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Increase in Plasma Phospholipid Docosahexaenoic and Eicosapentaenoic Acids as a Reflection of their Intake and Mode of Administration[1]

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ABSTRACT. The fatty acid, docosahexaenoic acid (DHA, 22:6n-3), is a major constituent of red blood cell phosphatidylethanolamine and phosphatidylserine at birth but declines in all phospholipid classes following preterm delivery unless the diet contains DHA. A bolus of fish oil prevented declines in DHA of red cell phospholipids (phosphatidylethanolamine, phosphatidylcholine, and phosphatidylserine) during 4 to 6 wk of feeding, with red blood cell DHA indistinguishable from that of infants fed human milk. The amount of DHA fed was almost an order of magnitude greater than usually provided by human milk, however, suggesting poor absorption of fish oil by preterm infants. The purpose of these studies was to determine if uptake of fish oil DHA could be improved by dispersion in preterm formula. Since plasma phospholipids rapidly reflect changes in dietary fatty acid composition, DHA uptake was assessed by fatty acid analysis of plasma phosphatidylethanolamine and phosphatidylcholine. All groups receiving fish oil (both bolus and dispersed) demonstrated a rise in plasma phospholipid phosphatidylethanolamine DHA. Infants receiving 11 mg/kg/day DHA from dispersed fish oil, however, appeared to absorb as much or more as those receiving 71 mg/kg/day DHA in a bolus. The lower intake of DHA provided only 0.2% of total dietary fatty acids (human milk typically provides 0.1 to 0.3%). This study, in conjunction with an earlier report, demonstrates the feasibility of 1) long-term maintenance of red cell membrane DHA by its inclusion in infant formula and 2) DHA maintenance by "physiological" intakes of DHA; *i.e.* the amount provided by human milk. Both factors are important in order to undertake studies of DHA function in preterm infants without undue concern for their safety. Fatty acids are designated as number of carbons:number of double bonds and family (n-6, derived from linoleic acid; n-3, derived from linolenic acid), thus 22:5n-6 contains 22 carbons, five double bonds, and is derived from linoleic acid. (*Pediatr Res* 22: 292–296, 1987)

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Abbreviations

DHA, 22:6n-3—docosahexaenoic acid
EPA, 20:5n-3—eicosapentaenoic acid
PE, phosphatidylethanolamine
PC, phosphatidylcholine
PS, phosphatidylserine
TG, triglyceride
BHT, butylated hydroxytoluene
SAS, statistical analysis system

Both mammalian brain gray matter and retinal membrane ethanolamine phosphoglyceride are enriched highly in docosahexaenoic acid (DHA, 22:6n-3) (1–5), an elongation-desaturation product of linolenic acid (18:3n-3). In the human infant most accumulation of DHA in these membranes occurs in the last intrauterine trimester (6, 7). Studies undertaken by Clandinin and coworkers (6, 8, 9) provided evidence that human milk could meet the theoretical needs for brain polyunsaturated fatty acids following premature delivery. A study of red blood cell phospholipids in preterm infants provided evidence that DHA status prior to birth is maintained by maternal/placental transfer, that DHA in human milk does indeed substitute in part for this accumulation, and that preterm infants have a relatively poor ability to convert 18:3n-3 to DHA (2). Other investigators have raised the question of the ability of the neonate to convert both linoleic (18:2n-6) and linolenic (18:3n-3) acids to the long-chain polyenoates concentrated in nervous tissue (9, 10) since humans appear to have a limited ability for Δ^4-desaturation (11), the final catalytic step in the formation of 22:5n-6 and 22:6n-3.

