Modification of Milk Formula to Enhance Accretion of Long-Chain n-6 and n-3 Polyunsaturated Fatty Acids in Artificially Reared Infant Rats

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ABSTRACT: Artificially reared infant rats were used to determine the effects of long-chain polyunsaturated fatty acid (LCPUFA) supplementation on blood and tissue concentrations of arachidonic acid (AA) and docosahexaenoic acid (DHA). Beginning at 7 d of age, infant rats were fed for 10 d with rat milk formulas supplemented with AA at 0, 0.5 and 1.0%, or supplemented with DHA at 0, 0.5 and 1.0% of total fatty acid. The supplementation of AA increased accretion of the fatty acid in tissue and blood phospholipids with a maximum increase of 9% in brain, 15% in liver, 25% in erythrocytes, and 43% in plasma above the values of unsupplemented infant rats. Rat milk formula containing 1.0% of AA had no added benefits over that containing 0.5% of AA. The supplementation of DHA increased phospholipid DHA by a maximum of 24% in brain, 87% in liver, 54% in erythrocytes, and 360% in plasma above the unsupplemented control. The increase in tissue and blood DHA was concentration-dependent on formula fatty acid. Brain phosphatidylcholine and phosphatidylethanolamine were similarly enriched with AA and DHA by supplementation of the corresponding fatty acids. In general the observed increase of AA was accompanied by a decrease in 16:0, 18:1n-9, and/or 18:2n-6, whereas the increased DHA was associated with a reduction of 18:1n-9, 18:2n-6, and/or 20:4n-6. Clearly, infant rats were more responsive to DHA than AA supplementation, suggesting a great potential of dietary manipulation to alter tissue DHA concentrations. However, the supplementation of DHA significantly decreased tissue and blood AA/DHA ratios (wt%/wt%), whereas there was little or no change in the ratio by AA supplementation. Although the physiological implications of the levels of AA and DHA, and AA/DHA ratios achieved under the present experimental conditions are not readily known, the findings suggest that artificial rearing could provide a suitable model to investigate LCPUFA requirements using various sources of AA and DHA in rats.

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Long-chain polyunsaturated fatty acids (LCPUFA), especially arachidonic acid (AA, 20:4 n-6) and docosahexaenoic acid (DHA, 22:6n-3) are found in high concentrations in neural tissues and retina (1–3). Although precise functions of LCPUFA are not completely understood, AA and DHA are currently considered essential for growth, development, and maturation of fetuses and infants (4–6). In addition, inadequate DHA level has been associated with impaired visual acuity, learning ability, and neural functions (7–11). Thus, adequate provision of LCPUFA and their precursors to ensure optimal growth and development of infants is obviously critical (6,12).

Human milk, which contains various concentrations of AA and DHA, is the preferred source of LCPUFA (13). Most infant formulas, on the other hand, are devoid of AA and DHA (13), but do contain linoleic acid (LA, 18:2n-6) and α-linolenic acid (LN, 18:3n-3), the precursors of LCPUFA (14). Preterm infants fed commercially available formulas had AA and DHA concentrations in plasma and erythrocytes that were lower than those of breast-fed counterparts (5,15,16). Similar relationships between infant formula feeding and blood LCPUFA levels were noted in full-term infants. Term infants fed conventional formulas were unable to maintain concentrations of blood AA and DHA seen in breast-fed infants during postnatal development (5,15,17–19). Coincident with the difference in plasma and erythrocyte DHA levels, the formula-fed infants had poorer visual acuity, slower information processing ability, and impaired neural functions compared to breast-fed infants (8–12). In an attempt to promote accretion of LCPUFA, DHA in the form of fish oil or LN in vegetable oil has been supplemented to infant formulas (16,18–22). Fish oil added to formula increased DHA in plasma and erythrocytes to a level comparable to that of breast feeding (16,21). Vegetable oil (e.g., soybean oil and canola oil) rich in LN also increased DHA levels in plasma and erythrocytes (10,18,22), and the extent of the increase was inversely related to LA/LN ratios in the diet (14,23). However, the maximum level of DHA achieved by dietary LN fell short of that observed in infants fed fish oil-supplemented formula or breast milk (20,23). Interestingly, the supplementation of DHA or LN in formulas has been shown to improve visual acuity and

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Abbreviations: AA, arachidonic acid; AR, artificial rearing; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; LN, α-linolenic acid; LCPUFA, long-chain polyunsaturated fatty acids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; RMF, rat milk formula.

neural functions to various degrees (10,12,19), although such benefits were not observed in other studies (22,24,25). It should be stressed that fish oil supplementation resulted in a reduction of AA in blood which was accompanied by growth retardation of preterm infants (26). Nonetheless, when fish oil high in DHA but low in eicosapentaenoic acid (EPA, 20:5n-3) was used in formula supplementation, no such adverse effects on blood AA concentration and growth were observed (9,16).

