. Future studies with higher DHA levels will be required to resolve this issue.

The amount of DHA in the supplemented formula was approximately twice that of the high end currently used in US formulas, as noted above. The greater potentiation for DHA than for ARA, shown in Table 4, the failure of supplemented preterm neonates to achieve brain DHA levels of breast-fed neonates, and the significant concentrations of efficient DHA precursors in breast milk suggest that formula DHA at 0.3%wt of FAs may not be adequate to support optimal brain DHA accretion. These data support an effort to examine yet higher levels of DHA supplementation in formula, perhaps as high as 1% of FAs, as was recently shown to improve cognitive function in 1-month-old rhesus monkeys (46). Higher levels would not be unphysiological, because human breast milk DHA ranges to levels in excess of 1% worldwide (25).

Yet another alternative explanation for the potentiation of precursor-derived LCP may be the relative concentrations of precursors. In this study, the LA-LNA ratio in the formulas and the breast milk were 9:7 and 12:8, respectively. This difference is not expected to yield major differences in LCP production, but normalization of the B group %Dose values should eliminate any small linear changes. Finally, unlike LCP, LA and LNA concentrations in human breast milk do not change appreciably in the first weeks of lactation (25). Thus, differences in precursor supply are unlikely to explain our results.

Finally, formula-fed neonates are not exposed to immunoglobulins and other biologically active compounds in breast milk. We speculate that altered and/or increased demands on formula-fed immune system may place increased demands on LCP, as ARA and the DHA precursor eicosapentaenoic acid are the precursors to eicosanoids. Although their specific effects on conversion are not known, it is likely that greater demand would induce more rapid turnover of LCP and, in turn, greater synthesis.

The observation of high conversion in the P+ group is consistent with high tissue LCP demand in the preterm neonate. During pregnancy, the fetus accumulates DHA in the liver and adipose tissue that can be used as a reservoir during postnatal life. Some authors estimate the difference in total body DHA between the preterm and term human infant to be as great as 4.5 g (1), and estimated stores for term human infants are ~1 g in body fat (47). It is likely that lower LCP stores in the preterm neonate increased overall LCP demand compared with that in the term breast-fed neonate.

The regression equations shown in Table 5 may be generally useful for estimating LCP synthesis in tracer studies of human infants that have access only to blood compartments. The correlation coefficients in Table 5 show that ~60–90% of variability in brain, retina, and liver LNA–derived DHA*, and 30–80% of that in LA–derived ARA*, is explained by plasma or RBC levels. Brain and retina are more highly correlated with RBC levels than is plasma, and liver is better correlated with plasma, in agreement with the liver's role as a major source of plasma FAs. The lesser slope coefficients for ARA* indicate that ARA* synthesis in brain, retina, and liver is less responsive to changes in ARA* synthesis in RBC or plasma and probably reflects tighter control of ARA synthesis compared with DHA. Most importantly, the slope coefficients indicate that incremental changes in plasma or RBC DHA and ARA do not reflect equivalent relative changes in tissues. One cautionary note is that these correlations are derived from a single time point, at 14 days after dose. Brain DHA* and ARA* are likely to be at a plateau at this time; however, the DHA* and ARA* in other tissues lose label at 14 days after dose at varying rates, which is expected to alter the correlation coefficients.

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In conclusion, these data demonstrate that feeding formula without LCP to term or preterm neonates potentiates the synthesis of DHA and ARA relative to breast-feeding and that for preterm neonates, this effect is only partially reversed by the inclusion of 0.3% of calories from DHA and 0.6% of calories from ARA in formula. No differences in ARA synthesis were detected in brain, liver, retina, and RPE among formula groups. Under these clinically relevant dietary regimens, precursor-derived DHA increased much more in tissue than did ARA due to formula-feeding, although total brain DHA in supplemented preterm neonates did not reach that of breast-fed neonates. RBC LCP* correlated well with brain and retina LCP*, whereas plasma LCP* was a better predictor of liver LCP*. These results strongly suggest that further attention to the optimal concentration of formula LCP, probably in excess of the levels studied here, is warranted.

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