No direct evidence is available to evaluate the degree of *de novo* synthesis of the 22-carbon polyunsaturates by developing infants. Indirect evidence that Δ^4-desaturation is limited in preterm infants is based on the steady decline in membrane DHA following birth early in the third trimester (2, 3). Decreases in red blood cell DHA in preterm infants may be indicative of limited DHA accumulation during the period when it normally accumulates in the central nervous system. Studies in the rat show that the DHA content of red blood cell phospholipids is related to those of the central nervous system during development (12). Poor accumulation of DHA during development has been associated with abnormalities in visual acuity and electroretinograms in the rhesus monkey (13, 14) and electroretinograms (15, 16) and behavior (17, 18) in the rat. DHA may be necessary during a specific developmental period as abnormal electroretinograms in rhesus monkeys and rats, respectively, were

not reversed by later normalization of membrane DHA with a source of dietary DHA (14, 15). Evidence contradicting a role for DHA in normal vision and retinal response to light has also been reported (19, 20).

As noted previously, the small amount of DHA in human milk (0.1 to 0.3% of total fatty acids) (21, 22) is an important source of red blood cell DHA in developing infants (2, 21). Since many preterm infants do not receive human milk, a fish oil triglyceride highly concentrated in both DHA and EPA (MaxEPA, R. P. Scherer, Troy, MI) was administered by orogastric tube (750 mg/kg/day). This dose prevented the decline in red blood cell phospholipid DHA (3) with treated infants having red blood cell phospholipid DHA like infants fed human milk (2, 21).

The present investigation was designed to determine if uptake of DHA could be improved by microdispersion of fish oil in preterm formula and consequent administration in multiple daily feedings. Our hypothesis was that microdispersion/multiple feedings would increase uptake compared to bolus fish oil in preterm infants. Since plasma phospholipids rapidly reflect changes in dietary fatty acid composition, DHA uptake was assessed by fatty acid analysis of plasma PE and PC. Triglyceride fatty acids were analyzed also. Infants were fed one of three formulas containing by analysis either 0, 0.2, or 0.5% DHA and 0, 0.34, and 0.8% EPA by weight. The fish oil source of DHA was microdispersed in a formula designed for preterm infants (Similac Special Care, Ross Laboratories, Columbus, OH) and administered in eight identical daily feedings (study 2). Data from these infants are contrasted with those from infants receiving daily either 6.5 or 2.6 times the intake of both EPA and DHA in a single bolus of fish oil (study 1).

MATERIALS AND METHODS

Study population. Infants enrolled in these studies weighed less than 1500 g at birth (range 560 to 1440 g). All were free of major congenital malformations and disease processes. Most infants received respiratory support for a short interval prior to entering the study with either continuous positive airway pressure or intubation and mechanical ventilation. Red blood cells were administered as necessary to maintain a hematocrit of 40% in infants until they reached 1500 g. The infants were discharged at approximately 1800 g. A total of 17 infants was recruited for study 2 in April and May of 1986, and all samples were collected by mid-June. Most of these patients were born in March and April 1986. During these 2 months a total of 44 infants weighing less than 1400 g at birth were admitted to the intensive care unit and survived. One infant enrolled did not complete the 2 wk of feeding because of feeding intolerance resulting in removal of enteral feedings for more than 24 h. This infant received the lower level of fish oil supplementation. Details of the period of enrollment and the number of infants enrolled, lost to the study, and available for enrollment during study 1 have been published (3).

Experimental design. The design of study 1 has been described (3). Infants in study 2 were enrolled after obtaining informed consent from a parent as approved by the Institutional Review Board of the University of Mississippi Medical Center. Study 2 infants were randomly assigned to receive one of three formulas. By calculation from the analysis of the individual components of Similac Special Care and MaxEPA and by gas chromatographic analysis of the sonicated formulas, these three formulas contained 0, 0.2, and 0.5% of total fatty acids as DHA and 0, 0.34, and 0.80% as EPA, respectively. Blood samples were drawn at 0 time (enrollment, start of treatment) and after 1, 7, and 14 days. The fatty acid composition of plasma phospholipids (PE, PC) and TG was analyzed by gas chromatography after purification of each lipid class and transmethylation of fatty acids with boron trifluoride-methanol.