Despite these observations there are concerns about the usefulness of fish oil to provide DHA for infants because of the potential deleterious effect of EPA (3). EPA may compete with AA for production of different series of prostaglandins and leukotrienes leading to metabolic abnormalities (27). The present study was therefore undertaken to test the use of microbial oils rich in either AA or DHA but devoid of EPA as sources of LCPUFA for infant rats. To this end, an artificial rearing (AR) model of infant rats was used throughout the study. The postnatal brain growth spurt in rats corresponds to the rapid growth of human brain during the perinatal period (28). Thus, the AR infant rats provide a useful model to study brain metabolism and development of preterm infants. One of the major advantages of the AR model is the precise control of quantity and composition of milk formula provided to each pup (29). Moreover, the model has been proven suitable for studying metabolic consequences or nutrient requirements of neonates by altering the composition and amount of milk (29–31). The results demonstrated that supplementation of AA and DHA was effective in enriching the respective fatty acids in blood and tissue phospholipids.

MATERIALS AND METHODS

Animals and artificial rearing. Pathogen-free pregnant Sprague-Dawley rats at 14 d of gestation were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and housed in individual plastic containers. They were fed *ad libitum* a nonpurified diet (Purina Rat Chow, Ralston Purina, St. Louis, MO) and had access to water at all times. For each study, newborns from four pregnant rats were delivered naturally, culled to 10 pups per dam within 12 h of birth, and were nourished by their dams until the beginning of AR. At 7 d of age, one to two weight-matched rat pups from each litter were assigned to one of three AR groups. In Study 1, the pups were fed rat milk formulas (RMF) that contained 0, 0.5, or 1.0% AA as total fatty acids by weight. In Study 2, the pups received RMF which contained 0, 0.5, or 1.0% DHA. Ten of the remaining pups comparable to those in the AR groups were assigned to one of the four dams. They were suckled by the dam throughout the lactating period and were used as models for growth rates. For artificial feeding, infant rats were anesthetized with diethyl ether for 90 s in a chamber for the purpose of permanent placement of intragastric cannulas using the nonsurgical technique of Hall (32). The cannulas were connected to plastic syringes on an infusion pump for automatic feeding for 15 min each hour (30). All pups were weighed daily, and AR care was provided, including antiseptic dressing, cleansing of the perianal area, and stimulation to eliminate (30). The daily milk intake of artificially reared rats was adjusted to match the growth rate of the mother-reared pups. The animal protocol was reviewed and approved by The Pennsylvania State University Animal Care and Use Committee.

RMF. RMF were prepared essentially according to the method of Auestad *et al*. (33). The formulas were modified from RMS-2A formula and were composed of carbohydrate, protein, fat, vitamins, and minerals that closely resembled those of rat milk (33). All formulas contained 11.8% fat (w/w) consisting of fat from milk formula base (3.4%), medium-chain triglyceride (2.0%), and corn oil (microbial oil + corn oil = 6.4%) (30). Microbial oils (Martek Bioscience, Columbia, MD) provided sources of n-6 and n-3 LCPUFA. Microbial oil A (triglyceride oil produced by *Mortierella alpina*) contained 17.7% AA, 16.3% 16:0, 15.1% 18:0, 23.4% 18:1n-9, 11.5% 18:2n-6, and small percentages of other fatty acids, but no DHA and EPA. Microbial oil B (triglyceride oil produced by *Crypthecodinium cohnii*) contained 41.9% DHA, 15.5% 14:0, 19.1% 16:0, 13.8% 18:1n-9, and small percentages of other fatty acids, but no AA and EPA. In Study 1, microbial oil A was added at the expense of corn oil to make RMF variations in AA concentrations of 0, 0.5, or 1.0%. Microbial oil B was used in Study 2 to prepare formulas containing 0, 0.5, or 1.0% DHA. All RMF were degassed with nitrogen and allocated for storage at −20°C. The formulas were rehomogenized daily before feeding.

Lipid extraction and fatty acid analysis. Infant rats were artificially fed for 10 d. At 17 d of age, the pups were anesthetized with pentobarbital (Anpro Pharmaceutical, Arcadia, CA) (5 mg/100 g body weight) for collection of blood by cardiac puncture. Livers and brains were excised, rinsed in 0.9% NaCl, blot-dried on filter paper, frozen in liquid nitrogen, and then stored at −80°C for subsequent lipid analysis. Plasma and erythrocytes were prepared by centrifugation and wash procedures described previously (34).

For analyses of fatty acids, lipid extracts were prepared from plasma, erythrocytes, brain, and liver according to the extraction procedures of Folch *et al.* (35), using chloroform/methanol (2:1, vol/vol) containing 50 μg butylated hydroxytoluene as the antioxidant. Lipid extracts were then subjected to thin-layer chromatographic separation of lipid classes using silica G plates and a hexane/ethyl ether/acetic acid (80:20:1, by vol) solvent (36). The phospholipid band remaining at the origin of the plate was scraped into an ampule for transmethylation by using 12% boron trifluoride in methanol (wt/vol) (37). The methylated fatty acids were analyzed by gas chromatography (Model 5980 Series II; Hewlett-Packard, Palo Alto, CA) equipped with an SP-2330 fused-silica capillary column (30 m × 0.25 mm i.d., 20 μm film; Supelco, Bellefonte, PA) and a flame-ionization detector. The gas chromatographic conditions were the same as those described elsewhere (38). For fatty acid analysis of phospholipid species, the lipid extracts were separated into phosphatidylcholine (PC) and phosphatidylethanolamine (PE) by thin-layer chromatography using silica G plates and a solvent mixture of chloro-