The design of study 2 differed from study 1 in that a) all infants received the identical preterm formula (Similac Special Care, Ross Laboratories, Columbus, OH) instead of any of several preterm formulas; b) all infants received 100 kcal/kg/day of Similac Special Care with intake adjusted for weight gain weekly instead of a range of intakes (60 to 120 kcal/kg/day); c) the daily dose of fish oil was dispersed in formula by ultrasound and administered in eight daily feedings instead of once daily as a bolus; and d) all study 2 infants were provided with daily supplementary vitamin E (25 IU, Aquasol E drops, Armour Pharmaceutical Co., Kankakee, IL, 15 IU/0.3 ml) and vitamin D (400 IU, Drisdol-Winthrop-Breon, New York, NY, 8000 IU/ml in propylene glycol).

Formula preparation. Infants in study 2 received Similac Special Care (20 kcal/oz) to which either 250 μl of soybean oil (0% DHA) or 100 or 250 μl of MaxEPA (0.2 and 0.5% DHA, respectively) was added to each 4-oz bottle. These oils were dispersed in the formula with a Sonicator Ultrasonic Processor W375 (Heat Systems Ultrasonics, Inc., Farmingdale, NY) using an output control of 7, a percent cycle of 75 and a ½-inch tip for four 20-s intervals. The formula was immersed in ice during sonication. The fatty acid composition of formula prepared in this manner remained homogenous for at least 24 h. New formula was prepared daily and refrigerated until use. All formulas were fed at 150 ml/kg/day and, including the added oils, provided 102, 101, or 102 kcal/kg/day for the formulas containing 0, 0.2, or 0.5% of dietary fatty acids as DHA. DHA and EPA intakes achieved for these formulas were 0 and 0, 11 and 19, and 27 and 49 mg/kg/day as compared to 71 and 125 mg/kg/day for study 1. These concentrations of fish oil were chosen after a pilot study in which supplemented infants (n = 2) received 81 mg/kg/day of DHA dispersed in formula. Plasma phospholipid EPA and DHA increased so markedly that the experiment was terminated at five days. Study 2 formulas were designed, therefore, to provide much lower amounts of DHA and EPA. Formulas provided 0, 0.2, and 0.5% DHA by weight of total fatty acids. The level of 0.2% was chosen because it represented the amount of DHA reported in preterm milk to maintain membrane phospholipid DHA (2). DHA was included at a higher concentration (0.5%) in the other experimental formula to compensate for possible limitations in digestion and absorption by preterm infants. All stated DHA and EPA intakes were calculated from analysis of the lot of MaxEPA fed in these studies (9.41% DHA and 16.72% EPA) and the density of MaxEPA (0.93 g/ml) and rounded to the nearest mg. [Note: the manufacturer reports MaxEPA to contain 12–14% DHA and 18–20% EPA (weight percent of total fatty acids).] These calculated values differ slightly from those determined using the weight percent of DHA and EPA determined in study 2 formulas.

Blood samples. One ml of blood was drawn from an arm vein and added to EDTA to prevent coagulation. Plasma was removed after centrifugation at 5° C and the red blood cells washed three times with a solution of 0.15 M NaCl, 1 mM EDTA. Both plasma and red blood cells were stored at −20° C in a nitrogen atmosphere until analysis. This was accomplished in 2 to 10 days for plasma from study 2 and the red blood cells from both studies. Plasma samples from infants in study 1 were analyzed after 3 to 4 months of storage.

Preparation and analysis of phospholipid and TG fatty acids. In both studies total lipids in plasma were extracted by the procedure of Dodge and Phillips (23) using chloroform and methanol. Methanol contained 50 mg/l of BHT as an antioxidant. The lipid extracts were washed with 0.15 M KCl according to Folch *et al.* (24) and the organic solvent phase vaporized under nitrogen and fractionated by thin layer chromatography [Silica gel G plates, 10 × 20 cm (width × height), Analtech, Inc., Newark, DE]. Plasma TG was separated by eluting with hexane:diethyl ether:acetic acid (80:20:1) and migrated with an R_f > 0.5.