form/methanol/petroleum ether (bp 35–60°C)/acetic acid /boric acid (40:20:30:10:1.8; vol/vol/vol/vol/wt) as described in a previous study (39). The isolated individual phospholipids were transmethylated and analyzed by gas chromatography (38). Fatty acid proportions were expressed as wt% of total fatty acid. The absolute content of individual fatty acids was measured by the addition of free heptadecanoic acid as an internal standard prior to transmethylation (34).

Statistical analysis. Data are expressed as means ± SD. The significant difference in comparing three dietary treatments was analyzed by one-way analysis of variance. Tukey's pairwise comparisons (40) using Minitab Statistical software (Minitab, State College, PA) was applied to determine where the difference existed.

RESULTS

Artificially reared infant rats grew at the same rate as the mother-reared counterparts. At the onset of the artificial feeding on day 7 of life, mean body weight for three groups of infant rats fed AA- supplemented formulas were 15.5 ± 1.0 g, 16.0 ± 1.1 g, and 15.7 ± 1.0 g compared with 15.3 ± 0.8 g for mother-reared offspring. After 10 d of feeding (i.e., at 17 d of age) the mean body weights of 28.7 ± 1.7 g, 29.0 ± 0.8 g, and 29.1 ± 2.0 g were not different from 29.6 ± 1.4 g of the mother-reared infant rats with the same age. Similarly, there was no difference in body weight between a mother-reared and artificially reared infant rats before and after the feeding of DHA-supplemented formulas.

Fatty acid analyses of RMF revealed that the actual weight percentage of AA (i.e., 0, 0.46, and 0.93%) and DHA (i.e., 0, 0.51, and 0.94%) in the formulas were close to the targeted levels of 0, 0.5, and 1.0% (Table 1). There was no difference in other fatty acids among the three formulas supplemented with DHA. Similarly, the wt% of other fatty acids were maintained constant except stearic acid (18:0) in the AA-supplemented formulas. All formulas contained medium-chain fatty acids (i.e., 8:0 and 10:0), although they were lower in DHA-supplemented than AA-supplemented formulas. Overall, the unsupplemented formulas contained major fatty acids, except 18:3n-3, similar to those of rat milk from various sources compiled by Auestad *et al*. (33). The absence of 18:3n-3 in the present formulas was designed to minimize the contribution of n-3 LCPUFA by endogenous synthesis.

The first series of experiments determined the effects of AA supplementation on blood and tissue fatty acid composition of phospholipids. As shown in Table 2, AA supplemented at 0.5 and 1.0% increased AA equally in plasma by 35–43% above the level of unsupplemented formula. The increases were accompanied by a lower weight percentage of 16:0 and 18:1n-9 than that of unsupplemented. The concentrations of DHA in plasma were not altered by AA supplementation. The AA supplementation also enriched the AA level in erythrocytes by 20–25% as compared with control unsupplemented infant rats, but lowered the weight percentages of 16:0, 18:0, and 18:1n-9. The erythrocyte DHA level was higher in supplemented than unsupplemented animals.

In the brain, the weight percentage of AA in phospholipids was 9% higher in the AA-supplemented groups at either 0.5 or 1.0% AA than in the unsupplemented group (Table 3). There were slight, but significant reductions in DHA by the two levels of AA supplementation. The weight percentages of 18:2n-6, 22:4n-6, and 22:5n-6 were lower in 0.5% AA-supplemented than in unsupplemented infant rats. All other brain fatty acids remained unchanged. The weight percentages of AA in liver phospholipids of infant rats fed supplemented formulas (both at 0.5 and 1.0%) were 10–15% higher than the control (Table 3). AA supplementation had no effect on weight percentage of liver DHA or other fatty acids. Brain PE contained markedly higher concentrations of AA and DHA than PC (Table 4). AA supplementation increased AA accretion in both phospholipid species with greater percentages increases in PC (i.e., 21–25% above the control) than those in PE (i.e., 5–8% above the control). The AA supplementation did not alter weight percentages of brain DHA, 16:0, 18:0, and 18:1n-9, but lowered 18:2n-6 and increased 22:4n-6 and 22:5n-6 in PC. In PE, the increase in weight percentage of AA was accompanied by a reduction in 16:0 and 18:0 when the

TABLE 1
Fatty Acid Composition of Rat Milk Formulas Supplemented with Arachidonic or Docosahexaenoic Acid[a]