Phospholipids remained at the origin during the first elution and were immediately fractionated into individual classes by

eluting again with chloroform:methanol:acetic acid:water (60:40:8.4:4.6). This solvent system is a slight modification of that of Zail and Pickering (25). The procedures used for identification and isolation of the phospholipid bands (PE, PC) have been published, along with details of the preparation of fatty acid methyl esters (26) and their identification and quantitation by gas liquid chromatography (3). Data are expressed here for only plasma PE DHA and EPA, but detailed information of other plasma fatty acids and red blood cell phospholipid (PE, PC, PS) fatty acids (study 2) may be obtained from the authors. Red blood cell phospholipid analysis from study 1 has been published (3).

Statistical analysis. Fatty acids were analyzed following the multivariate analysis of variance approach of Cole and Grizzle (27) with significant treatment differences explored using the student Newman-Keul's multiple range test. For each fatty acid analyzed, significant treatment, time or time × treatment interactions were possible. Calculations were performed on the SAS (28) data package.

RESULTS

Bolus orogastric administration of fish oil (study 1). Infants in study 1 had body weights at birth ranging from 600 to 1440 g with means (±SD) in unsupplemented and supplemented groups of 963 ± 209 and 944 ± 191, respectively. Infants ranged in age from 10 to 53 days at enrollment with means (±SD) of 28 ± 14 and 25 ± 10 days in the two respective groups. These differences were not significant.

Figure 1 demonstrates graphically the effect of both time and treatment on plasma PE DHA and EPA. Infants receiving preterm formula only had a mean biweekly decline in PE DHA which differed from enrollment values at 4 and 6 wk ($p < 0.01$). With bolus fish oil both PE DHA and EPA increased by 2 wk ($p < 0.01$). The mean for PE DHA and EPA after 2, 4, and 6 wk of supplementation did not differ significantly. Although DHA constituted ≤2% of plasma PC fatty acids and ≤0.4% of plasma TG fatty acids, the same relative time and treatment effects occurred in these lipid classes ($p < 0.01$) as in PE DHA. Furthermore, as with PE DHA, additional significant increases did not occur beyond 2 wk of fish oil administration. By analysis of variance time × treatment interactions for EPA (20:5n-3) and DHA (22:6n-3) were significant in plasma phospholipids (PE, PC, $p < 0.001$) and TG ($p < 0.02$).

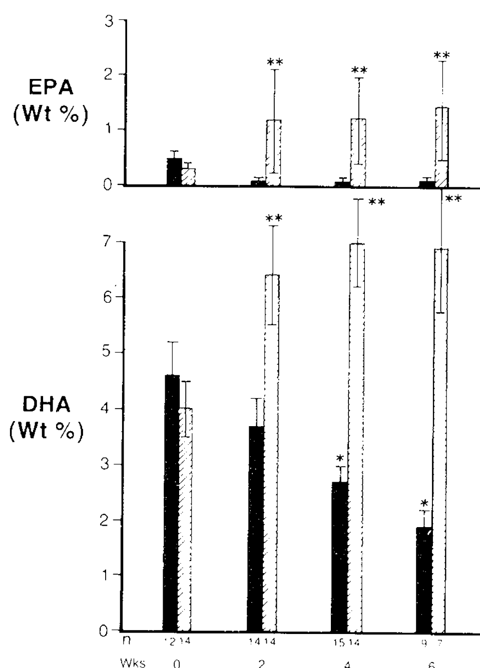

Fig. 1. Study 1. Plasma phosphatidylethanolamine EPA and DHA (weight percent of total fatty acids) at 0 (enrollment), 2, 4, and 6 wk of study. ■, preterm formula only; ▧, preterm formula and single daily bolus of fish oil (750 mg/kg) containing approximately 71 mg/kg DHA and 125 mg EPA. *Error bars* indicate SEM. Time by treatment, $p < 0.0010$. * Differs from unsupplemented at enrollment, $p < 0.01$; ** differs from supplemented at enrollment, $p < 0.01$.

Other than DHA and EPA, only 22:5n-6 showed a highly significant effect of treatment. Lower values were observed in both PE ($p < 0.0001$) and PC ($p < 0.0002$) in supplemented compared to unsupplemented infants. After four weeks PE arachidonate (20:4n-6) showed a less significant ($p < 0.04$) treatment effect. The weight percent in unsupplemented declined from 17 to 14.2% while in supplemented infants the decline was from 13.4 to 12.2%.

Several fatty acids in phospholipids changed significantly with time (*i.e.* independent of treatment). In two of the three (PE, PC, TG) plasma lipid fractions a significant increase in linoleic acid (18:2n-6) ($p < 0.001$) and decrease in 20:4n-6 ($p < 0.05$) occurred with time. In addition, plasma PC 16:0 ($p < 0.004$), 20:3n-6 ($p < 0.006$), and 22:5n-6 ($p < 0.003$) decreased significantly with time while 18:0 ($p < 0.001$) and 22:5n-3 ($p < 0.05$) increased with time. Despite the decline in DHA in unsupplemented infants, 22:5n-6 did not increase as would have been expected with normal Δ^4-desaturase activity.

Administration of microdispersed fish oil (study 2). Infants in study 2 had body weights at birth ranging from 560 to 1390 g with means (±SD) in unsupplemented ($n = 6$), low fish oil-supplemented (11 mg/kg/day DHA, 0.2% of fatty acids, $n = 4$), and high fish oil-supplemented (27 mg/kg/day DHA, 0.5% of total fatty acids, $n = 6$) of 974 ± 300, 932 ± 314, and 938 ± 98 g. At enrollment infants ranged in age from 14 to 49 days except for one infant in the high supplement group who was 71 days old. The mean age at enrollment was 29 ± 11 (18–47) days, 28 ± 11 (21–44) days, and 41 ± 21 (14–49, 71) days for the respective groups. These differences were not significant.

Figure 2 indicates the effects of time and the two levels of fish oil supplementation on plasma PE DHA and EPA. Only the formula containing 0.2% DHA at 14 days differed significantly

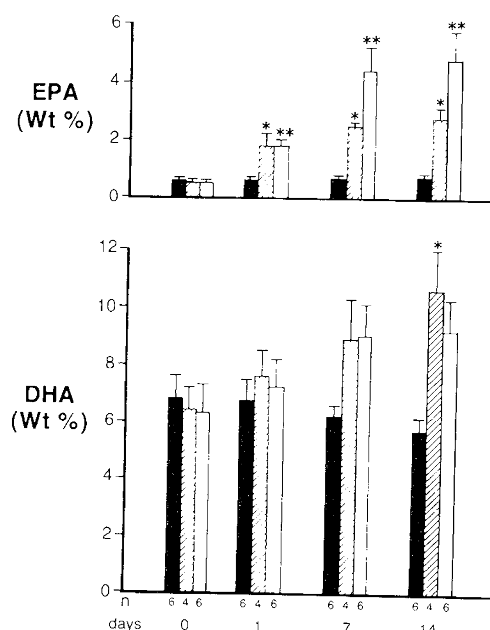

Fig. 2. Study 2. Plasma phosphatidylethanolamine EPA and DHA weight percent of total fatty acids) at 0 (enrollment), 1, 7, and 14 days. Formula with 0% DHA ■; 0.2% DHA ▧; 0.5% DHA □. *Error bars* indicate SEM. Time by treatment, $p < 0.02$. * Differs from 0.2% DHA at enrollment, $p < 0.02$; ** differs from 0.5% DHA at enrollment, $p < 0.01$.

from enrollment using the Student's t test although the overall time × treatment effect on PE DHA was significant ($p < 0.02$). Furthermore, supplemented (both 0.2 and 0.5% DHA) infants had significantly greater PE DHA compared to unsupplemented infants after seven and 14 days of feeding ($p < 0.05$). The two doses of DHA (0.2 or 0.5%) resulted in similar plasma PE DHA.