	Formula			Formula		
Fatty acid	0% AA	0.5% AA	1.0% AA	0% DHA	0.5% DHA	1.0% DHA
8:0	8.5 ± 0.7	8.3 ± 1.2	7.8 ± 1.7	5.9 ± 1.4	5.9 ± 1.1	7.0 ± 0.8
10:0	7.3 ± 0.4	8.0 ± 1.0	7.0 ± 0.6	4.6 ± 0.3	5.0 ± 0.5	4.7 ± 0.2
12:0	1.1 ± 0.2	1.3 ± 0.3	1.2 ± 0.3	1.0 ± 0.3	1.0 ± 0.1	1.1 ± 0.1
14:0	3.5 ± 0.6	4.2 ± 1.3	4.0 ± 1.0	3.4 ± 1.1	3.5 ± 0.2	3.6 ± 0.5
16:0	15.8 ± 1.2	16.9 ± 2.8	16.7 ± 1.9	16.2 ± 2.0	16.1 ± 0.4	16.0 ± 1.0
18:0	3.9 ± 0.6[a]	5.7 ± 1.3[b]	5.6 ± 0.8[b]	5.3 ± 1.1	5.2 ± 0.2	5.2 ± 0.6
18:1n-9	21.5 ± 0.5	21.9 ± 1.4	22.3 ± 0.9	23.6 ± 0.3	23.1 ± 0.5	22.9 ± 0.3
18:2n-6	35.2 ± 2.2	30.9 ± 3.4	32.6 ± 3.3	38.8 ± 5.9	38.6 ± 1.1	37.4 ± 2.2
20:4n-6	—	0.46 ± 0.03[a]	0.93 ± 0.04[b]	—	—	—
22:6n-3	—	—	—	—	0.51 ± 0.01[a]	0.94 ± 0.05[b]

[a]Values (wt%) are means ± SD for four samples. Fatty acids included only major saturated and unsaturated (n-9, n-6, and n-3) species, and hence did not add up to 100%. Values for each supplement with different superscript roman letters in the same row are significantly different at $P < 0.05$. AA = arachidonic acid, DHA = docosahexaenoic acid.

TABLE 2
Fatty Acids of Plasma and Erythrocyte Phospholipids in Infant Rats Fed Formulas Supplemented with AA[a]

	Plasma[a]			Erythrocyte[a]		
Fatty acid	0% AA	0.5% AA	1.0% AA	0% AA	0.5% AA	1.0% AA
16:0	28.5 ± 1.5[a]	26.8 ± 1.7[a,b]	24.9 ± 1.5[b]	32.6 ± 2.1[a]	29.4 ± 2.1[b]	29.2 ± 2.5[b]
18:0	25.2 ± 2.1	24.3 ± 1.1	24.4 ± 0.9	13.7 ± 0.2[a]	12.5 ± 1.0[b]	12.2 ± 1.3[b]
18:1n-9	9.6 ± 0.9[a]	8.2 ± 0.5[b]	8.5 ± 0.6[b]	10.9 ± 0.6[a]	9.3 ± 0.2[b]	8.4 ± 2.6[b]
18:2n-6	24.5 ± 2.8	25.3 ± 2.0	25.9 ± 2.9	9.7 ± 0.5[a,b]	10.8 ± 1.0[a]	9.4 ± 0.5[b]
20:4n-6	7.4 ± 0.7[a]	9.9 ± 0.8[b]	10.5 ± 1.4[b]	18.0 ± 1.0[a]	21.6 ± 0.9[b]	22.4 ± 1.6[b]
22:4n-6	0.3 ± 0.5	0.4 ± 0.4	0.4 ± 0.5	1.8 ± 1.5	2.9 ± 0.3	2.9 ± 0.2
22:5n-6	—	—	—	1.7 ± 0.2[a]	2.3 ± 0.3[b]	2.3 ± 0.5[b]
22:6n-3	2.3 ± 0.5	2.8 ± 0.8	2.4 ± 0.3	4.5 ± 0.4[a]	6.2 ± 1.0[b]	6.4 ± 1.1[b]
AA/DHA	3.3 ± 0.8[a]	3.7 ± 1.0[a,b]	4.4 ± 0.7[b]	4.0 ± 0.2	3.6 ± 0.5	3.6 ± 0.5

[a]Values (wt%) are means ± SD for four samples. Fatty acids included only major saturated and unsaturated (n-9, n-6, and n-3) species, and hence did not add up to 100%. Values for plasma or erythrocyte with different superscript roman letters in the same row are significantly different at $P < 0.05$. For abbreviations see Table 1.

formula was supplemented with 1.0% AA. The weight percentage of DHA was not affected, but the weight percentages of 22:4n-6 and 22:5n-6 were slightly increased by the AA supplementation. Consequent to the AA supplementation, AA/DHA ratios were increased in plasma and brain total phospholipids but were not changed in erythrocyte and liver total phospholipids or in brain PC and PE (Tables 2–4).

In the second series of experiments, RMF supplemented with DHA were fed to artificially reared infant rats. The supplementation of DHA markedly increased accretion of DHA in plasma phospholipids to 2.7–4.6-fold of the unsupplemented level (Table 5). The increase in DHA was accompanied by reduction of 18:0 (4–10 wt%), 18:1n-9 (13–16 wt%), and 16:0 (11 wt% only at high DHA-supplemented group). The weight percentage of plasma AA was not affected by the supplementation. Erythrocyte DHA levels were 27–54% higher in infant rats fed formula supplemented with DHA than the control (Table 5). There was a concomitant reduction in weight percentages of 18:2n-6 (5–8%) and AA (7–13%). No other fatty acids were affected by the DHA supplementation.

The supplementation of DHA also enhanced the accretion of DHA in tissue phospholipids. In the brain, weight percentages of DHA of the supplemented animals were 20–24% higher than those of unsupplemented counterparts (Table 6). Brain AA, 16:0, 18:0, and 18:2n-6 levels were not changed by DHA supplementation, but there were significant decreases in weight percentages of 18:1n-9 and 22:4n-6. Liver phospholipid DHA levels were highly enriched by the DHA supplementation (Table 6). In the supplemented groups, weight percentages of DHA were 45–81% higher than those of unsupplemented infant rats. On the other hand, the weight percentages of 18:1n-9 and 18:2n-6 were lower in the supplemented than in the unsupplemented group. There were no differences in AA, 16:0, and 18:0 among the groups. A similar trend of changes in fatty acid composition of brain PC and PE was noted. In PC, DHA supplementation increased weight percentages of DHA by 17–33% with little changes in the other fatty acids (Table 7). The enrichment of DHA in PE was more pronounced than in PC and was accompanied by slight but significant reduction in 18:1n-9, 18:2n-6, AA and 22:4n-6.

The supplementation of DHA in formulas reduced AA/DHA ratios in blood and tissues. This reduction resulted from increased DHA together with either decreased or unchanged AA. The most pronounced decrease in the ratio was seen in plasma, followed by erythrocyte phospholipids. The ratios decreased from 8:1 in the control to 3:1 and 2:1 in the supplemented groups in plasma, and from 6:1 in the control

TABLE 3
Fatty Acids of Brain and Liver Phospholipids in Infant Rats Fed Formulas Supplemented with AA[a]

	Brain			Liver		
Fatty acid	0% AA	0.5% AA	1.0% AA	0% AA	0.5% AA	1.0% AA
16:0	33.9 ± 0.9	35.6 ± 0.6	35.0 ± 1.1	20.2 ± 1.3	19.2 ± 1.7	20.8 ± 1.8
18:0	21.6 ± 0.4	22.1 ± 0.7	21.8 ± 1.0	25.8 ± 2.8	24.6 ± 1.5	26.6 ± 2.9
18:1n-9	14.4 ± 0.1[a]	15.1 ± 0.2[b]	14.7 ± 0.4[a,b]	11.2 ± 1.6	10.5 ± 1.3	11.0 ± 1.4
18:2n-6	1.3 ± 0.1[a]	1.2 ± 0.03[b]	1.3 ± 0.1[a,b]	17.9 ± 2.5	17.7 ± 2.7	15.7 ± 2.6
20:4n-6	13.8 ± 0.4[a]	15.0 ± 0.4[b]	15.1 ± 0.5[b]	13.9 ± 0.7[a]	16.0 ± 0.8[b]	15.2 ± 0.6[b]
22:4n-6	2.4 ± 0.1[a]	2.1 ± 0.1[b]	2.0 ± 0.9[a,b]	0.9 ± 0.3	1.4 ± 0.4	1.1 ± 0.4
22:5n-6	1.4 ± 0.2[a]	1.1 ± 0.1[b]	1.3 ± 0.1[a]	1.3 ± 0.8	1.1 ± 0.8	0.8 ± 0.4
22:6n-3	8.3 ± 0.4[a]	6.5 ± 0.7[b]	7.5 ± 0.6[c]	6.1 ± 0.9	7.0 ± 1.8	5.5 ± 1.3
AA/DHA	1.3 ± 0.03[a]	1.4 ± 0.1[b]	1.4 ± 0.1[b]	2.4 ± 0.4	2.4 ± 0.6	2.8 ± 1.4

[a]Values (wt%) are means ± SD for four samples. Fatty acids included only major saturated and unsaturated (n-9, n-6, and n-3) species, and hence did not add up to 100%. Values for each tissue with different superscript roman letters in the same row are significantly different at $P < 0.05$. For abbreviations see Table 1.

TABLE 4
Fatty Acids of Brain Phosphatidylcholine and Phosphatidylethanolamine in Infant Rats Fed Formulas Supplemented with AA[a]