EPA had already increased significantly by 24 h in the two fish oil-supplemented groups. PE EPA in fish oil-supplemented infants differed significantly from 0 (enrollment) at 1, 7, and 14 days ($p < 0.01$). Furthermore, PE EPA was significantly higher in infants receiving the higher compared to the lower dose of fish oil at both 7 and 14 days ($p < 0.05$).

The only other time × treatment effect which was significant in study 2 was PC 20:4n-6 ($p < 0.05$). PC 20:4n-6 decreased from 8.4 to 6.9% in infants receiving the higher level of fish oil supplementation over 14 days but did not change in infants without supplementation (8.9 to 8.7%) or receiving the low level of supplementation (8.2 to 8.2%).

Several other fatty acids changed significantly with time irrespective of treatment: 22:5n-3 increased in PE, PC, and TG ($p < 0.0002$, 0.003, and 0.002), 18:1 decreased in these same respective lipids ($p < 0.04$, 0.002, and 0.003), and 22:5n-6 decreased in PE ($p < 0.002$) and PC ($p < 0.009$). These changes were not obviously altered by treatment except in the case of 22:5n-3 which appeared to increase more in the supplemented groups.

Study 1 infants received daily 71 mg/kg/day of DHA and 125 mg/kg/day of EPA. This amounted to 6.5 and 2.6 times as much of each fatty acid as provided by the formula containing 0.2 and 0.5% DHA fed in study 2. Despite the much lower intakes in study 2, the difference between PE DHA of unsupplemented and supplemented infants was as great or greater after 14 days of feeding: 2.8% (study 1) and 5.0 and 3.5% (0.2 and 0.5% DHA, study 2) (Fig. 3). Similarly, the difference in PE EPA was 1.3% in study 1 compared to 2.1 and 3.7% in study 2 (Fig. 3). In PC and TG, study 2 infants also had DHA and EPA as high or

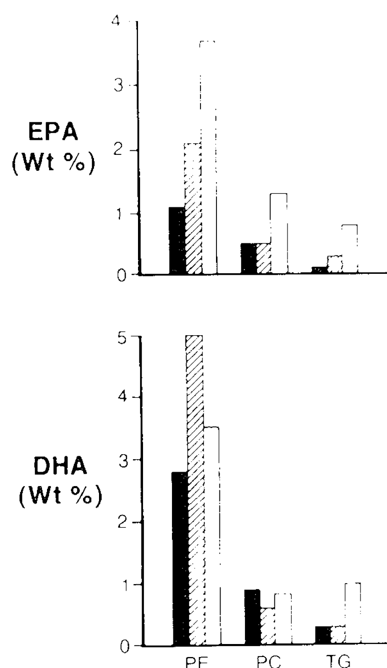

Fig. 3. Increase ($\bar{X}$ supplemented − $\bar{X}$ unsupplemented) in plasma EPA and DHA with fish oil supplementation after 14 days of treatment. ■, 125 mg/kg/day EPA and 71 mg/kg/day DHA, once daily bolus (study 1); ▨, 19 mg/kg/day EPA and 11 mg/kg/day DHA, microdispersed in formula and administered in 8 feedings/d (study 2); □, 49 mg/kg/day EPA and 27 mg/kg/day DHA, microdispersed in formula and administered in eight feedings/day (study 2).

higher than study 1 infants (Fig. 3). b) Figure 3 also graphically demonstrates the enrichment of plasma PE > PC > TG with DHA and EPA following fish oil administration.

Red blood cell phospholipids of all study 2 infants were analyzed over the course of the 14-day study although previous experience suggested that treatment effects would not occur by 14 days (3). Nevertheless, PE and PC EPA (20:5n-3) did demonstrate a significant time × treatment interaction ($p < 0.02$ and < 0.04, respectively) in this short period of time. Furthermore, in at least two of the phospholipid classes analyzed (PE, PC, and PS), significant increases in 18:2n-6 and decreases in 20:4n-6 and 22:5n-6 occurred with time. The effects of fish oil supplementation on red blood cell phospholipid DHA and EPA in study 1 have been published (3).