	Phosphatidylcholine[a]			Phosphatidylethanolamine[a]		
Fatty acid	0% AA	0.5% AA	1.0% AA	0% AA	0.5% AA	1.0% AA
16:0	61.6 ± 0.7	60.3 ± 1.2	60.4 ± 1.2	10.4 ± 0.6[a]	10.0 ± 0.6[a,b]	9.5 ± 0.5[b]
18:0	8.5 ± 0.4	8.7 ± 0.4	8.4 ± 0.3	34.9 ± 0.8[a]	33.4 ± 1.6[a]	31.8 ± 0.7[b]
18:1n-9	18.2 ± 0.5	18.4 ± 0.1	18.1 ± 0.2	9.4 ± 0.5	9.3 ± 0.5	8.9 ± 0.5
18:2n-6	1.8 ± 0.02[a]	1.3 ± 0.1[b]	1.4 ± 0.1[b]	0.7 ± 0.04	0.7 ± 0.1	0.7 ± 0.1
20:4n-6	3.9 ± 0.1[a]	4.7 ± 0.5[b]	4.8 ± 0.7[b]	17.2 ± 0.6[a]	18.2 ± 0.8[b]	18.6 ± 0.4[b]
22:4n-6	0.4 ± 0.04[a]	0.4 ± 0.1[a,b]	0.5 ± 0.1[b]	5.5 ± 0.2[a]	5.7 ± 0.2[a]	6.3 ± 0.2[b]
22:5n-6	0.2 ± 0.03[a]	0.2 ± 0.1[a,b]	0.3 ± 0.1[b]	2.3 ± 0.1[a]	2.4 ± 0.2[a]	2.9 ± 0.2[b]
22:6n-3	1.2 ± 0.1	1.4 ± 0.3	1.5 ± 0.4	17.5 ± 0.9	18.0 ± 1.1	19.1 ± 1.8
AA/DHA	3.2 ± 0.2	3.4 ± 0.5	3.4 ± 0.5	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1

[a]Values (wt%) are means ± SD for four samples. Fatty acids included only major saturated and unsaturated (n-9, n-6, and n-3) species, and hence did not add up to 100%. Values for each phospholipid with different superscript roman letters in the same row are significantly different at $P < 0.05$. For abbreviations see Table 1.

to 4:1 and 3:1 in the supplemented infant rats in erythrocytes (Table 5). The ratios in the liver decreased from the unsupplemented 4:1 to 3:1 and 2:1 in the supplemented groups (Table 6). There was slight but significant reduction of the ratios in brain total phospholipids, PC, and PE (Tables 6 and 7).

The increased weight percentages of AA and DHA in tissue and blood resulting from the supplementation of the corresponding AA and DHA in milk formulas led to significant changes in the absolute amounts of the fatty acids. In infant rats who received unsupplemented formula, absolute amount of total fatty acids in phospholipids of plasma was 1.03 ± 0.86 mg/mL, of erythrocytes 0.52 ± 0.05 mg/mL, of brain 9.03 ± 0.57 mg/g, and of liver 2.97 ± 0.33 mg/g. The concentrations were not altered in those fed formulas supplemented with either AA or DHA.

DISCUSSION

Long-chain polyunsaturated fatty acids, especially DHA, have been suggested as conditionally essential and should be provided to infants (41,42). Although infants are capable of desaturation and elongation of LN and LA to form DHA and AA, respectively (14,43), the synthesis may not be sufficient to meet AA and DHA requirements during the rapid phase of growth and development in infants (20,23,43). Supplementation of DHA in formula has been proven effective in enriching and maintaining DHA levels in the circulation to levels that are achieved by breast-feeding (16,19–21). More importantly, the supplementation of DHA improved visual function to a greater extent than LN supplementation in preterm infants (44,45). However, the effectiveness of LN supplementation in improving visual acuity is controversial. Makrides *et al.* (46) and Jorgensen *et al.* (11) have independently demonstrated that infants fed formulas with LN levels as high as or higher than human milk had lower visual acuity than that of breast-fed counterparts. To the contrary, most recent studies of Auestad *et al.* (22) and Innis *et al.* (24,25) showed that there was no difference in visual acuity in infants fed either breast milk or formula containing LN. Clearly, the benefits of n-6 and n-3 fatty acid supplementation require further investigation. In this context, it is pertinent to point out that supplementation of DHA from various sources has been extensively investigated in human infants (12,16,18,22), and newborn animals (30,47–49). The present study was designed to evaluate the potential use of Microbial oil A, rich in AA but devoid of DHA and EPA, and Microbial oil B, rich in DHA but devoid of AA and EPA, to enrich LCPUFA in infant rats.

The results clearly demonstrated that supplementation of

TABLE 5
Fatty Acids of Plasma and Erythrocyte Phospholipids in Infant Rats Fed Formulas Supplemented with DHA[a]

	Plasma[a]			Erythrocyte[a]		
Fatty acid	0% DHA	0.5% DHA	1.0% DHA	0% DHA	0.5% DHA	1.0% DHA
16:0	22.4 ± 0.6[a]	22.2 ± 1.3[a]	19.9 ± 1.6[b]	32.4 ± 1.3	33.7 ± 1.0	33.6 ± 0.7
18:0	27.2 ± 0.6[a]	26.0 ± 0.7[b]	24.6 ± 0.7[c]	15.8 ± 0.7	15.7 ± 0.4	15.8 ± 0.5
18:1n-9	8.8 ± 0.4[a]	7.6 ± 0.8[b]	7.4 ± 0.5[b]	10.5 ± 0.4	10.5 ± 0.3	10.2 ± 0.2
18:2n-6	28.3 ± 1.1	29.0 ± 1.2	29.4 ± 2.3	13.2 ± 0.5[a]	12.1 ± 0.6[b]	12.5 ± 0.3[b]
20:4n-6	10.6 ± 0.6	10.3 ± 1.0	11.8 ± 1.1	19.4 ± 1.8[a]	18.0 ± 0.8[a]	16.9 ± 0.4[b]
22:4n-6	0.5 ± 0.1	0.5 ± 0.04	0.4 ± 0.1	1.0 ± 0.1	0.9 ± 0.03	0.8 ± 0.2
22:6n-3	1.2 ± 0.1[a]	3.3 ± 0.8[b]	5.4 ± 0.4[c]	3.4 ± 0.4[a]	4.4 ± 0.4[b]	5.3 ± 0.4[c]
AA/DHA	7.9 ± 0.7[a]	3.3 ± 0.6[b]	2.2 ± 0.2[c]	5.7 ± 0.2[a]	4.2 ± 0.3[b]	3.2 ± 0.2[c]

[a]Values (wt%) are means ± SD for four samples. Fatty acids included only major saturated and unsaturated (n-9, n-6, and n-3) species, and hence did not add up to 100%. Values for each plasma or erythrocyte with different superscript roman letters in the same row are significantly different at $P < 0.05$. For abbreviations see Table 1.

TABLE 6
Fatty Acids of Brain and Liver Phospholipids in Infant Rats Fed Formulas Supplemented with DHA[a]

	Brain[a]			Liver[a]		
Fatty acid	0% DHA	0.5% DHA	1.0% DHA	0% DHA	0.5% DHA	1.0% DHA
16:0	35.2 ± 1.4	34.2 ± 0.4	35.0 ± 1.6	21.0 ± 0.9	21.8 ± 0.4	21.2 ± 1.2
18:0	25.3 ± 0.5	24.1 ± 0.9	24.4 ± 0.5	27.9 ± 0.5	27.1 ± 0.7	28.0 ± 1.2
18:1n-9	13.3 ± 0.2^{a}	12.8 ± 0.4^{b}	11.8 ± 0.2^{b}	9.0 ± 0.8^{a}	7.9 ± 0.7^{a,b}	7.5 ± 0.6^{b}
18:2n-6	1.7 ± 0.1	1.8 ± 0.1	1.7 ± 0.1	16.8 ± 0.9a	15.4 ± 1.5^{a,b}	14.3 ± 0.7^{b}
20:4n-6	12.2 ± 0.6	13.3 ± 0.9	13.3 ± 0.6	16.0 ± 0.5	16.3 ± 1.2	17.3 ± 1.5
22:4n-6	0.2 ± 0.01^{a}	0.2 ± 0.01^{b}	0.1 ± 0.1^{b}	0.8 ± 0.01	0.7 ± 0.1	0.9 ± 0.2
22:5n-6	1.2 ± 0.03	1.2 ± 0.1	1.2 ± 0.01	0.9 ± 0.3	1.2 ± 0.4	1.1 ± 0.8
22:6n-3	8.8 ± 0.9^{a}	10.6 ± 0.7^{b}	10.9 ± 0.8^{b}	4.2 ± 0.1^{a}	6.1 ± 0.1^{b}	7.6 ± 1.0^{c}
AA/DHA	1.4 ± 0.1^{a}	1.3 ± 0.02^{b}	1.2 ± 0.1^{b}	3.9 ± 0.2^{a}	2.7 ± 0.2^{b}	2.3 ± 0.4^{b}

[a]Values (wt%) are means ± SD for four samples. Fatty acids included only major saturated and unsaturated (n-9, n-6, and n-3) species, and hence did not add up to 100%. Values for each tissue with different superscript roman letters in the same row are significantly different at $P < 0.05$. For abbreviations see Table 1.

AA increased accretion of the fatty acid in total phospholipids of plasma, erythrocytes, brain and liver (Tables 2, 3). Similar increases in brain PE and PC were observed (Table 4). The extent of increase of AA, however, was relatively small, ranging from ≤9% for brain, ≤15% for liver, ≤25% for erythrocytes, to ≤43% for plasma above the values of the infant rats fed unsupplemented formula. Furthermore, an increase in AA content from 0.5 to 1.0% in milk formulas did not further increase tissue and blood accretion of AA. The increase of AA in most cases was accompanied by a decrease in 18:2n-6, 18:1n-9, and/or 16:0. DHA provided in milk formulas was effective in enriching the fatty acid in the circulation and tissues (Tables 5–7), and the extent of the increase above the control was greater than that of AA enrichment. Phospholipid DHA was increased up to 360% in plasma, 87% in liver, 54% in erythrocytes, and 24% in brain above that of the unsupplemented group. There was a concomitant reduction of 18:1n-9, 18:2n-6, and/or 20:4n-6 associated with these changes. Moreover, the accretion of DHA increased with increasing DHA content in milk formulas in almost all tissue and blood phospholipids. The reason for the different degree of enrichment between AA and DHA is not known. Contrary to LA and LN, both AA and DHA are not readily oxidized for energy (7). Instead, they are mostly esterified to glycerolipids (50). Therefore, the competition between β-oxidation and incorporation into complex lipids may not explain the difference. It is possible that phospholipids in various blood components and tissues are initially saturated with AA more than DHA, and the degree of saturation may determine the incorporation of LCPUFA into phospholipids. This notion is supported by the facts that weight percentages of AA are higher than weight percentages of DHA in plasma, erythrocytes, brain, and liver of unsupplemented infant rats. Thus, the data indicate that infant rats are more responsive to dietary supplementation of DHA than AA, suggesting a great potential of dietary manipulation to alter tissue DHA concentrations.