DISCUSSION

The results of plasma phospholipid fatty acid analysis are presented for preterm infants following supplementation with fish oil highly concentrated in both DHA and EPA. The primary purpose of these studies was to determine if uptake of DHA and EPA could be improved by microdispersion of fish oil in preterm formula and consequent administration in multiple daily feedings (study 2) compared to a single daily orogastric bolus administered with formula (study 1). Since plasma phospholipids rapidly reflect changes in dietary fatty acid composition, DHA and EPA uptake were assessed by fatty acid analysis of plasma PE, PC, and TG.

The data suggest that the amount of fish oil absorbed was as great or greater in study 2 as in study 1 infants despite much lower absolute intakes in study 2: a) The increase in DHA and EPA after 2 wk of supplementation was as great or greater in study 2 compared to study 1 infants (Fig. 3). b) Red blood cell PE and PC EPA demonstrated a significant time by treatment effect at 14 days in study 2 but not until 4 wk in study 1 (3) despite significant increases in plasma EPA (and DHA) by 2 wk (Fig. 1). Plasma PE DHA increased by 2 wk and, thereafter, remained unchanged during 2 to 6 wk of continuous supplementation (Fig. 1), while red blood cell PE DHA remained high during this same period (3). Together these studies suggest the feasibility of long-term maintenance of red cell membrane DHA by its inclusion in infant formula.

Since intakes of fish oil from the formulas fed in study 2 were only 15% (0.2% DHA) and 38% (0.5% DHA) of those in study 1, the proportion of fish oil absorbed must have been increased dramatically in the second study. Preterm infants are recognized to have low levels of pancreatic lipase and bile salts which can limit their ability to digest and absorb dietary fat. Dispersion of the fat in microparticles may have enhanced the efficiency of lipase and bile salts which were available. Roy *et al.* (29), in what may be an analogous situation, demonstrated dramatically improved lipid digestion when rats deprived of salivary lipase were fed lipids microdispersed by ultrasonication. The proportional uptake of fish oil fatty acids may have been improved also by multiple rather than single daily addition(s) to the diet.

When comparing the two levels of supplementary fish oil in study 2, 0.2% DHA enriched PE DHA as well as 0.5% DHA. Fortuitously, 0.2% DHA is in the range of DHA provided by human milk [0.1–0.3% (21, 22)]. The lower level of DHA supplementation also produced a smaller enrichment of PE EPA and did not decrease plasma phospholipid arachidonic acid. Potent metabolic effects have been shown to occur as a result of dietary changes which alter the ratio of arachidonic acid to EPA. Decreases in this ratio decreased synthesis of prostaglandin E_2 and thromboxane A_2 (30, 31). Fish oils enriched in EPA and DHA fed at levels (g/kg/day) only slightly higher than received by our study 2 infants administered 0.5% DHA reduced human leukotriene B_4 generation and the chemotactic response of neutrophils to leukotriene B_4 by 50 to 70% (32).

Ideally, DHA included in formula at a physiological concen-

tration could provide long-term maintenance of membrane phospholipid DHA in preterm infants. Long-term studies of DHA function in preterm infants could then be undertaken without undue concern for infants' safety. Measurement of plasma lipid DHA after bolus administration of fish oil, combined with earlier studies of membrane phospholipid DHA in these same infants (3), indicate levels of plasma DHA which prevent declines in membrane phospholipid DHA. Dispersion of fish oil in formula produced equivalent or greater elevations in plasma lipid DHA. Together these observations suggest that declines in membrane phospholipid DHA following preterm delivery may be prevented by prolonged consumption of DHA in amounts typically consumed by infants fed human milk.

Our hypothesis is that preterm infants may require supplemental DHA for normal functional development of the central nervous system and retina. If lesions exist due to inability of the newborn to synthesize DHA from dietary precursors, preterm infants would likely be more vulnerable than term infants. Accordingly, we have undertaken studies in preterm infants with the goal of measuring development of visual acuity in supplemented and unsupplemented infants during infancy.

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