Coinciding with the disproportionate incorporation of dietary AA and DHA into tissues and blood, DHA supplementation significantly decreased the ratio of AA/DHA, whereas there was little or no change in the ratio by AA supplementation. Although the beneficial effects of DHA enrichment on retinal and neural functions have been widely recognized (7–9), the consequences of the decrease in AA/DHA ratio resulting from DHA supplementation is not known. It is reasonable to hypothesize that a decrease in the ratio may be deleterious to infant growth as has been shown by Carlson

TABLE 7
Fatty Acids of Brain Phosphatidylcholine and Phosphatidylethanolamine in Infant Rats Fed Formulas Supplemented with DHA[a]

	Phosphatidylcholine[a]			Phosphatidylethanolamine[a]		
Fatty acid	0% DHA	0.5% DHA	1.0% DHA	0% DHA	0.5% DHA	1.0% DHA
16:0	60.2 ± 0.1^{a}	61.2 ± 0.4^{b}	59.7 ± 0.6^{b,c}	7.7 ± 0.3	7.8 ± 0.2	7.4 ± 0.4
18:0	10.0 ± 0.2	9.8 ± 0.4	9.5 ± 0.7	28.2 ± 0.7	27.6 ± 0.6	27.9 ± 1.0
18:1n-9	17.7 ± 0.3^{a}	17.3 ± 0.2^{a,b}	16.9 ± 0.6^{b}	7.9 ± 0.5^{a}	7.5 ± 1.0^{a,b}	6.6 ± 0.5^{b}
18:2n-6	1.6 ± 0.1^{a}	1.4 ± 0.01^{b}	1.6 ± 0.1^{a,b}	1.8 ± 0.5^{a}	1.7 ± 0.7^{a}	1.0 ± 0.1^{b}
20:4n-6	3.7 ± 0.1^{a}	3.4 ± 0.1^{b}	4.3 ± 0.6^{c}	24.0 ± 0.4^{a}	22.9 ± 0.4^{b}	22.5 ± 0.6^{b}
22:4n-6	0.3 ± 0.01	0.3 ± 0.02	0.3 ± 0.1	6.9 ± 0.1^{a}	6.4 ± 0.3^{b}	6.4 ± 0.1^{b}
22:5n-6	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	2.1 ± 0.1	2.2 ± 0.1	2.1 ± 0.1
22:6n-3	1.2 ± 0.6^{a}	1.4 ± 0.6^{b}	1.6 ± 0.6^{c}	18.7 ± 1.2^{a}	21.9 ± 1.2^{b}	24.0 ± 1.5^{c}
AA/DHA	3.1 ± 0.1^{a}	2.4 ± 0.1^{b}	2.7 ± 0.2^{b}	1.3 ± 0.1^{a}	1.1 ± 0.1^{b}	0.9 ± 0.1^{c}

[a]Values (wt%) are means ± SD for four samples. Fatty acids included only major saturated and unsaturated (n-9, n-6, and n-3) species, and hence did not add up to 100%. Values for each phospholipid with different superscript roman letters in the same row are significantly different at $P < 0.05$. For abbreviations see Table 1.

et al. (26), who found that preterm infants fed formula supplemented with fish oil had increased DHA and decreased AA levels of plasma and erythrocytes which were accompanied by poor growth. Therefore, any attempt to increase accretion of LCPUFA must take into consideration the balance between n-3 and n-6 fatty acids. Whether this can be achieved by simultaneous supplementation of AA and DHA is currently under investigation.

It is worth noting that, although supplementation of DHA consistently enriched the fatty acids in blood and tissues, the degree of enrichment of DHA in plasma and erythrocytes was markedly higher than that of brain total phospholipids, PE, and PC. If one examines the adequacy of DHA accretion in brain, the levels of fatty acid determined in plasma or erythrocytes may not serve as a completely accurate index. This notion is in agreement with a recent suggestion by Innis (41) that AA and DHA in circulating lipids are not a good index of organ LCPUFA status. Since circulating lipid, especially erythrocyte phospholipid AA and DHA, has been widely used to reflect LCPUFA status in infants (41,51,52), further studies to establish the validity for use of erythrocyte lipids are warranted.

Despite the significant increase in accretion of AA and DHA in brain phospholipids, it is not known whether the levels achieved under the present experimental conditions are adequate for growth and development since optimal concentrations of LCPUFA have not been established (7). The requirements of AA and DHA for infants, estimated from either milk fatty acid composition or infant blood lipid profile, have been suggested but none has been agreed on (7). The present study shows that an AR system using AA and DHA derived from microbial sources for supplementation may provide a useful model to establish LCPUFA requirements for infants.

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